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UNRAVELLING SALT STRESS TOLERANCE: PHYSIOLOGICAL, MORPHOLOGICAL AND GENETIC COMPONENTS IN CROP SPECIES AND MODEL PLANTS

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FOREWORD

Salinity is one of the most critical abiotic stresses affecting crop yield and quality in various agricultural systems. Based on recent estimates, the cost of salinity to agriculture is approximately $US 12 billion a year. Scientists have therefore approached different aspects of salinization related to both soil and plant issues. For the latter, much effort in the last decades has been dedicated to understanding the fundamental biology of plant stress adaptation with the ultimate objective of identifying key stress tolerance functions that could be transferred via traditional breeding and/or trans-gene technology to crop plants. Salinization of soils is a natural phenomenon occurring in areas of the world where evaporation exceeds precipitation. Biological systems, however, have shown wide adaptation to environmental stresses including salt, and plants can be found growing in saline environments and indeed in seawater. Hence, plant growth is not incompatible with salt, even though this level of salinity would be toxic to most crop plants currently cultivated. Although, most of our current knowledge on plant response to salinity has been obtained through a thorough characterization of the molecular basis of stress adaptation in model plants (Arabidopsis), the transfer of the *acquired knowledge* to improve salinity tolerance of crop species has been slow. One reason for this is possibly the absence of a complete correspondence between tolerance mechanisms in model plants and crop species. Moreover the elucidation of the fundamental physiology of salt tolerance using model systems has revealed different facets of a complex scenario, which is not always controlled by single genetic components. Based on these facts, in this work we considered different approaches to study plant response to salinity: 1) the use of model species (Arabidopsis stress tolerant wild type relatives) to identify new relationships between morphological/physiological

characteristics and salt tolerance traits; 2) the study of the physiological basis defining diverse stress tolerance performances in two basil ecotypes; 3) the analysis of possible mechanisms of cross-talk between abiotic and biotic plant stress adaptation. The overall objective of this thesis was to define a new level of complexity in salt stress tolerance that is often overlooked when we move from models to crop plants and to identify new strategies that could be pursued in order to direct recent advances in the field of molecular biology to their application to crop species.

ABSTRACT

This PhD Thesis address fundamental mechanisms of salt stress response in plants from an agronomic, physiological, morphological and genetic point of view. Specifically we considered salt stress tolerance performances of eleven wild species of *Cruciferae*, closely related to *Arabidopsis thaliana* (Chapter 1). In the second chapter, salt tolerance has been related to main morphological and physiological traits of two cultivars of sweet basil (Genovese and Napoletano). Finally (Chapter 3) we considered how salinity stress tolerance of tomato may be affected by a constitutive over-expression of genes involved in wounding responses. Part of this research has been conducted at Purdue University (Indiana - USA) and at the experimental station of the University of Bologna, located in Teresina (PI – Brazil).

Riassunto

Il presente elaborato analizza, sotto l'aspetto fisiologico, morfologico, genetico e produttivo, i principali meccanismi di risposta a stress salino di alcune specie vegetali. In particolare, l'indagine ha riguardato la caratterizzazione della risposta a stress salino in undici specie di *Brassicacee* evolutivamente vicine ad *Arabidopsis thaliana* e caratterizzate da diversi livelli di tolleranza (Capitolo 1). Lo studio ha, altresì, valutato, da una prospettiva morfologica e fisiologica la tolleranza al sale di due cultivar di basilico (Genovese e Napoletano) con spiccate differenze nella risposta allo stress salino (Capitolo 2). Infine, sono stati indagati alcuni possibili meccanismi di cross-talk tra adattamento a stress abiotici e tolleranza indotta dalla sovra-espressione costitutiva di geni attivati da danni meccanici (da ferita) in piante di pomodoro (Capitolo 3). Parte della ricerca è stata effettuata presso l'Università di Purdue (Indiana, USA) e presso la stazione sperimentale dell'Università di Bologna locata in Teresina (Piaui, Brasile).

CHAPTER I.

EVOLUTION OF SALT TOLERANCE IN PLANTS: THE LONG PATH FROM HALOPHYTES TO GLYCOPHYTES. A COMPARATIVE STUDY OF SALT TOLERANCE IN ELEVEN WILD RELATIVES OF *ARABIDOPSIS THALIANA*.

1.1 INTRODUCTION

Saline water covers 70 percent of the surface of the earth. NaCl concentration in the oceans is roughly 500 mM. If such a high salty solution was used to irrigate crops, the result would be fatal to plants. However, many marine plants and algae have developed ways to survive such conditions. In the evolution of terrestrial plants the ability to cope with an extremely saline medium has been largely lost, or was never acquired. It is not an essential adaptation in an environment rinsed by rain. However, there are terrestrial ecosystems that are saline. They are habitat of halophyte species, enabled to exist in their stressful environment through an array of structural, physiological and biochemical adaptations, always including several that minimize

water loss (Goyal *et al.*, 2003). According to this, there is no fundamental biological incompatibility between salinity and plant life.

An intricate and controversial topic in understanding salt tolerance is the origin of the typically terrestrial halophytes. There are reasons to believe that glycophytes developed from halophytes, rather than the other way around. The primacy of the halophytes follows logically from the concept that when life originated in the water, the oceans were salty bodies of water. As suggested by Kovda in the early seventies (Kovda, 1973), comparative research into the physiology of salt tolerance in marine, shoreline and typically terrestrial plants is therefore of major theoretical and practical interest. Studies in this field may help trace the development from marine plants to typically terrestrial halophytes and glycophytes.

Arabidopsis (*Arabidopsis thaliana*) is a widely spread small annual weed of the mustard family (*Cruciferae*), that is native to Europe and Central Asia (Koornneef *et al.*, 2004). Its reported low salt tolerance (Gong *et al.*, 2005) and its adoption as the major model plant, make it ideal for studying natural variation of adaptive traits (Koorneef *et al.*, 2004). Comparative studies between salinity stress adaptation in Arabidopsis and its relative halophyte *Thellungiella halophila* are reported in literature (Inan *et al.*, 2004; Gong *et al.*, 2005). The high genome similarity between the two, both belonging to the *Brassicaceae*/*Cruciferae* family, together with a different salt response, makes the study of *Thellungiella* extremely interesting. The *Brassicacee*/*Cruciferae* family is large, consisting of about 340 genera and more than 3350 species (Al-Shebaz, 1984). This family is of particular interest from a genome evolution perspective because it has nuclear DNA contents that anchor the low range of angiosperm values (Johnston *et al.*, 2005). In order to be a genetic model system, a plant must have desirable

botanical traits (small size, short life cycle, ability to self pollinate and to produce seeds in high number), as well as suitable genetic traits (small genome and easy transformation and mutagenesis capability).

Among *Cruciferae*, other species have been tested for performances under abiotic stresses. Species of *Hirschfeldia*, *Capsella*, *Thlaspi* and *Lepidium* have repeatedly been reported to well perform for phyto-remediation (Aksoy *et al.*, 1999; Davies *et al.*, 2004; Fisherova *et al.*, 2006; Fuentes *et al.*, 2006; Gisbert *et al.*, 2006; Jiménez-Ambriz *et al.*, 2007; Madejon *et al.*, 2005; Madejon *et al.*, 2007; Pedras *et al.*, 2003).

Comparison of salt tolerance traits between different species may contribute to define physiological mechanisms important in salt tolerance. As reported by Glenn *et al.* (1999), the existence in plants of variability in response to salt stress may suggests strategies for improving crop salt tolerance via genetic engineering (Lauchli, 1999; Mc Neil *et al.*, 1999).

Tightly controlled uptake of Na^+ and Cl^- ions is closely correlated with growth in halophytes (Inan *et al.*, 2004). Nevertheless whether growth is limited by the ability to rapidly accumulate ions or whether the growth rate determines the physiology of salt accumulation still remains to be established (Flowers *et al.*, 1986; Munns, 2002). Possibly, plants that are capable of both tightly-control cell uptake and high rates of vacuolar sequestration are able to maintain fast growth under high salinity conditions, presumably by taking advantage of the osmotic potential of accumulated ions. Regulation of water fluxes has emerged as another important feature of halophytes. They normally have reduced stomatal conductance compared to glycophytes and transpiration is often further decreased with increased exposure to salinity (Flowers *et*

al., 1986; Inan *et al.*, 2004). Tolerance to cold stress has been also reported to be higher in halophytes (Inan *et al.*, 2004), which indicates a possible cross-talk between stress adaptation mechanisms (Zhu, 2001).

In this study we compared the response to abiotic stresses in several species of *Arabidopsis* close relatives. Growth parameters, water and hormone homeostasis were primarily considered to link morphological/physiological modifications to stress adaptation mechanisms.

1.2. MATERIAL AND METHODS

Eleven wild relatives of *Arabidopsis thaliana*, belonging to the *Brassicaceae/Cruciferae* family were collected in salty/dry environments (e.g. seaside, desert land) and their taxonomy identified with the help of Prof. Al Sheebaz (Missouri Botanical Garden, USA). The studied species were *Thellungiella halophila*, *Hirschfeldia incana*, *Malconia triloba*, *Thellungiella parvula*, *Descurainia pinnata*, *Lepidium virginicum*, *Lepidium densiflorum*, *Capsella bursa pastoris, Barbarea verna, Thlaspi arvense,* and *Sysimbrium Officinale* (Table 1). As controls, we used *A. thaliana (Col-0)*, and two *A. thaliana* lines, with an over-expressed or disrupted *SOS1* function, respectively. The SOS1 gene encodes for a plasma membrane Na^+-H^+ antiporter responsible for Na^+ cellular exclusion and it has been reported to improve or reduce salt tolerance when is over-expressed transgenic plants or disrupted, respectively (Qiu *et al.*, 2004)

1.2.1. SALT TOLERANCE

✓ *Test 1 – Effects of salt on plant growth*

Seeds of *A. thaliana*, *T. halophila*, *H. incana*, *M. triloba*, *T. parvula*, *D. pinnata*, *L. virginicum*, *L. densifloru, C. bursa pastoris* and *S. officinale* were sown on plastic trays filled with commercial soil. All seedlings were germinated after six days. Plants were transplanted on 3'' plastic pots filled with Turface® eleven Days After Sowing (DAS). Four salt treatments were applied, respectively 0, 60, 150 and 300 mM NaCl, starting from 25 DAS, and 7 plants per treatment were considered, with 3 replications. The salt treatment lasted thirty days. At the end of the experiment, plants were collected to perform measures of root length and leaf area, using Image J® software (Abramoff *et al.,* 2004).

Test 2 – Determination of L50

The ten species considered were *A. thaliana*, *T. halophila*, *H. incana*, *M. triloba*, *T. parvula*, *D. pinnata*, *L .virginicum*, *L. densiflora*, *C. bursa pastoris* and *S. officinale*. Plants were sown on plastic trays filled with commercial soil. Transplant was conducted twenty DAS, when all plants had at least two not-cotyledonal leaves fully expanded. Eleven salt treatments were imposed, respectively 0, 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 mM NaCl, starting from thirty DAS. The experiment lasted thirty days with periodical counting of survived plant material.

- ✓ *Test 3 –Effects of salt on germination*

Seeds of *H. incana*, *T. parvula*, *D. pinnata*, *T. halophila* and *A. thaliana* sos1-OE (piante overesprimenti il gene sos1, caratterizzate de migliorata tolleranza a stress salino) and *A. thaliana* sos1-KO (piante nelle quali l'espressione di sos1 è eliminate, caratterizzate da scarsa tolleranza allo stress) were sterilized and sowed on Petri dishes containing either MS agar medium or MS medium supplemented with 0, 60, 150 and 300 mM NaCl. Plant material was stratified at 4°C for 4 days and transferred to a growth chamber with 16 h of light at 22 °C and 8 h of darkness at 18 °C. The number of germinated seeds was assessed seven and fourteen days after sowing.

- ✓ *Test 4 – Effects of salt on root growth*

Seeds of *H. incana*, *M. triloba T .parvula*, *D. pinnata*, *T. halophila*, *C. bursa pastoris, A.thaliana* sos1-OE and *A. thaliana* sos1-KO were sterilised and plated on MS agar covered with a membrane. Ten plants per line were sown in each plate and we considered 12 plate repetitions. sos1 KO and sos1 OE were used as control in every dish. Dishes were then placed vertically in the growth chamber. After one week, the membranes with germinated seeds were moved and placed on new Petri dishes for salt

treatments, 0, 60, 150 and 300 mM NaCl, respectively. Plates were kept standing vertically and turned upside down, so the root would bend making an hook while growing (Verslues *et al.*, 2006). Salt treatments lasted 10 days for all species. Afterward, photographs of Petri dishes were collected using a transmissive scanner. Roots were then measured using Image J software (Abramoff *et al.*, 2004).

1.2.2. WATER LOSS

✓ *Test 1 – Characterisation of species-specific water loss*

Seeds of *A. thaliana, T. halophila*, *H. incana*, *M. triloba*, *T. parvula*, *D. pinnata*, *L. virginicu*m, *L. densiflorum* and *S. officinale* were sown on plastic trays filled with commercial soil. After complete expansion of the first non cotyledonal leaves, all plants were moved into plastic pots filled with commercial Turface®. Fourty days after sowing, each pot was sealed with a plastic film to prevent water loss from the soil surface, leaving the shoot protruding from the film. Each plant was then placed on an electronic balance under a light intensity of 140 µmol m^2 s^{-1} at 25 °C, and the weight loss was automatically measured every hour for 24 h using a PC software. Water loss values were normalized for plant dry weights taken at the end of the experiment.

✓ *Test 2 – Determination of salt influence on water loss*

Plants of *A. thaliana*, *T. halophila*, *T. parvula*, *D. pinnata*, *L. virginicum* were prepared as reported above. Before starting the experiment, plants were watered adopting two salt treatment 0 (control) and 300 mM NaCl. Weight variations were then recorded for the next 5 days to monitor influence of salt on leaf transpiration. Four plants per treatment were considered.

1.2.3. COLD TOLERANCE

✓ *Test 1 – Characterisation of species-specific cold tolerance*

Plants of *A. thaliana*, *T. halophila*, *T. parvula*, *D. pinnata*, *L. virginicum*, *L . densiflorum* were grown on plastic pots filled with turface. At thirty DAS, ten plants per species were moved into a cold chamber set at 4°C and acclimated for 15 days. Afterward, all plants underwent a 24 hour cold treatment at -15°C and then moved back to the refrigerated chamber, according to the procedure described in Verslues *et al.* (2006). Five days after the treatments, images of the plants were collected and tolerance was evaluated by visual scoring by two independent evaluators. Scoring varied from 0 (extreme sensitivity) to 5 (no visible effects).

1.2.4. ABA SENSITIVITY

✓ *Test 1 – Characterisation of influence of ABA and salt treatment on root growth*

Seeds of *L. virginicum*, *T. parvula*, *D. pinnata*, *C. bursa pastoris* and *A. thaliana* sos1 KO were sown on Petri dishes containing MS medium. Seed germination was recorded after 7 days. The experiment considered two salt treatments (0 and 150 mM NaCl) and three ABA treatments (0, 0.5 and 1 μM NaCl). At 14 DAS, seedlings were moved to salinised plates, considering 10 plants per species and 3 repetitions per treatment. At 25 DAS, survived plants were counted. Photo pictures of the plates were taken. Measures of root length was performed by using Image J software.

1.2.5. STOMATAL SIZE AND DENSITY

Counting and sizing of stomata were performed using a bright-field light microscope. Leaf surface imprints were obtained by using transparent nail polish. Leaves selected for sampling were of uniform age. Leaf samples from ten plants were taken from the middle portion of the blade between the midrib and leaf margin.

1.3. RESULTS

1.3.1. Morphology and life cycles

The species selected in this study share many important features with *Arabidopsis*. All plant material belongs to the *Cruciferae* (Table 1). Life cycles can be completed in 6 to 12 weeks. Some of the species (*T. halophila*, *D. pinnata*) showed slower growth of seedlings, compared to *Arabidopsis*, while most of them (*L. virginicum*, *L. densiflorum*, *H. incana*, *M. triloba*, *C. bursa pastoris*, *T. parvula*) displayed higher growth rate and reached a much bigger size than *Arabidopsis*. Differences in leaf pubescence (Tab. 1, Fig. 1) among species were observed ranging from pubescent species (*H. incana*, *C. bursa pastoris* and *S. officinale*) to glabrous species (*D. pinnata*; *L. virginicum*; *L. densiflorum*; *B. verna*; *T. parvula*; *T. halophila*; *M. triloba*; *T. arvense*).

Differences in stomatal size and density were also found among the analyzed species (Fig. 2). A smaller stomatal size (Fig. 3a) was detected in *T. parvula* and *T. halophila*, whereas bigger stomata were found in *L. Virginicum*, *C. bursa pastoris*, *A. thaliana* and *D. pinnata*. Interestingly, the lower stomatal size was correlated to higher stomatal density (Fig. 3b).

1.3.2. Response to Salt Stress

T. halophila is an halophyte able to tolerate very high NaCl concentrations (Inan *et al.*, 2004). *Arabidopsis* and *Thellungiella halophila* (salt cress) were then adopted as extreme models to assess salt responses among other close relatives. At 150 mM NaCl,

root and leaf area growth rates in *M. triloba*, *L. virginicum* and *T. parvula* were comparable to *T. halophila* (Fig. 4 A and B).

To confirm these results, root growth under saline treatments was evaluated by the root bending essay (Verslues *et al.*, 2006). At 300 mM NaCl, values comparable to *T. halophila* were observed in *T .parvula*, *H. incana* and *M. triloba* (Fig. 5).

Identification of the NaCl dose lethal to 50% of the population is crucial in determining species-specific tolerance to salt. Being 500 mM NaCl the threshold for T. *halophila* as described by Inan *et al.* (2004), comparable responses were observed in *L. virginicum*, *L .densiflorum*, *M. triloba* and *T. parvula* (Fig. 6).

Inan *et al.* (2004) reported that despite the observed tolerance of *T. halophila* to salt at plant stage, its germination is extremely sensitive to salt. Germination sensitivity to elevated salinity has been reported also in other halophytes (Flowers *et al.*, 1986). Thus, it is not surprising that best performers for growth under salt are not germinating in a saline environment (Fig. 7). Apparently, seeds dormancy could have been enhanced by a salty environment as a consequence of endogenous production of ABA, which has been proved to dramatically reduce *T. halophila*, *A. thaliana* and *L. sativum* germinability (Inan *et al.*, 2004; Muller *et al.*, 2006).

1.3.3. Stomatal control in halophytes and glycophytes

Halophytes typically exhibit reduced transpiration rates compared to glycophytes (Lovelock and Ball, 2002). The decreased stomatal apertures of halophytes prevent excessive water loss and, more importantly, reduces the movement of ions into the shoots during salt exposure (Lovelock and Ball, 2002). Three different responses could be detected in our experiments (Fig. 8). Some species showed a particularly low

transpiration rate, below 1 g H_2O loss g^{-1} DW h^{-1}, namely *H. incana*, *S .officinale*, *D. pinnata*, *L. virginicum* and *T. halophila*. We ranked these species all together under group 1. Transpiration rates were just over 1 g H_2O loss g^{-1} DW h^{-1}, in *M. triloba* and *T. parvula*. These species gave best performances under salt stress and were ranked as group 2. Finally, high transpiration rates, over 3 g H_2O loss g^{-1} DW h^{-1} (group 3), were recorded for *A. thaliana* (Fig. 9).

In general, lower water loss values were found in species showing a certain tolerance to salt (higher than *A. thaliana*). At the same time, these species were not the best performers under salt stress. In contrast high salt tolerant species (*M .triloba* and *T. parvula*), showed enhanced transpiration rates compared to the previous ones, though still below *Arabidopsis thaliana* (Fig. 10 A and B). Although Lovelock and Ball (2002) reported a reduced transpiration in halophytes compared to glycophytes, apparently this may not be the case of *extremophiles* for which multiple adaptation pathways may be involved in addition to that controlling ion uptake and homeostasis.

1.3.4. Stomatal control and salt stress

Control of transpiration fluxes through stomata regulation is critical in hyperosmotic environment. Differences were found among different species (Fig. 11 and 12). High reduction of water loss in response to salt stress was recorded in both *A. thaliana* and *D. pinnata*, leading, by the end of the experiment, to plant death. In halophytes, though, two different responses were observed. While in *L.virginicum* the effects of salt on transpiration rates were minimal, great reduction were observed in *T. halophila* and *T.parvula*, although no visible symptoms were observed at the end of the treatment in either of these species.

1.3.5. Cold tolerance

Plants of *A. thaliana*; *T. halophila*; *T. parvula*; *D. pinnata*; *L. virginicum* and *L. densiflorum* were acclimated at 4°C for 2 weeks and subsequently brought to -15°C. *Arabidopsis* plants were killed by a 24 h -15°C treatment, since they could not recover upon transferring in the greenhouse. Significant damages were also observed in *T. parvula* plants, while milder injuries were recorded in *T. halophila*, *D. pinnata*, *L. virginicum* and *L. densiflorum. D. pinnata* was the best performer under cold stress, with a moderate cold injury (Fig. 13). The shoot apex and young expanding leaves were always more tolerant to subfreezing temperatures than mature leaves.

The relation between salt and cold tolerance is reported in Fig. 14. Most salt tolerance species are sensitive to cold, even if they perform better than *Arabidopsis*.

1.3.6. Effects of ABA on salt tolerance

Results with salt and cold stress experiments indicated that *T. parvula*, had the highest salt tolerance, a moderate stomatal closure and reduced performances under cold stress. It has been reported that ABA plays an important role on both inducing stomatal closure under salt stress in halophytes (Desikan *et al.*, 2004) and enhancing plant tolerance to cold (Chinnusamy *et al.*, 2004). In this experiment, seedling of *L. virginicum*; *T. parvula*; *D. pinnata*; *C. bursa pastoris* and *A. thaliana* sos1 KO were sown on MS medium and, after germination, moved to plates containing different concentrations of NaCl and ABA. At the end of the experiment, root length was measured and the relative growth was compared (Fig. 15 a and b).

Based on our data, no effects were attributable to ABA on control sos1 KO plants, whereas a 150 mM NaCl treatment was fatal. Two different responses could be

detected in other species. ABA treatment alone dramatically reduced root length. When salt was added, most species (*L. virginicum*, *D. pinnata* and *C. bursa pastoris*) were not affected by increased ABA on plates. However, ABA reduced the root length of *T. parvula*.

1.3.7. Genetic analysis of stress tolerance determinants

In a first approach towards the identification of stress tolerance components that could be associated to different levels of stress tolerance, we attempted to isolate the genetic counterparts of the Arabidopsis *SOS1, NHX, AKT,* genes involved respectively in cytoplasmic Na^+ exclusion, vacuolar Na^+ accumulation and K^+ homeostasis. Primers specific to each gene were designed within conserved regions identified on cDNA sequences available for different species. The list of primers used is reported in Table 2. Six week old plants of *A. thaliana*, *T. halophila*, *D. pinnata*, *T. parvula*, *C. bursa pastoris, S. officinale* were treated with 150 and 300 mM NaCl. Samples were collected after 1, 2, 4 and 8 hours for RNA extraction.

Partial sequences of PCR fragments (Fig. 16) were cloned in the pGEM vector (Invitrogen) and sequenced to confirm the presence of SOS1, NHX, AKT homologues in wild relatives of *A. thaliana.* The PCR amplified fragments will be used as probes in Northern blots for expression studies and/or to screen cDNA libraries to isolate full length genes. Possibly, the follow-up of this study should allow the identification of specific relationships between gene sequences and tolerance phenotypes.

1.4. DISCUSSION

1.4.1. Comparative analysis of stress-response

Plants ability to cope with abiotic stresses involves morphological as well as physiological adjustments. As reported by Munns (2002), plant adaptation to salt stress consists in a sudden reduction of plant growth (with the effect of reducing plant water requirement and adjusting osmotic potentials), which is then restored once plant normal functions resume. In our experiments, after thirty days of severe salt treatment, leaf and root growth was efficiently restored only in most tolerant species, namely *T. parvula*, *L. virginicum*, *M. triloba*, *T. halophila* and *H. incana*.

Leaf pubescence was not associated with plant salt tolerance. We recorded a higher number and lower size of stomata in the most tolerant species, *T. parvula* and *T. halophila*, compared to the other ones. This result was consistent with previous studies by Inan *et al.* (2004), who also documented morphological adjustments usually found in halophytes, including a reduced stomata size, which is probably associated to a more efficient control of leaf gas exchanges and higher water retention capacity.

1.4.2. Ranking abiotic stress responses

According to Munns (2002) salt tolerance in plants may be assessed based on two indexes: the percent biomass production in saline versus non-saline control conditions over a prolonged period of time, or the assessment of the survival rate. Best performances for these two indexes are usually found in nature among halophytes. Our results also demonstrate that some of the studied species exhibit growth and other physiological properties typical of halophytes, i.e. a rapid growth at moderate NaCl

concentrations and an increased survival at extremely high salt conditions, including near-seawater concentrations. Based on these results, we classified the studied species into four main groups:

1. Extremely salt sensitive species: *A. thaliana*;
2. Salt sensitive species: *H. incana*; *S. officinale*; *D. pinnata*; *C. bursa pastoris*; *T. arvense*; *B. verna*;
3. Salt tolerant species: *L .virginicum*; *L. densiflorum*; *T. halophila*;
4. Extremely salt tolerant species: *T .parvula* and *M. triloba*.

In a recent study by Inan *et al.* (2004), *T. halophila* was described as an extremophylic higher plant. The experiments presented in this paper showed that *T. halophila* was able to survive to high salt stress and low-temperature stress. These results were confirmed by our tests. The ability of withstanding several abiotic stresses should be interpreted as a general feature of halophytes. Conceivably, halophytes should have a reduced stomatal opening to reduce water loss and allow physiological adjustment, for plant growth maintenance. Nevertheless, *T. parvula*, which was the best performer under salt, suffered more than other species under cold treatment and showed a high water loss rate, which was slightly reduced under salt treatment. *L. virginicum*, another species with halophytic behaviour, displayed relatively low stomatal closure when treated with salt.

These results indicate that the physiological bases of stress adaptation in halophytes cannot be restricted to water homeostasis control, yet it involves more complex mechanisms. Indices of tolerance may vary among different species and comparison should consider a wide number and type of experiments. As reported by Zhu (2001), the mechanisms of salt tolerance in halophytes are substantially the same as

those known to exist in glycophytes and subtle differences in regulation result in large variations in tolerance or sensitivity.

1.4.3. Role of ABA in modulating plant response to salt

ABA is involved in plant response to abiotic stresses such as low temperature, drought and salinity as well as the regulation of plant growth and development, including embryogenesis, seed dormancy, leaf transpiration, shoot and root growth (Leung and Giraudat, 1998; McCourt, 1999; Rock, 2000).

According to Chinnusamy *et al.* (2004), however, two gene-induction pathway are activated upon osmotic stress. While an ABA-dependent pathway binds to both MYB/C Responsive Sequences (MYB/C RS) and ABA Responsive Sequences (ABRE), DREB2 transcription factors induce ABA-independent transcription of stress responsive genes. The effect of these pathways are concurrent and lead to plant stress tolerance.

According to our data, we could suppose that ABA treatment in *L.virginicum*, *D.pinnata* and *C.bursa pastoris* grown at 150 mM NaCl did not affect root growth because the threshold ABA level required to activate stress adaptation responses was already triggered by osmotic stress. Indeed, *T.parvula* seedling root growth was reduced by ABA treatment even at 150 mM NaCl. Most likely, relatively lower amount of ABA were produced as a consequence of the salt treatment. According to this, the higher salt tolerance score found in *T.parvula* could be possibly related to the ABA-independent pathway rather than the ABA-dependent.

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TABLES

Species	Origin	Leaf Pubescence	Leaf area	Root length	Salt tolerance
			$cm^2\ plant^{-1}$	cm	
Arabidopsis thaliana	Europe, Asia	Glabrous	22.9±1.85	9.5±0.48	Sensitive ++
Barbarea verna	Indiana - USA	Glabrous	83.0±30.99	16.6±3.32	Sensitive
Capsella bursa pastoris	Indiana – USA	Pubescent	88.4±28.96	22.1±6.35	Sensitive
Descurainia pinnata	Indiana – USA	Glabrous	57.5±2.72	18.8±2.40	Sensitive
Hirschfeldia incana	Central Turkey	Pubescent	79.0±8.16	13.4±1.60	Sensitive
Lepidium densiflorum	Byron Bay – Australia	Glabrous	9.7±0.43	11.0±0.98	Tolerant
Lepidium virginicum	Byron Bay – Australia	Glabrous	9.8±0.68	11.2±0.63	Tolerant
Malconia triloba	Central Turkey	Glabrous	27.2±1.91	6.5±0.10	Tolerant ++
Sysimbirium officinale	Southern Italy	Pubescent	150.8±31.96	22.6±1.38	Sensitive
Thellungiella halophila	China	Glabrous	4.2±0.02	8.8±0.69	Tolerant
Thellungiella parvula	Central Turkey	Glabrous	7.9±0.72	9.3±0.16	Tolerant ++
Thlaspi arvense	Indiana - USA	Glabrous	66.1±6.00	14.8±1.22	Sensitive

Table 1. List of the species considered in the experiments. Values of Leaf area, root length and fresh weight refers to 6 weeks old plants Mean values ± std errors.

Primer set	Forward sequence	Reverse sequence
Insitu	F: 5'- GATTTCCTTTGGCAGGAAAGTGC-3'	R: 5'- GTACACATTAACCATGCTGCCG-3'
Thsos1	F: 5'- TCAGGTTCTCCGTGTTGTGA -3'	R: 5'- GCAGCGAGTGATTGTTGCTT -3'
sos1Th	F: 5'- GGGACTCTACGAAGTCCTCA-3'	R: 5'- CCCACCGAAACTTACACTCCTT-3'
Psos1	F: 5'- CCCTGATGAATGATGGGAC -3'	R: 5'- ACCATTTCCCAGAAGTGATG – 3'
Qpcr	F: 5' - TGAACGAGCGATGCAACTTA -3'	R: 5'- GTTATCTTGTGCCTTGTTATTGTTCA -3'
At3181S1	F: 5'- CGGCAGCATGGTTAATGTGTAC -3'	R: 5'- CATAGATCGTTCCTGAAAACG -3'
Th2891s1	F: 5'- GCAAGAGTAATCATCTTCAAC -3'	R: 5'- CATAGATCGTTCCTGAAAACG -3'
Th328s1	F: 5'- CGATGGAAGTTCACCAGATCAAG -3'	R: 5'- GTAGCTCACTGCAATCGTAAGAG -3'
Th915s1	F: 5'- CATCACTTCTGGGAAATGGT -3'	R: 5'- GGATCCATTAACTATCAGAG -3'
Pesos1	F: 5'- CCCTGATGAATGATGGGAC -3'	R: 5'- GGATCCATTAACTATCAGAG -3'
Sos1le	F: 5'- GGATGTGGAACGAACTGG -3'	R: 5'- GTAGCTCACTGCAATCGTAAGAG -3'
HKT1	F: 5'- ATTCGGACAGTTCCATCGAG -3'	R: 5'- ATTTTGCCTTTCGGTGATTG -3'
SOS1	F: 5'- GGCGATTGTTGTTTTCCAGT -3'	R: 5'- CAGGTCCTAGCTCCTCATCG -3'
NHX1	F: 5'- CGAATTCGCCTCTCTGTTTC -3'	R: 5'- TCACCCAAGTCAAAGGTTCC -3'

Tab. 2. Primers used on cDNA of wild relatives of A. thaliana in order to identify SOS1, HKT and NHX1 genes.

FIGURES

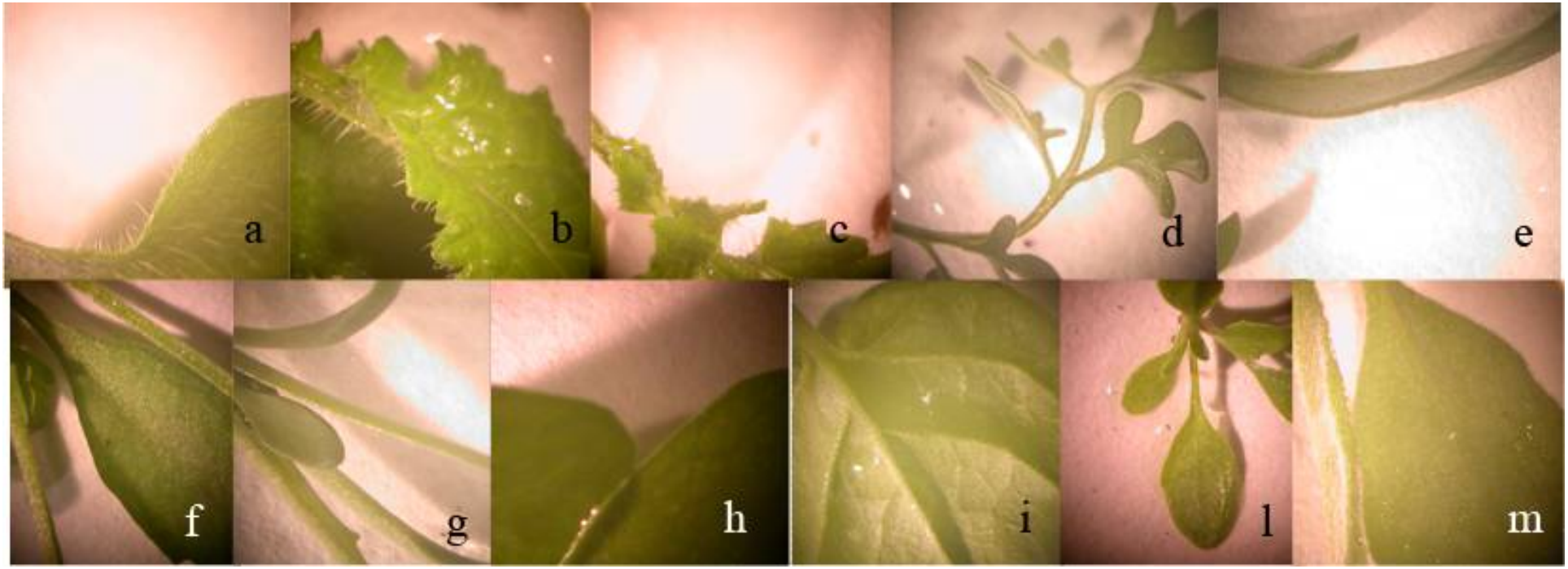

Fig. 1. Images of leaves of the tested species taken with optical microscopy. a) *H. incana*; b) *C. bursa pastoris*; c) *S. officinale*; d) *D. pinnata*; e) *L. virginicum*; f) *L. densiflorum*; g) *T. parvula*; h) *T. harvense*; i) *B. verna*; l) *T.halophila*; m) *M. triloba*.

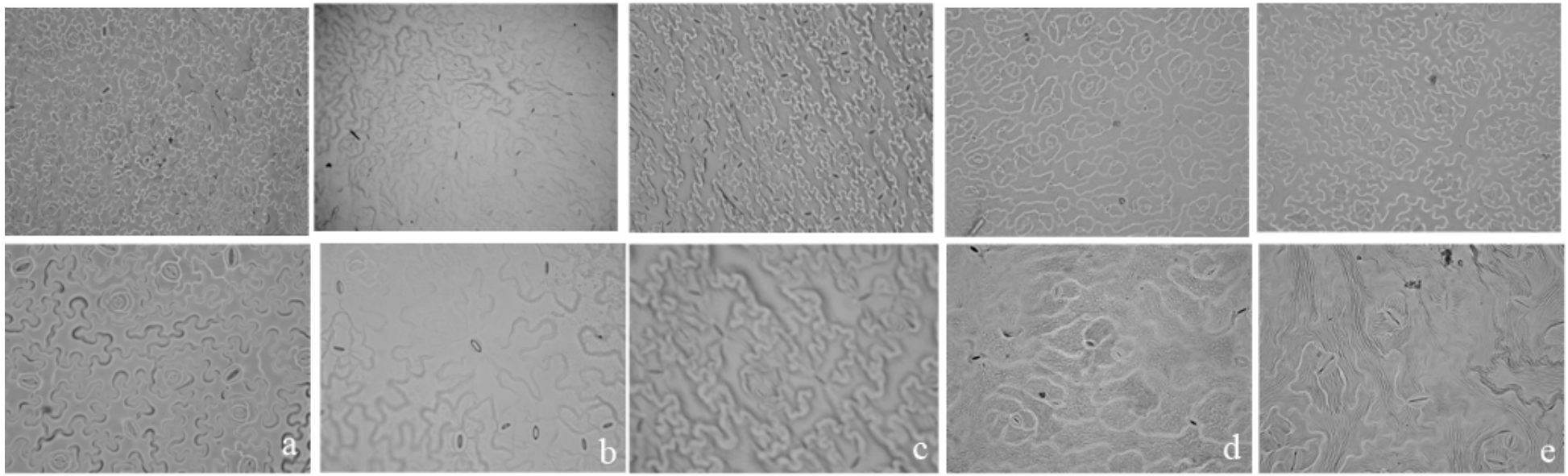

Fig. 2. Images of stomata of the tested species taken through optical microscopy. a) *C. bursa pastoris*; b) *A. thaliana*; c) *T.halophila*; d) *T. parvula*; e) *L. virginicum*. Top row 20x, lower row 60x.

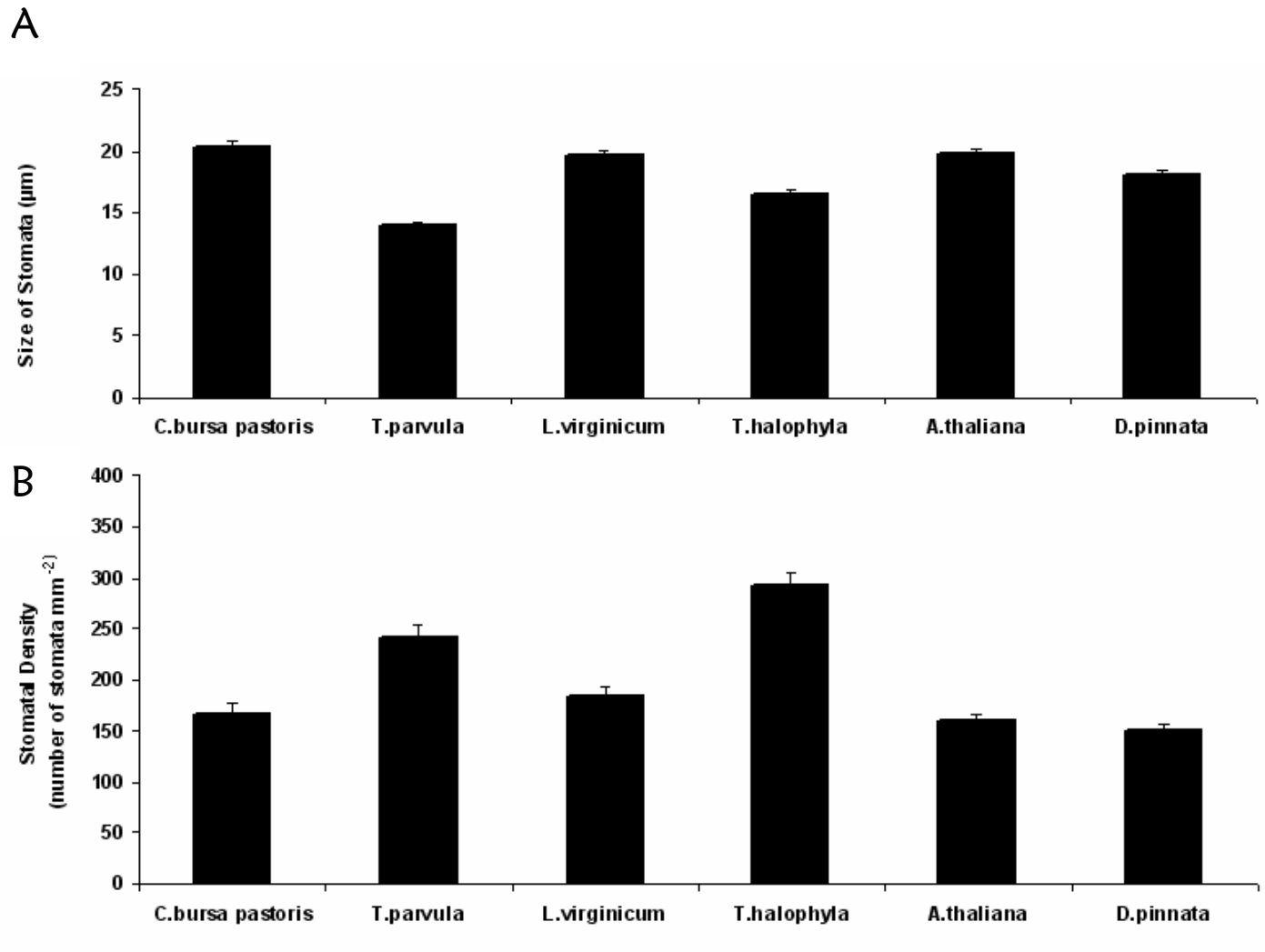

Fig. 3. Stomatal size (A) and density (B) as measured by electron microscopy. Values refer to 20 independent measures per leaf on three leaves per species. Values ± standard errors.

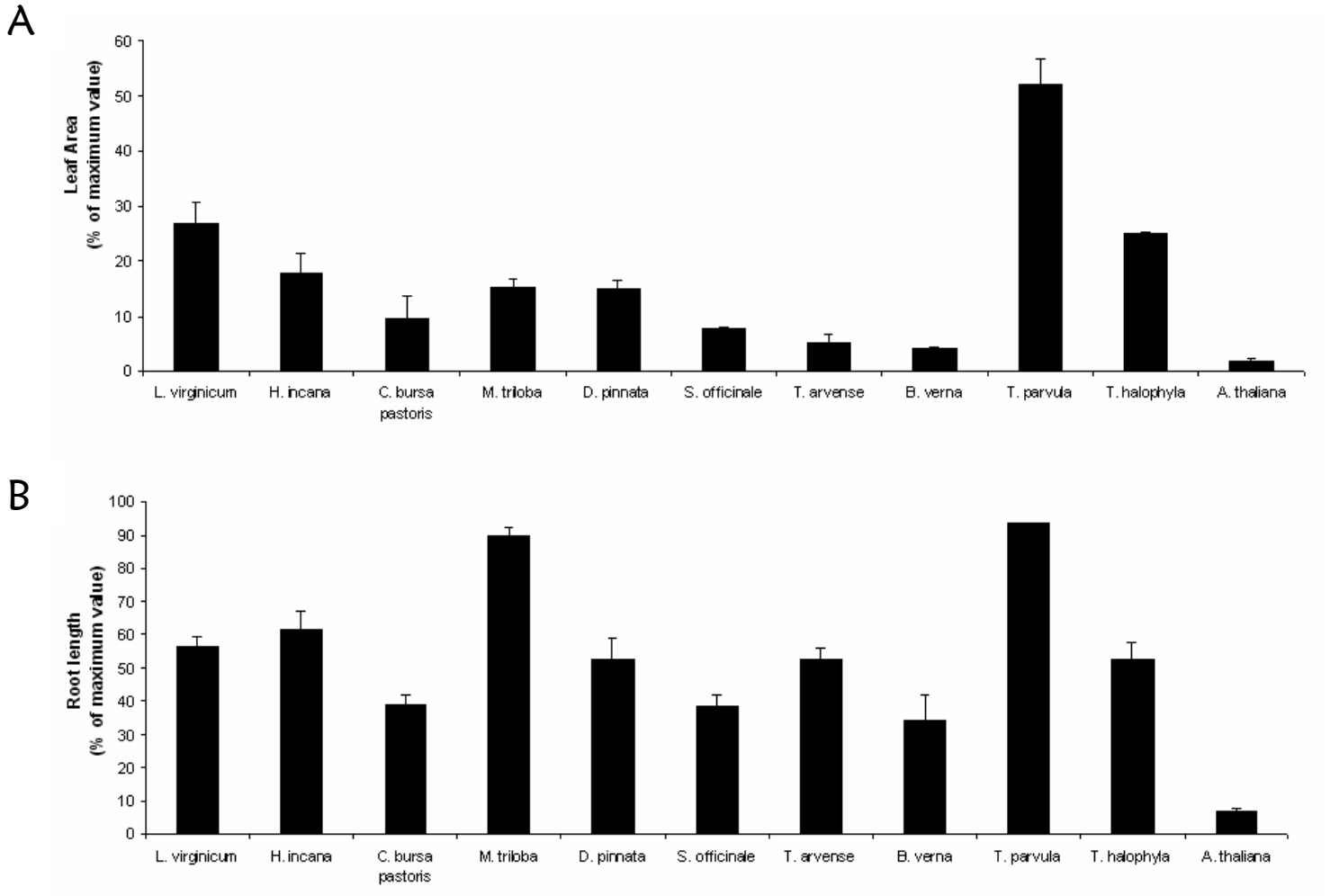

Fig. 4. Leaf area (A) and Root growth (B) as influenced by 150 mM NaCl. Salt treatment started 25 DAS and lasted 30 days. Values ± standard errors.

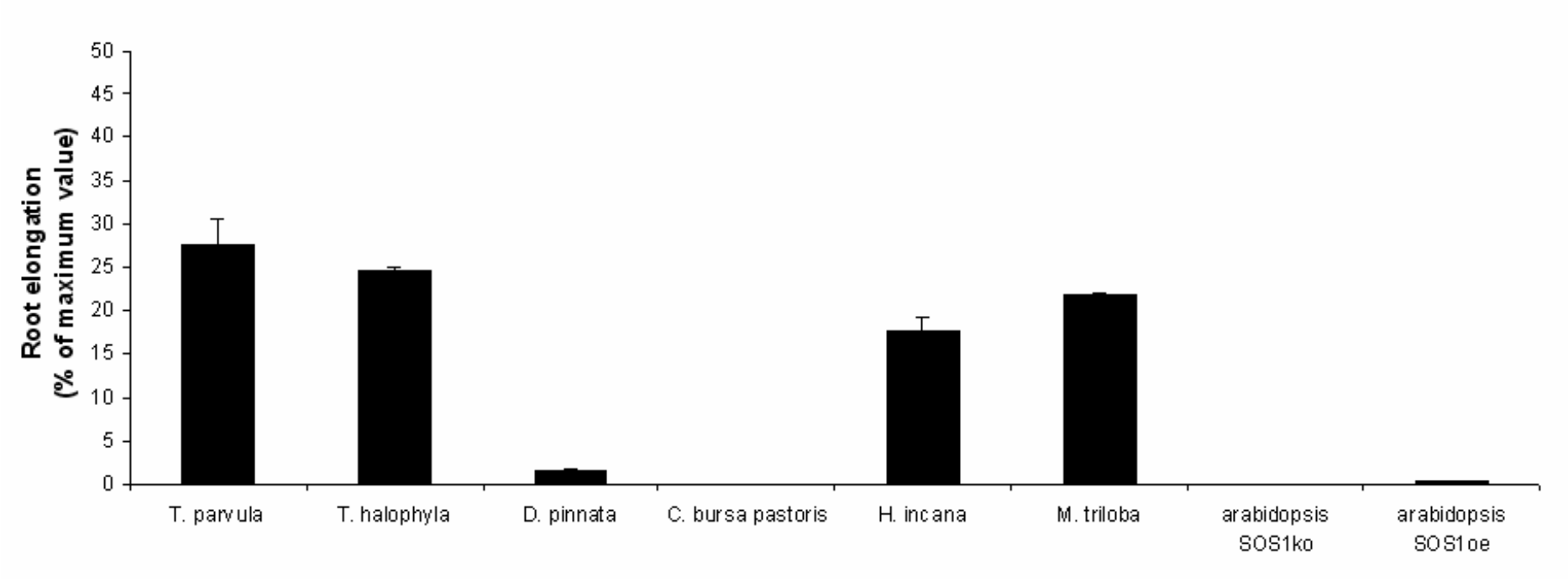

Fig. 5. Root elongation as observed by Root Bending Essay on plants undergoing 300 mM NaCl treatment. After germination seedlings were moved into salt-treated plates and placed vertically, with roots directed upward. Measures of the hook on the roots were performed after 10 days. Values ± standard errors.

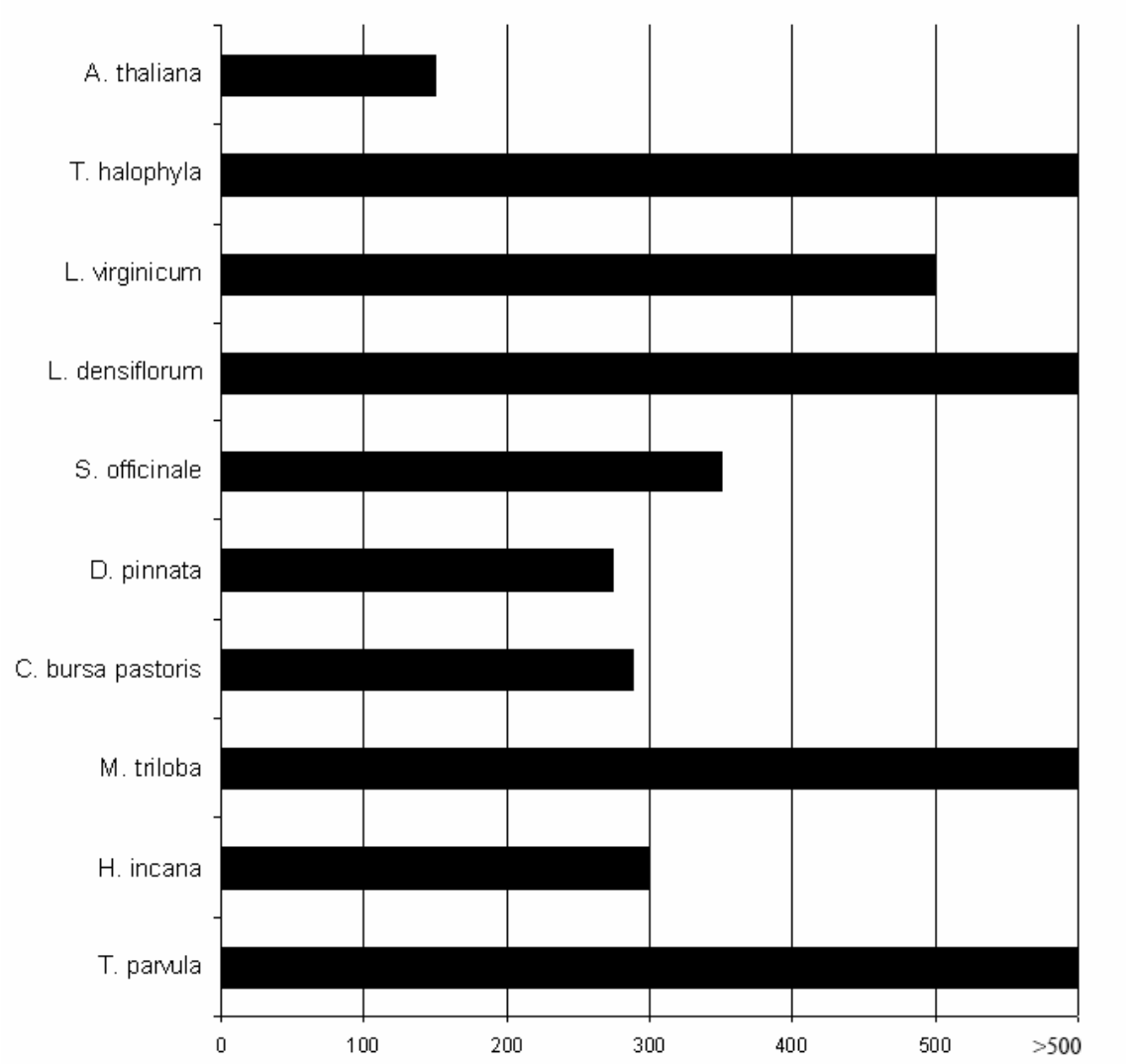

Fig. 6. Species-specific L50. Salt treatment started 30 DAS and lasted 30 days. Salt treatment ranged from 0 to 500 mM NaCl. Rate of survival over a sample of twenty plants replicated three times was assessed at the end of the experiment.

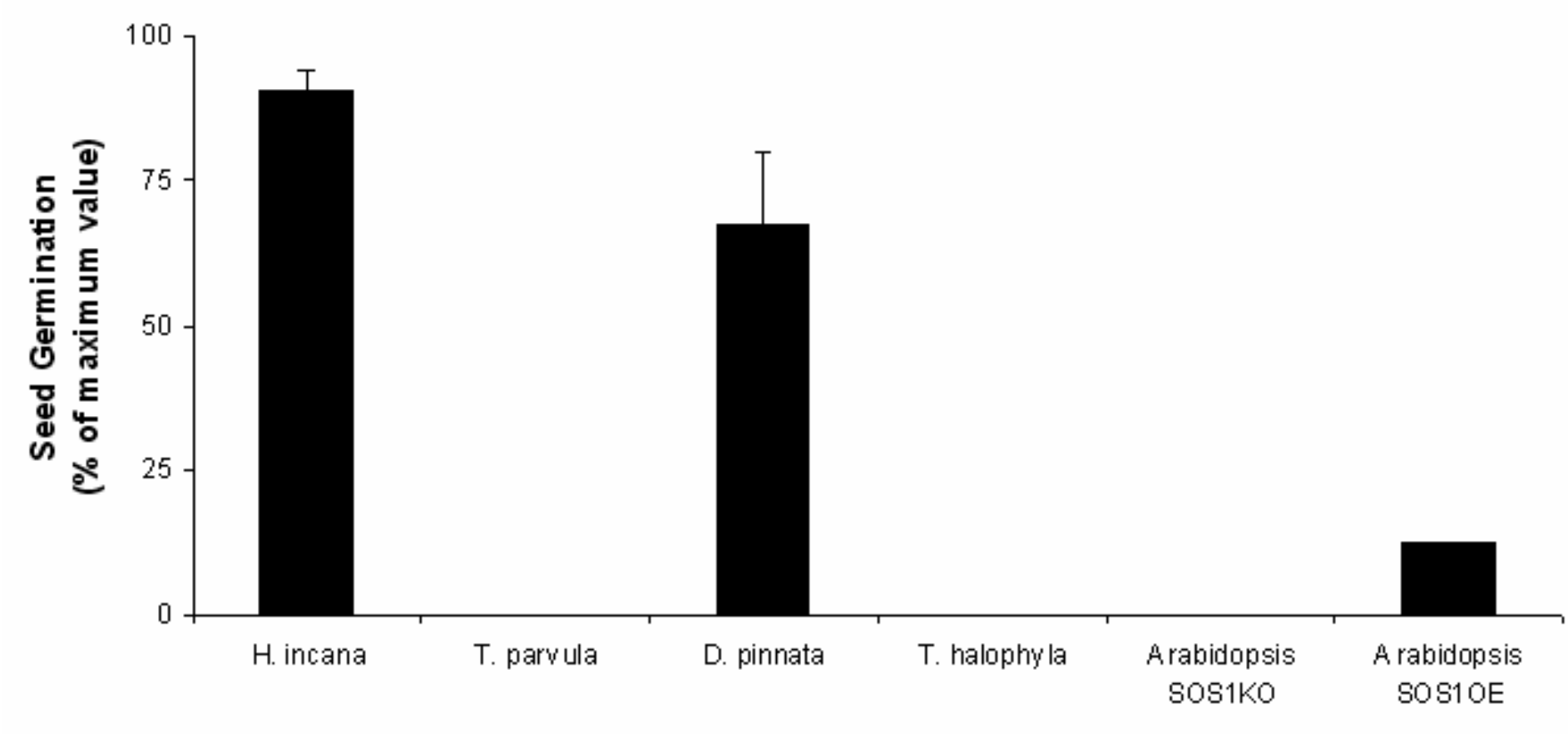

Fig. 7. Seed germination on MS medium enriched with 150 mM NaCl. Germination rate was determined at 14 DAS. Values ± standard errors.

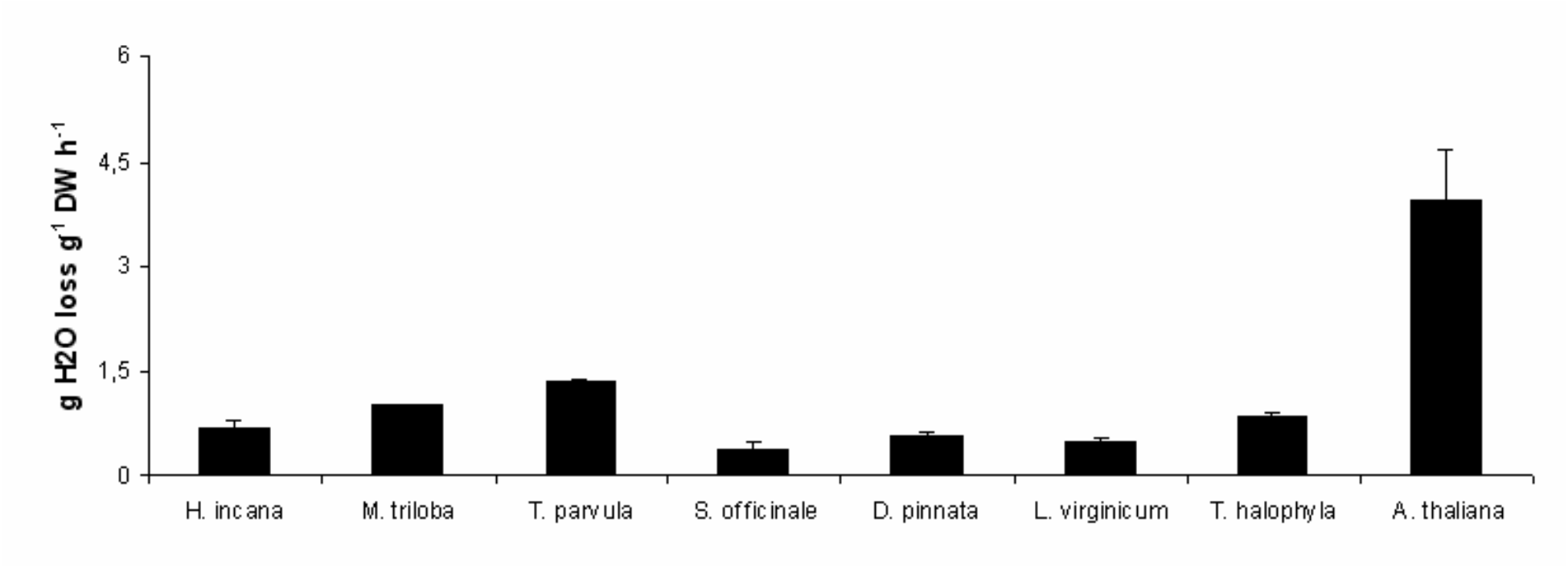

Fig. 8. Four-week-old seedlings grown under long-day conditions with cool-white fluorescent lighting were used for measurements of whole-plant water loss. Single plants were grown in 9-cm pots, which were sealed in plastic wrap and placed on electronic balances. Weight was determined every 60 min for 5 d. Values are means of transpiration rate throughout the experiment of 4 plants. Values ± standard errors.

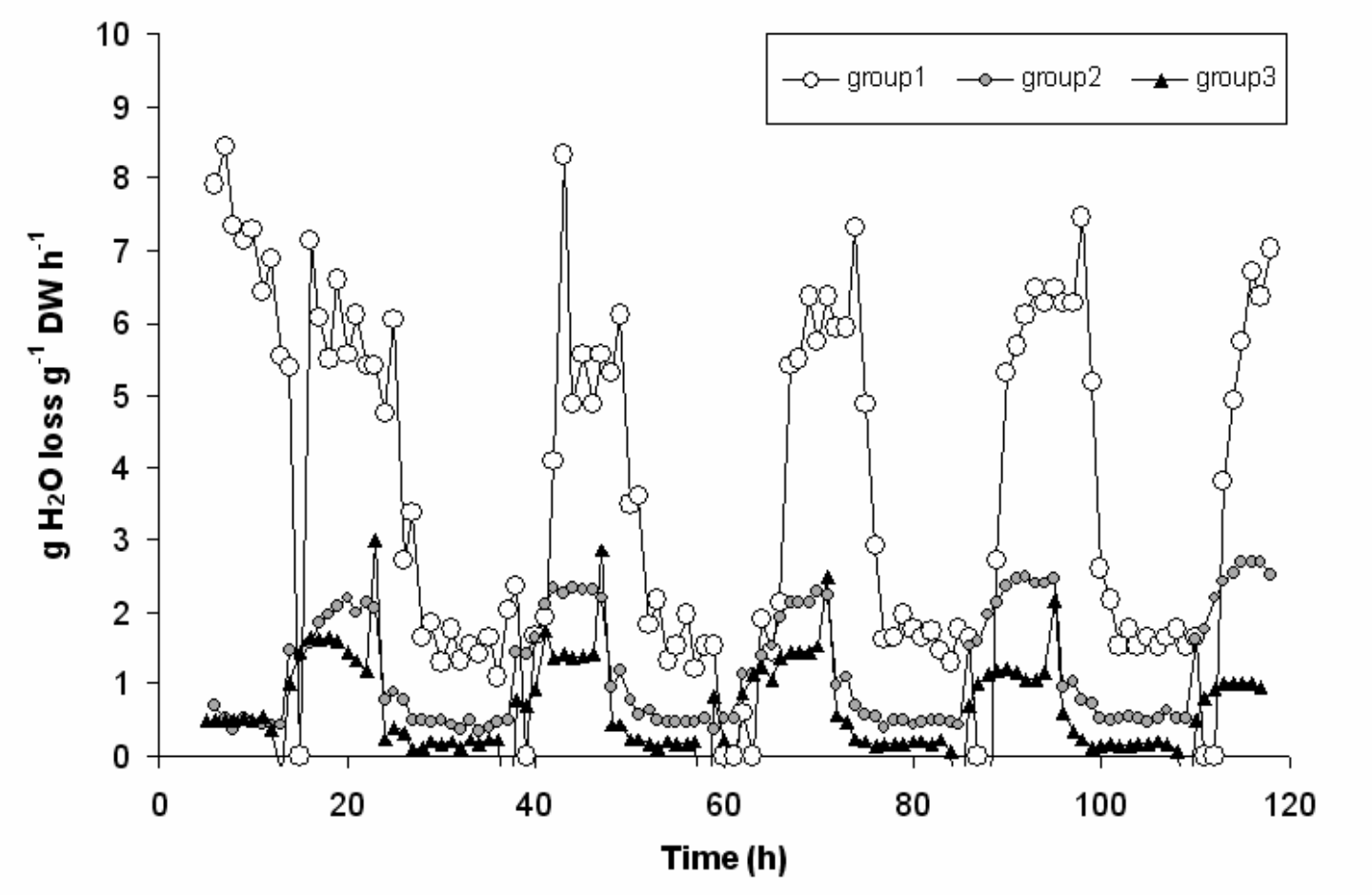

Fig. 9. Four-week-old seedlings grown under long-day conditions with cool-white fluorescent lighting were used for measurements of whole-plant water loss. Plants were grown singularly in 9-cm pots, which were sealed in plastic wrap and placed on electronic balances. Weight was determined every 60 min for 5 d. Values are means of 4 plants. White circles, group 1 (*A.thaliana*), grey circles, group 2 (*M.triloba* and *T.parvula*) and black circles, group 3 (*H.incana*, *S.officinale*, *D.pinnata*, *L.virginicum* and *T.halophila*).

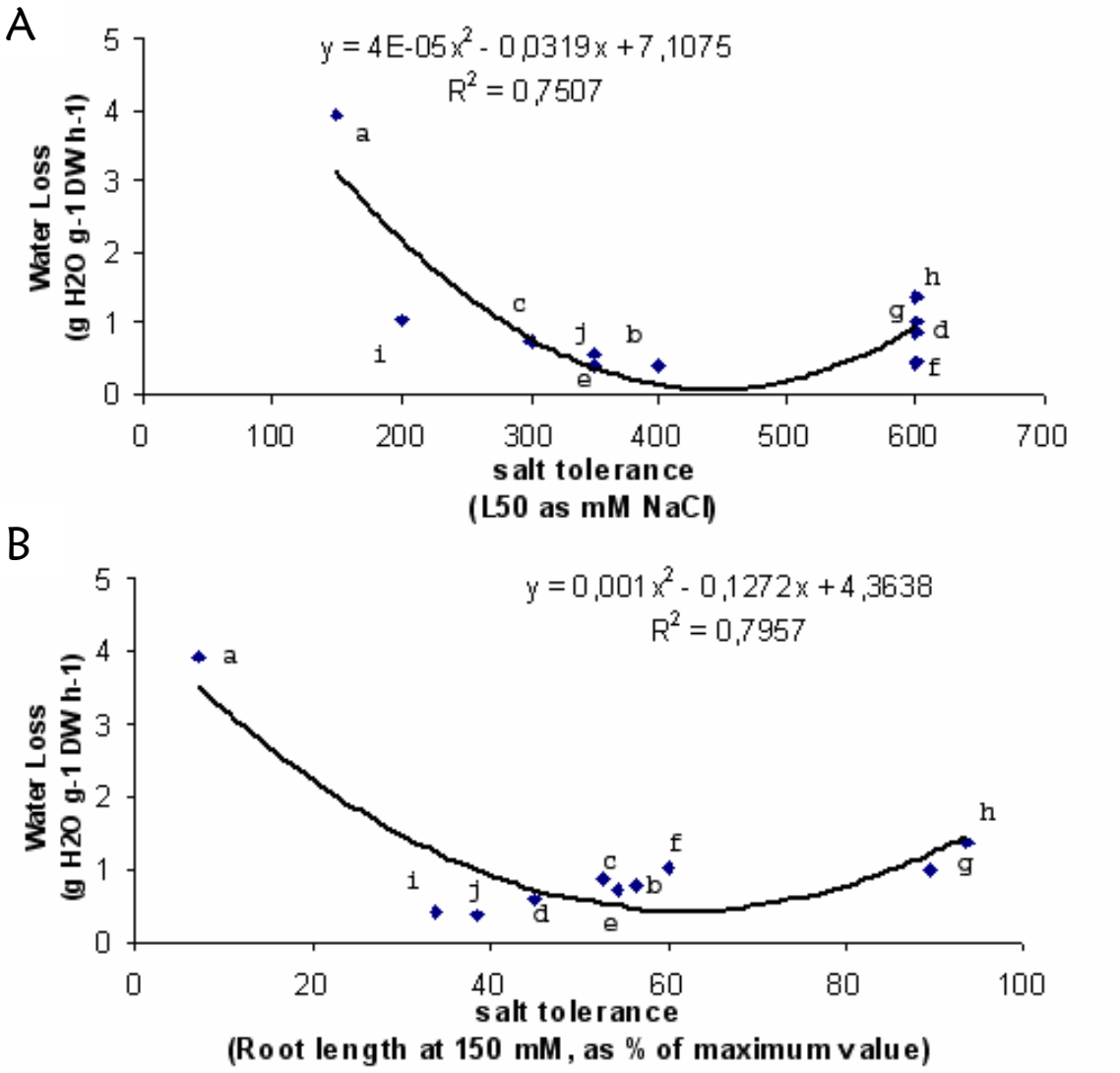

Fig. 10. Water loss (g H2O loss g-1 DW h-1) and salt tolerance (expressed as L50, (A), or root elongation (B) at 150 mM NaCl) of tested species. In the figures: **a) *A. thaliana*; b) *D. pinnata*; c) *H. incana*; d) *T. halophila*; e) *S. officinale*; f) *L. virginicum*; g) *M. triloba*; h) *T. parvula*; i) *B. verna*; j) *C. bursa pastoris*.**

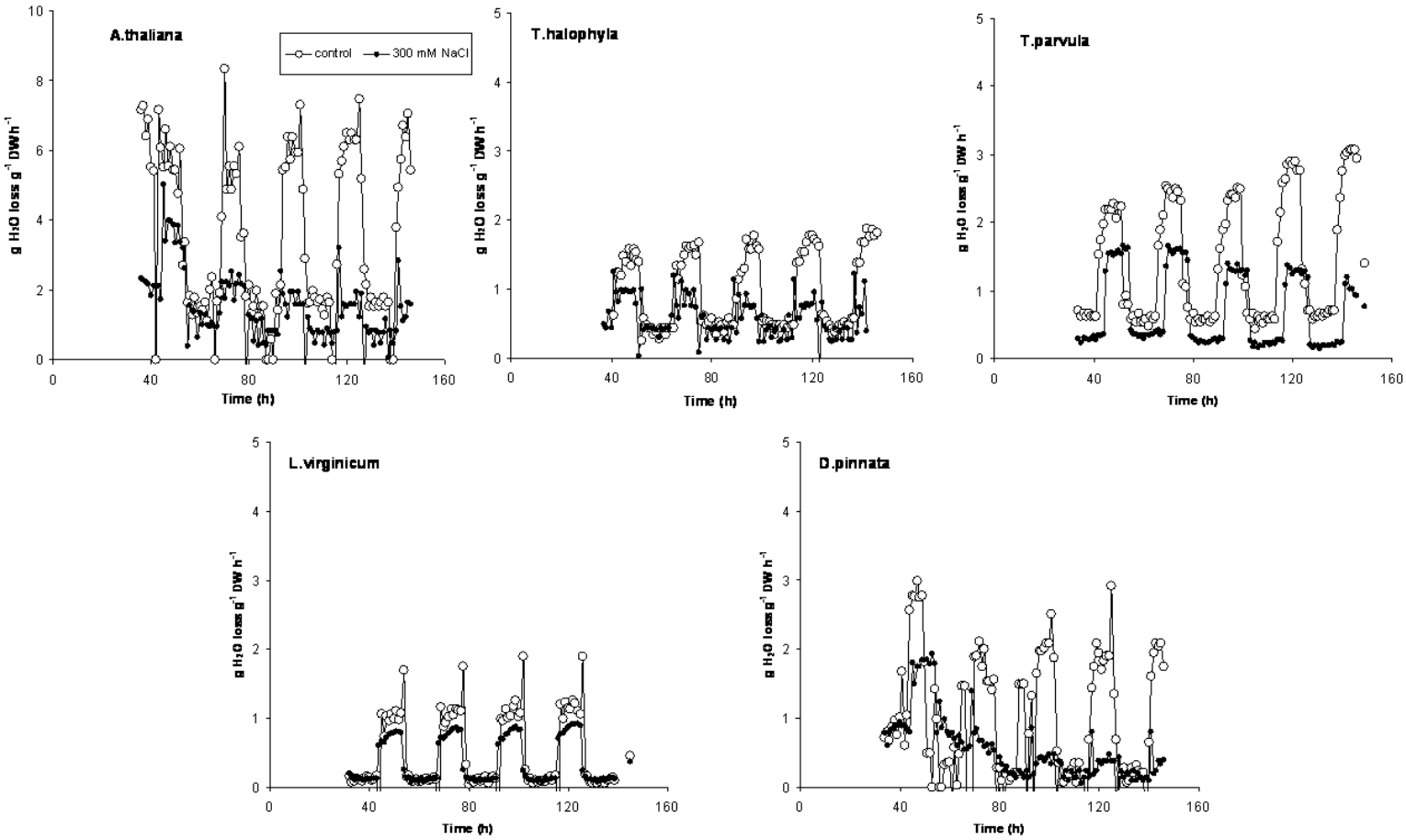

Fig. 11. Water loss (g H2O loss g-1 DW h-1) was determined in *A.thaliana*; *D.pinnata*; *T.halophila*; *T.parvula* and *L.virginicum*. Four-week-old seedlings grown under long-day conditions with cool-white fluorescent lighting were used for measurements of whole-plant water loss. Plants were grown singularly in 9-cm pots, which were sealed in plastic wrap and placed on electronic balances. Weight was determined every 60 min for 5 d. Values are means of transpiration rate throughout the experiment of 4 plants. White, control, Black 300 mM NaCl.

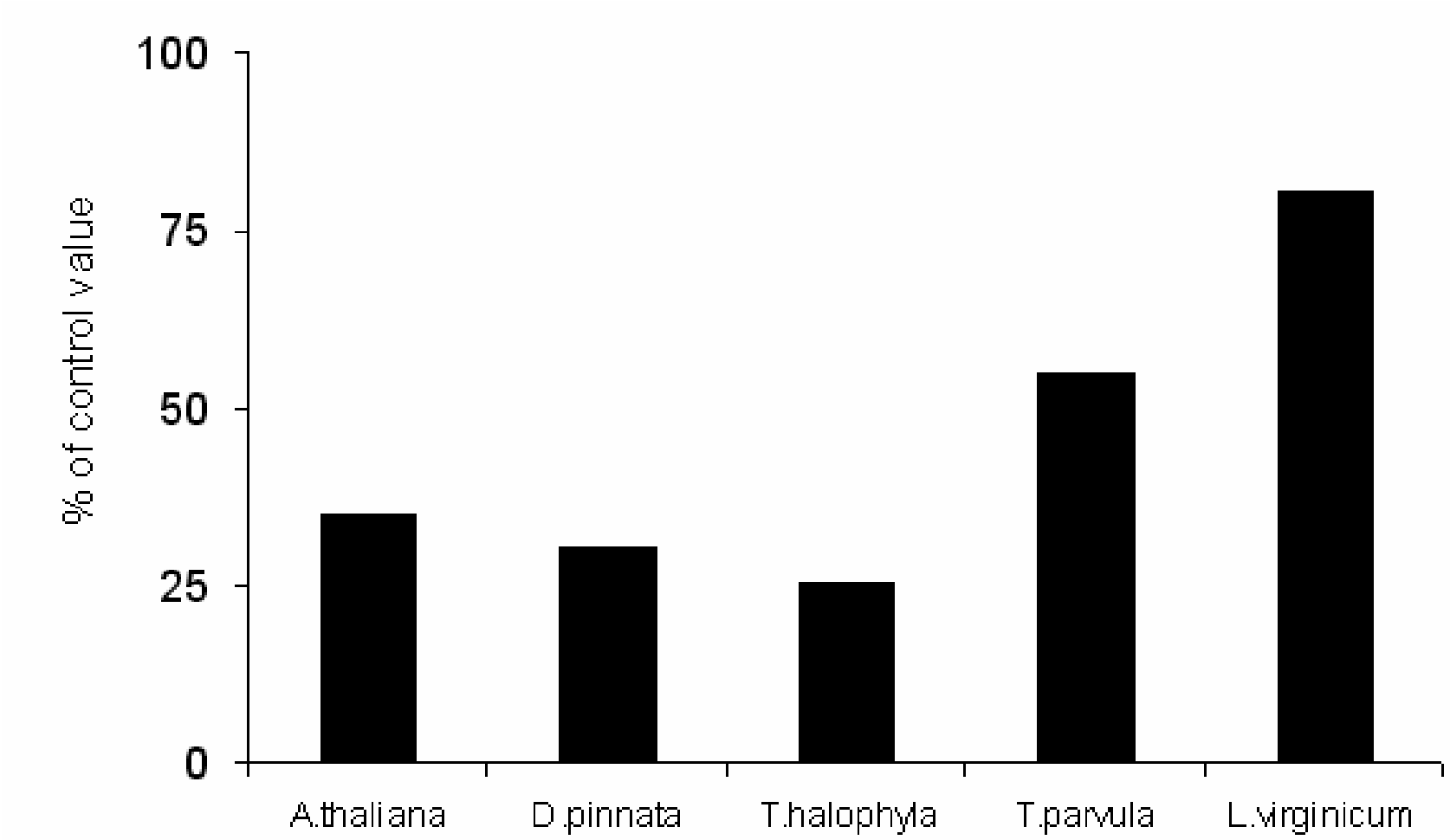

Fig. 12. Relative water loss in 300 mM NaCl stressed plants, as compared with control values.

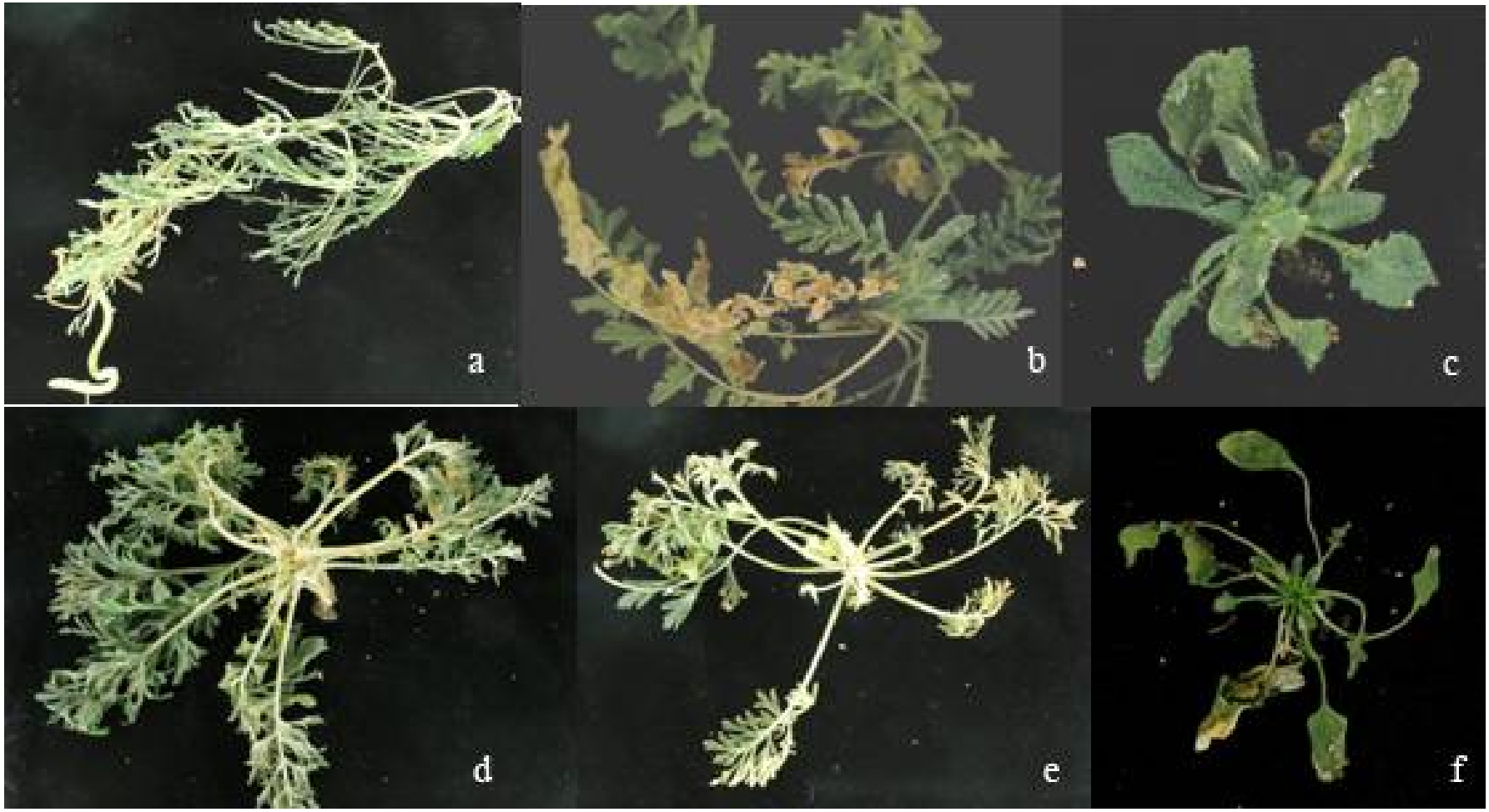

Fig. 13. Images of plants after cold stress. Plants were acclimated for 15 days at 4°C and then transferred at -15°C for 24 hours, and subsequently moved back to the refrigerated chamber. The plants reported are a) *T. parvula*; b) *D. pinnata*; c) *A.thaliana*; d) *L. virginicum*; e) *L. densiflorum*; f) *T. halophila*.

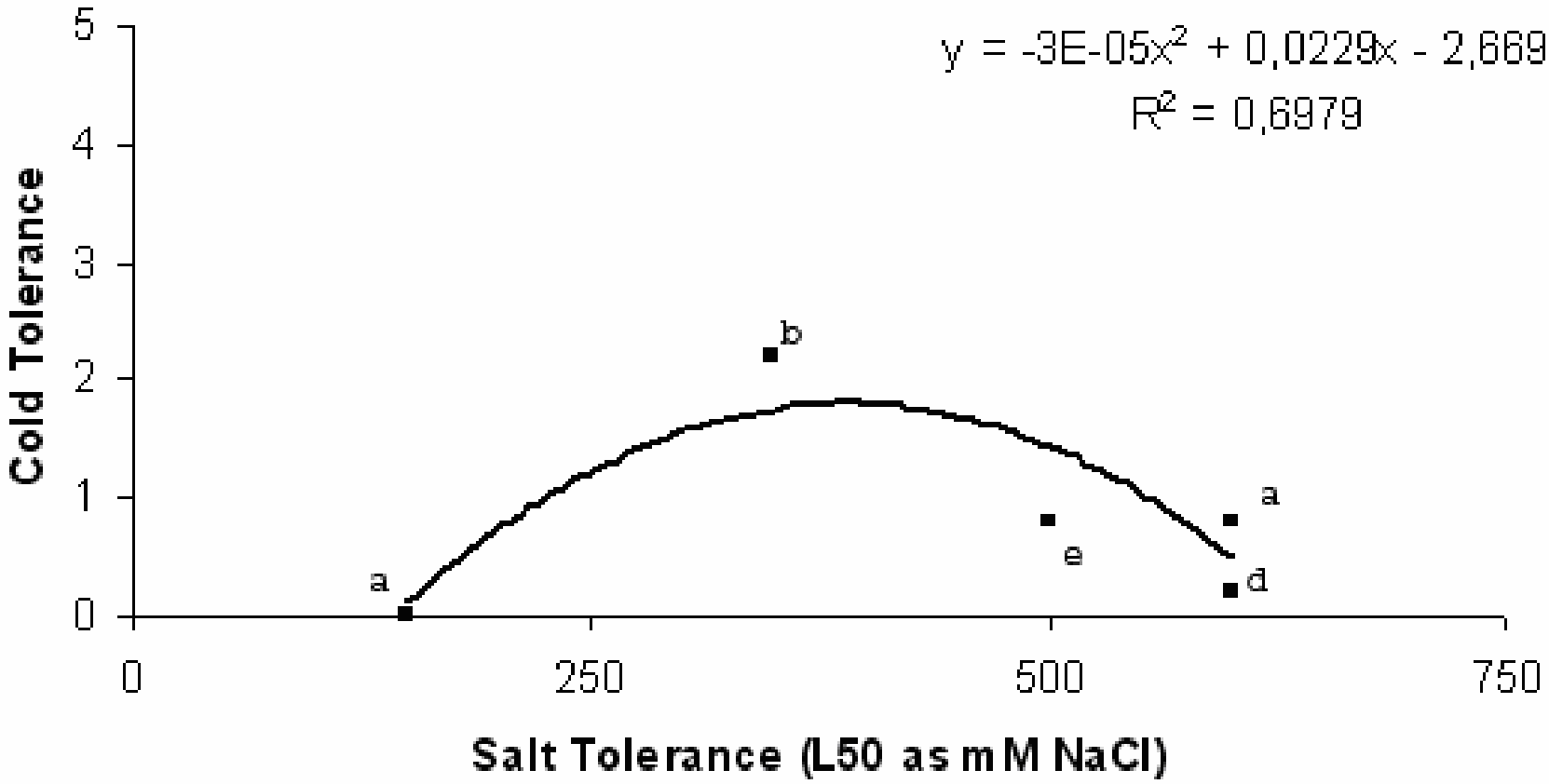

Fig. 14. Cold tolerance (score) and salt tolerance (expressed as L50) of tested species. In the figure: **a) *T. parvula*; b) *D. pinnata*; c) *A.thaliana*; d) *L. virginicum*; e) *L. densiflorum*; f) *T. halophila.***

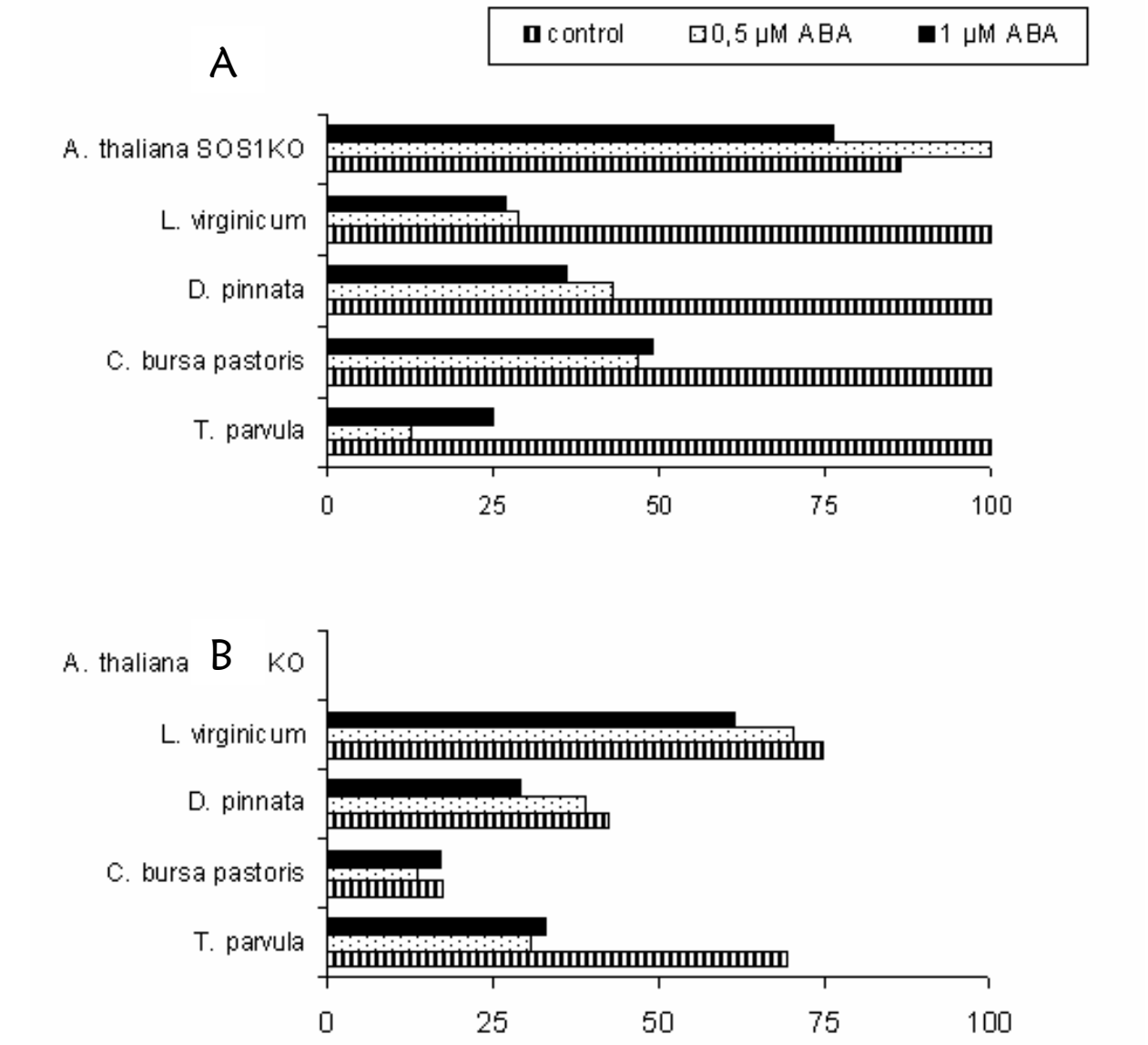

Fig. 15. Influence of ABA treatment and salt stress on Relative Root elongation. After germination seedlings were moved to ABA-treated plates (A) and ABA-treated plates containing MS+150 mM NaCl (B). Measures of root length were performed after 15 days. Values are shown as percentage of maximum length.

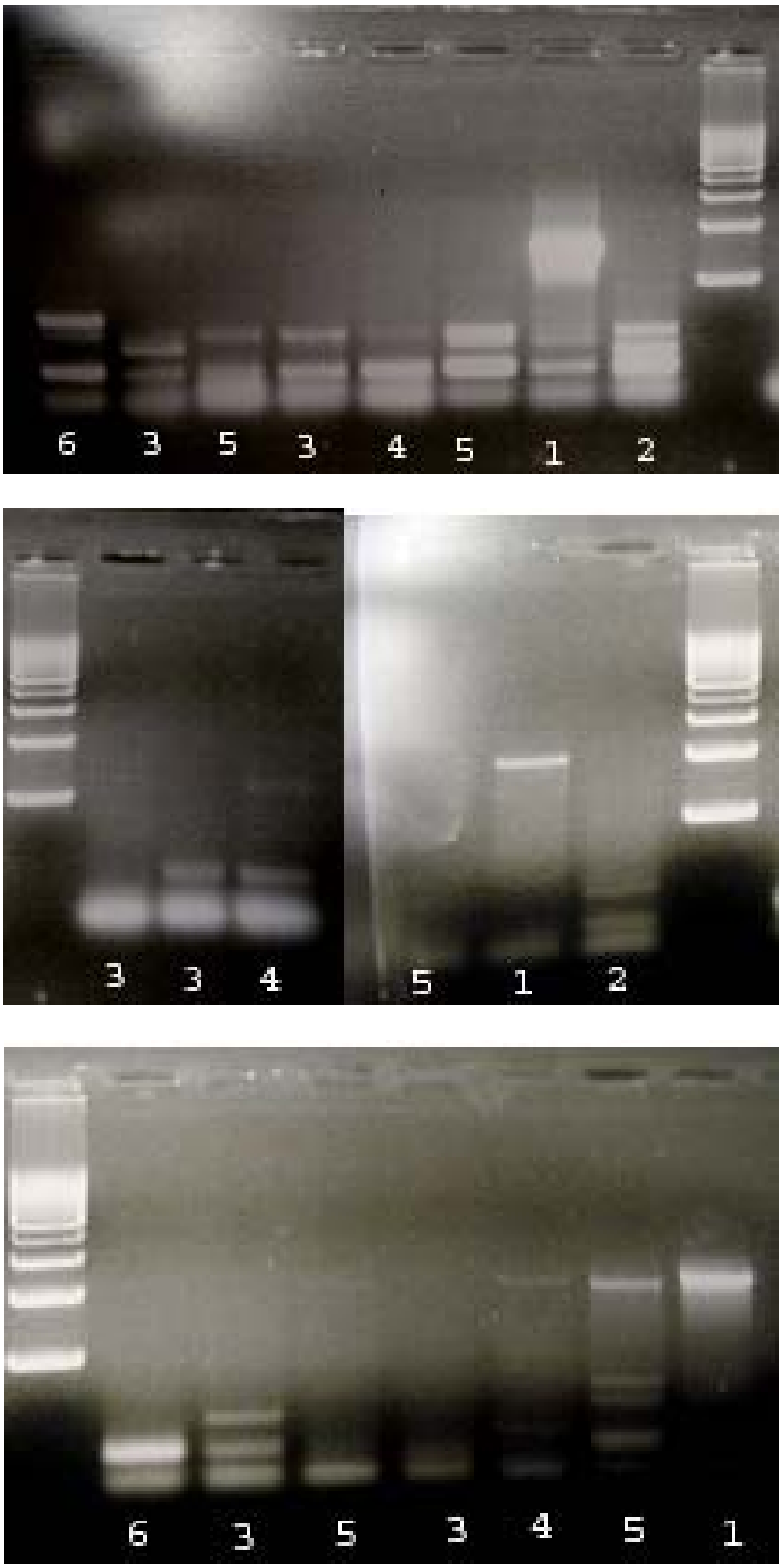

Fig. 16. Preliminary identification of NHX (A), HKT1 (B) and SOS1 (C) homologues in 1) A. thaliana, 2) T. halophila, 3) D. pinnata, 4) T. parvula, 5) C. bursa pastoris; 6) S. officinale. Agar gel showing DNA amplified fragments that will be used as probes in Northern blots analyses for expression studies and/or screening of cDNA libraries to isolate full length genes. Further studies will consent to define a specific relationship between gene sequences and/or expression patterns and tolerance phenotypes.

CHAPTER II.

FROM CROP SPECIES TO MODEL PLANTS: SALT STRESS EXPERIMENTS WITH SWEET BASIL (OCIMUM BASILICUM L.).

2.1. INTRODUCTION

Establishing a link between basic mechanisms of salt tolerance and functional traits that may actually improve crop production in saline environments is a major task. High salt stress disrupts homeostasis in water potential and ion distribution both at a cellular and at whole plant level. Furthermore, prolonged salt stress may lead to molecular damage, growth arrest and even death. Salt tolerance is achieved in many plants through three interconnected mechanisms (Zhu, 2001). First, damage may be prevented or alleviated (*detoxification*). Second, homeostatic conditions should be re-established in the new stressful environment (*homeostasis*). Third, growth must resume, although at a reduced rate (*growth regulation*). Extreme salt stress is responsible for damages of cellular structures, as well as the inhibition of enzymatic activities, nutrient uptake and photosynthetic functions. Most of these events are generally associated to the generation of reactive oxygen species (ROS) which can signal and/or exacerbate the occurrence of a stressful event. To recover from the effects of toxic molecules and ions, plants respond with the synthesis of stress proteins and compatible osmolytes, which are likely to be involved in plant *detoxification* (Zhu *et al.*, 1997). Most of the improvements in plant salt tolerance via trans-gene technology has been achieved by enhancing this detoxification strategy (Rus *et al.*, 2004). In addition to their

involvement in osmotic adjustment, several osmolytes including mannitol, fructans, trehalose, ononitol, proline, glycinebethaine and ectoine have been reported to be active in scavenging ROS (Shen *et al.*, 1997). This is consistent with their relatively low concentration in plant tissues that has been found in response to various stresses, which could not explain exclusively an osmotic function. Furthermore, osmolytes-producing transgenic plants often possess improved performance not only in saline environment, but also in various other stresses including chilling, freezing, heat and drought, all generating ROS (Kalir and Poljakoff-Mayber, 1981) but not always presenting osmotic disturbances.

Another strategy for achieving greater salt-stress tolerance is to help plants re-establish both ionic and osmotic *homeostasis* in stressful environments. The terminal determinants of such mechanisms are various ion transporters that re-allocate/distribute toxic ions in plants at both the cellular and organ level (Zhu, 2001). The accumulation of high Na^+ levels in the cytoplasm inhibits many enzymes and therefore its entry into the cell should be avoided or at least reduced. An important objective of salt tolerance studies is to determine which transporters are involved in Na^+ entry into the cells. This would be the way to block its influx and therein increase salt tolerance. A major gene involved in Na^+ transport in plants is SOS1, which has been shown to encode a putative plasma membrane Na^+-H^+ antiporter (Shi *et al.*, 2000). Mutations of SOS1 render *Arabidopsis* plants extremely sensitive to Na^+ stress. Overexpression of SOS1 reduces shoot Na^+ concentration and improved salt tolerance in *Arabidopsis* (Zhu, 2001).

Finally, salt stress, like many other abiotic stresses inhibits *plant growth*. Slower growth may be an adaptive feature for plant survival under stress because it could allow plants to save resources, restore damaged structures and restart physiological functions. Some plants are excessively responsive to a mild stress and they almost cease growing. On the other hand, plants that do not have a sufficiently prompt response to the prevailing stress conditions may not be able to survive (Wilkinson and Davies, 2002; Farnsworth, 2004; Yang *et al.*, 2005). In natural ecosystems, the extent of salt or drought tolerance often appears to be inversely related to the growth rate

(Munns, 2002). Among the causes for growth-rate reduction under salt stress there is an inadequate photosynthesis due to stomata closure and consequently limited carbon dioxide uptake. Nevertheless it has been demonstrated that salt stress and adaptation mechanisms may directly involve cell division and expansion (Maggio *et al.*, 2002; Zhu, 2001).

Studies on the effects of salt stress on crop plants have traditionally been based on an assessment of plant response to increasing salinity levels (Maggio *et al.*, 2004). Although this approach may provide practical indications for growing crop plants in saline environments, it does not allow to elucidate the basic physiology underlying salt tolerance. For this purpose, model plants such as *Arabidopsis* have been successfully introduced in recent years to understand the fundamental mechanisms that confer salt tolerance (Zhu, 2000). To date, many fundamental components of stress tolerance have been revealed, however it sill remains to translate the acquired knowledge in practical implication/applications for agricultural plants, a process that turned out to be more complex than expect. In this respect, the individuation of agricultural model plants is advisable and sought after the so-called post-*Arabidopsis* revolution (Zhu, 2001), which will allow overtaking the limits of studying only traits possessed by *Arabidopsis* or its close wild relatives. Searches for such potential models have already begun. Several species have served as genetic models such as tomato (*Lycopersicon esculentum*) (Giovannoni, 2004), snapdragon (*Antirrhinum majus* L.) (Saedler, 1994), and maize (*Zea mays* L.) (Sachs, 2005). The Gramene (www.gramene.org) database studies comparatively several species within the grass family, such as rice (*Oryza sativa* L.), maize, sorghum (*Sorghum bicolour* L.), barley (*Hordeum vulgare* L.), wheat (*Triticum aestivum* L.), and oat (*Avena sativa* L.) (Ware *et al.*, 2002). In these cases there are certainly many traits where mutants are already available (Bressan, 2001). One important issue that come up by using model plants is that most tolerant traits found in Arabidopsis do not necessarily have their counterpart in crop species. In contrast, less amenable model plants that may have an agricultural value may present some interesting stress tolerance traits that may deserve further attention. In the following section, we will provide an example in which we identified stress

tolerance determinants in basil that may have a counterparts in Arabidopsis. In this respect, we followed a sort of *reverse* thinking process from *crop plant* to *model species*. The potential implications of this approach in stress tolerance research is discussed. Two cultivars of sweet basil (*Ocimum basilicum* L.), characterised by a different response to salt stress, were compared for their main agronomic and physiological features. Major results were also referred to other plants belonging to the *Lamiaceae* (or *Labiatae*) family, such as mint (*Mentha spp.* L.), rosemary (*Rosmarinus officinalis* L.), sage (*Salvia officinalis* L.), savory (*Satureja spp.* Tourn. ex Mill.), marjoram (*Origanum majorana* L.), oregano (*Origanum vulgare* L.), thyme (*Thymus spp.* L.), lavender (*Lavandula spp.* L.), and perilla (*Perilla frutescens* Shiso), for which salt-stress resistance mechanisms have been documented, yet poorly understood.

2.2. MATERIAL AND METHODS

Two experiments were carried out at the University of Naples Federico II (40°49' N, 14° 15' E, 30 m.a.s.l.) in a cold glasshouse, in the summer 2007. Two cultivar of sweet basil (*Ocimum basilicum*), Napoletano and Genovese, were sown in 4 m^2 polystyrene containers filled with sterilised soil and grown in floating systems. Each growing unit was filled with 1.000 L of aerated nutrient solution, replaced every week. Oxygenation of the solution was maintained with electric pumps. Planting density was 100 plants m^{-2}, as commercially adopted for hydroponic production of basil. The first cycle was conducted in June (seed-to-harvest time was 25 days), while the second in September (seed-to-harvest time 30 days).The composition of the standard nutrient solution adopted in all experiments was N-NO_3^- 14 mM; N-NH_4^+ 6 mM; Cl^- 3.0 mM; PO_4^- 3.5 mM; S 6.0 mM; Ca 5.0 mM; Mg 3.7 mM; K 10.5 mM; Na 2.2 mM; Bo: 0.02 mM; Fe 0.04 mM, (Pimpini *et al.*, 2001; Marschner, 1995). Plant stress was imposed as follows:

- Experiment A*:* starting from sowing, plants were exposed to 0, 50, 100 and 200 mM NaCl. The experimental design was a randomized block with four replications.
- Experiment B: starting from 10 DAS, plants were exposed to a salt stress of 0, 100, 200 and 300 mM NaCl. The experimental design was a randomized block with four replications.

Leaf area was measured with a scanner and the images were analysed using the ImageJ software (Abramoff *et al.*, 2004). Fresh and dry yield were measured at harvest and after drying at 60°C, respectively. Stomatal conductance (expressed in cm s^{-1}) was measured in two events (at 15 and 25 DAS) for each growing cycle on the abaxial surface of the youngest fully expanded leaves with a diffusion porometer (AP-4, Delta-T Devices, Cambridge). At least 20 measurements were done per each treatment. Stomatal size and density were assessed in leaves using a digital microscope. Leaf water potentials (Ψ_t) were determined using a dew-point psychrometer (WP4, Decagon Devices, Washington). Osmotic Potential (Ψ_π) was measured on frozen/thawed leaf samples and Pressure Potential (Ψ_p) was estimated as the difference between Ψ_t and Ψ_π, assuming a matric potential equal

to 0. Leaf osmotic adjustment (OA) was determined as the difference $\Psi_{\pi o} V_o - \Psi_\pi V$, where $\Psi_{\pi o} V_o$ is the product of (osmotic potential)x(osmotic volume) of unstressed plants and $\Psi_{\pi o} V_o$ is the product of (osmotic potential)x(osmotic volume) of leaves from plants under salt treatment. For each measurement, the osmotic volume was approximated by the corresponding RWC value calculated as: RWC = (leaf fresh weight - leaf dry weight)/(leaf saturated weight – leaf dry weight) (Morgan, 1984). ABA determinations were performed on crude extracts of the youngest fully expanded leaves using an immunoassays kit (Hormondetek-ISCI Research Institute for Industrial Crops, Bologna, Italy) (Quarrie *et al.*, 1988). Proline was determined on leaves of plants of 0, 20 and 40 mM NaCl treatments, according to Claussen *et al.* (2005). Nitrate contents were measured on leaf extracts by spectrophotometric (HACH DR/2000 spectrophotometer) determination after cadmium reduction. Two different cation assays were utilized to measure the antioxidant activity of hydrophilic (HAC, Hydrophilic Antioxidant Capacity) and lipophilic (LAC, Lipophilic Antioxidant Capacity) fractions on lyophilized leaf samples. The antioxidant activity was measured on the water-soluble fraction using the DMPD (N, N-dimethyl-pphenylenediamine) method and expressed in μmol of trolox equivalents per g of dry weight. The ABTS [2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid)] method was utilized to assess the antioxidant activity of water-insoluble fractions expressed as μmol of Ascorbic Acid (AA) equivalents per g of dry weight (De Pascale *et al.*, 2006). Ascorbic acid (AsA) and dehydroascorbate (DAsA) were determined by using an assay based on the reduction of Fe^{3+} to Fe^{2+} by AsA and spectrophotometer detection of Fe^{2+} reaction with 2,2'dipiridyl method (Kampfenkel *et al.*, 1995).

Chloride, phosphate and potassium concentrations were measured on dried and ground tissue samples from fully expanded, not-senesced leaves from the first truss (here defined as mature leaves) and the second truss (here defined as young leaves) of nine plants per each treatment (three plants per each replication. The determination was carried out by colorimeter titration after extraction with water (Walinga *et al.*, 1995).

Plant water use was measured by using an automated electronic balance system. Seeds were sown on plastic trays filled with commercial soil and, after complete expansion of the first non-cotyledonal leaves, plants were moved into plastic pots. At day 20 after sowing, each pot was covered with a plastic film with the sealed shoot protruding outside the film. This system was used to avoid water loss from the soil surface. Each plant was then placed on an electronic balance under a light intensity of 140 μmol m^2 s^{-1} at 25 °C, and the weight loss was automatically measured every hour for 24 h using a PC software (A&D WinCT Data Communication Software**)**. Water loss values were normalized for the plant leaf area.

Data were analyzed with ANOVA and means were compared by the LSD test.

2.3. RESULTS

2.3.1. Growth response

ANOVA results are reported in Table 1. Plant development was affected by the salt concentration of the nutrient solution (Fig. 1). The fresh weight of both cultivars decreased at increasing salinization, although it decreased less in Genovese (GEN) plants compared to Napoletano (NAP) at 100 mM NaCl treatment. Specifically, the fresh weight was reduced to 50% and 75% compared to control plants, in GEN and NAP respectively. Similarly, the leaf area decreased upon salinization whereas no differences were observed in absence of salt and at 50 mM treatments between the two cultivars. At 100 mM the development of NAP was significantly affected more than GEN plants.

Non-stressed GEN plants had a leaf number three times higher than NAP (22±2.4 vs 7±0.4, respectively). However, in response to salinization, the leaf number decreased in GEN and at 200mM the leaf number was halved compared to 0 NaCl level. The leaf number in NAP was not affected by salt. As shown in Fig. 1, the specific leaf area (SLA) of NAP was twice that measured in GEN without stress, yet at 100 mM the SLA was similar for the two cultivars. The dry matter of both cultivars increased upon salinization. In unstressed NAP plants, the stomatal conductance was higher than GEN (Fig. 2). However these results were reversed in stressed plants where the stomatal conductance was higher in GEN compared to NAP plants. These differences were amplified on relative terms (data not shown). At 50 mM, the stomatal conductance was reduced more in NAP relatively to GEN, where no reduction from non-stressed conditions were observed. This response was observed in GEN also at 100 mM, although differences between the two cultivars were cancelled at higher salinization. In unstressed NAP plants, the water loss was 30% higher respect to GEN and such differences were confirmed also for diurnal/nocturnal water loss measurements. In this respect, NAP transpiration was always higher than GEN even though differences were reduced upon salinization (Fig. 3).

In absence of stress, the stomatal density of GEN was 40% lower than NAP. Interestingly, upon salinization, the stomatal density was significantly reduced in NAP, while no variations were observed in GEN. The size of stomata was higher in GEN than in NAP and no effects were attributable to salinization. The leaf chlorophyll content (Fig. 4) decreased under salt stress. However, the lower concentration was greater in NAP. Interestingly, the total chlorophyll content in GEN was reduced of 50% at 50 mM NaCl and it did not further decrease at higher salinity.

ABA contents (Fig. 5) varied respect to both cultivar and salinization. In absence of stress, the ABA level was very low (0.7 μg ABA g DW^{-1}) with no difference between cultivars. However salt stress moderately increased ABA in GEN whereas it greatly enhanced it in NAP.

2.3.2. Accumulation of compatible solutes and ROS scavengers

Salt stress increased the proline content of both cultivars. An increase in proline accumulation among cultivars was observed between 100 and 200 mM NaCl. At 300 mM NaCl, a further increase in proline content was observed only in NAP (Fig. 5). Both hydrophilic (HAC) and lipophilic (LAC) antioxidant capacity were significantly enhanced upon salt stress, with no differences observed among cultivars. Interestingly, upon extreme salinization (300 mM NaCl), LAC was higher in GEN compared to NAP. In unstressed plant, the polyphenol oxidase activity (PPO) showed similar value among cultivars. PPO activity in GEN was not reduced at increasing NaCl concentration. In contrast, the PPO activity in NAP decreased of approximately 6 fold already at 50 mM.

2.3.3. Water relations and ion accumulation

Leaf water and osmotic potentials were reduced upon salinization. Furthermore, the pressure potential reached its highest value in GEN at 100 mM NaCl (Tab. 2). Consistently, the leaf osmotic adjustment (LOA) increased upon salinization . Highest LOA values were achieved in GEN at 100

mM NaCl. The presence of NaCl in the rooting medium induced an increase in Cl^- concentration in leaves in both cultivars. The K^+ concentration of the NAP leaves gradually decreased in response to salt treatment, whereas the K^+ content in GEN was not affected upon salinization, suggesting that the K^+ uptake was not compromised by increasing the Na^+ concentration in the nutrient solution.

The nitrate content in leaves of both cultivars decreased at increasing salinity. In absence of salt, the nitrate content was higher in NAP while differences disappeared upon salinization. Both cultivars experienced a decrease of phosphate content upon salinization (Fig. 6).

2.4. DISCUSSION

2.4.1. A NaCl concentration threshold of 100 mmol differentiates Genovese from Napoletano performances

The response of Genovese at 100 mM NaCl revealed a higher tolerance of these plants to salinity compared to Napoletano. Nevertheless, a higher salt treatment had a similar detrimental effect on both cultivars (Fig. 1 and Tab. 1). Below 100 mmol NaCl, we observed in Napoletano plants a great biosynthesis of ABA and proline, a sudden stomatal closure and reduction of leaf transpiration. These responses were coupled to a significant decrease of stomatal density, leaf area, plant biomass and specific leaf area, respect to control conditions. Moreover, up to this salt concentration (100 mmol NaCl), we observed significant changes in nitrate, phosphate and potassium contents, as well as the decrease of both polyphenol-oxidase activity and chlorophyll content in leaves. Such harsh responses were only partially observed in Genovese, which apparently had a better control of the stress experienced. Interestingly, a great increase in pressure potential, at this salt concentration, was observed in Genovese, but not in Napoletano, indicating that the former was able to better adjust to the hyperosmotic environment.

2.4.2. Physiology of salt stress response in basil: control of water and ion homeostasis

Our results indicate that the two cultivars studied may use different strategies to adapt to high salinity. Napoletano dramatically reduced its growth rate by activating a prompt stomatal closure in response to stress. This is typical of a *short-term* stress response strategy which, over the whole cycle, will result in poor agronomic performances (low yield). In contrast, Genovese was able to adapt more efficiently to a stressful environment with a resulting reduced growth to an agronomically acceptable limit.

A different control of ion uptake and translocation was observed between the two ecotypes. Potassium content did not vary upon salinization in Genovese leaves. Indeed, a great reduction of such ion was observed in Napoletano leaves already at 100 mM NaCl. In comparative studies among the glycophyte *A.thaliana* and its salt-resistant relative *T.halophila*, Volkov *et al.* (2003) suggested the presence of specific ion-channel features supporting K^+/Na^+ homeostasis under salinity stress in the halophyte (Volkov and Amtmann, 2006). Genovese seems to possess selective mechanisms that are able to cope with the Na/K competition however such mechanisms are often linked to a less efficient uptake and/or allocation of other important nutrients. Consistently, in Genovese plant tissues we found lower nitrate and phosphate contents in absence of salt compared to Napoletano, even though their reduction upon salinization was always lower than in Napoletano.

Under control conditions, stomatal conductance and leaf transpiration were higher in Napoletano plants. However, following salinization, the stomatal conductance was drastically reduced in Napoletano, whereas little variation was observed in Genovese. A reduced transpiration limits salt loading to the shoots and may be useful in short-term stress adaptation. In the longer term, however, this response will limit nutrient supply and growth of the shoots and is therefore required to restore normal plant functions. The role of ABA in mediating stomatal closure under salt stress and drought is well known (Zhu *et al.*, 1997). However, it appears that in some halophytes (such as *T. halophila*), ABA signalling under the same conditions is either absent or modified (Volkov *et al.*, 2003). Therefore, it becomes of great interest to understand whether ABA functions may be different even between two cultivar within the same species with so diverse salt stress response.

Considering the high concentration of Na and Cl ions found in leaves that are still functioning normally, these ions have to necessarily be sequestered in the cellular vacuole, upon salinization, to guarantee normal metabolic functions (Munns, 2002). Although obtaining direct experimental evidence for compartmentalization is technically difficult, in tobacco cells growing in

salt medium was confirmed the higher vacuolar concentration of Na and Cl despite of the cytoplasmatic concentration of both ions (Munns, 2002).

It is widely accepted that during osmotic adjustment the cells tend to compartmentalize most of the absorbed ions in the vacuoles at the same time that they synthesize and accumulate compatible organic solutes in the cytoplasm in order to maintain the osmotic equilibrium between these two compartments (Serrano and Gaxiola, 1994; Hare *et al.*, 1998; Hasegawa *et al.*, 2000). The osmotic regulation contributes to maintain water uptake and cellular turgor, which are essential to sustain physiological processes such as cell expansion, stomatal opening, photosynthesis, and many others plant functions (Zhang *et al.*, 1999). Both salt stress and endogenous signals control the development, density and opening of stomata: these factors influence both water loss by transpiration and net photosynthesis (Chaerle *et al.*, 2005). The main response to water stress is the reduction of transpiration through stomatal closure, even though it could also be optimized through the control of stomatal size and density (Woodward *et al.*, 2002). Actually, it was shown that a lower stomatal index enhances drought (Aharoni *et al.*, 2004) and salt tolerances (Bray and Reid, 2002). Consistently with the published literature, the lower stomatal density of GEN compared to NAP at 100 mM, could allow the plant to optimize water loss vs. photosynthesis and consequently to cope with salt stress more efficiently than NAP.

We found also other differences between the two ecotypes that could be associated to the different level of stress tolerance observed. The total chlorophyll content was reduced upon salinization, but this decline was less evident in GEN compared to NAP. According to Aslam *et al.* (1984), chlorophyll content decreases upon salinization, and therefore we could suggest that GEN would be facing lower stressful conditions compared to NAP.

2.4.5. ABA: one signal, many stimuli

The ABA content in NAP dramatically increased from 0 to 100 mM NaCl, whereas no increases were observed in GEN. The role of ABA on many feature of salt stress response has been

widely explored in literature (Chinnusamy *et al.*, 2004; LeNoble *et al.*, 2004; Desikan *et al.*, 2004; Ruggiero *et al.*, 2004). Studies on ABA-mutants have mainly considered *Arabidopsis* accessions (Savouré *et al.*, 1997; Xiong *et al.*, 2002), even though there are few cases of studies on horticultural crops such as, for instance, tomato (Burbidge *et al.*, 1999; Mäkelä *et al.*, 2003; Mulholland *et al.*, 2003). ABA controls many stress adaptation responses, including stomatal closure (Hetherington, 2001; Neill *et al.*, 2003), activation of genes involved in osmotic adjustment (Savouré *et al.*, 1997), ion compartmentalization (Verslues and Zhu, 2005), regulation of shoot versus root growth and modifications of root hydraulic conductivity properties (Ruggiero *et al.*, 2004;). The ABA production in Genovese did not vary at increasing salt stress conditions (Fig. 7). Consistently, a large stomatal closure was experienced by Napoletano plants undergoing salt stress, whereas little conductance reduction was observed in Genovese.

Furthermore, we observed a decrease in plant leaf area in both cultivars upon salinization. Such reduction was either attributable to a reduced number of leaves (Genovese) or to their specific area (Napoletano). Since ABA may inhibit cell-division (Chaerle *et al.*, 2005), we may suggest that the reduced expansion of leaves in Napoletano is a response to an increased ABA level. Apparently, a different (and more effective) strategy was put in order by Genovese.

2.4.6. Proline accumulation: *activator* or *indicator* of stress responses?

Many plants accumulate high levels of proline in response to osmotic stress, and proline is thought to play an adaptive role during osmotic stress (Delauney and Verma, 1993). Proline content increased upon salinization, and its accumulation was higher in Napoletano. However, up to 100 mM NaCl treatment, proline accumulation in plant tissue did not vary among the two cultivars, even though ABA biosynthesis was much higher in Napoletano than in Genovese. Savouré *et al.* (1997) reported on the presence of two alternative path for proline biosynthesis as a consequence of plant stress, respectively ABA-dependent and ABA-independent. Even though an ABA transduction cascade may possibly be involved in the expression of the proline biosynthesis genes

(Yoshiba *et al.* 1995), several *Arabidopsis* genes induced by osmotic stress (Gosti *et al.* 1995) or cold treatments (Gilmour and Thomashow 1991; Nordin *et al.* 1991) are not regulated by endogenous ABA. The characterisation of ABA biosynthetic mutants revealed that the induction of a particular gene by exogenously applied ABA does not necessarily imply that the regulation of gene expression is ABA-dependent upon stress (Giraudat *et al.* 1994). Therefore, ABA-independent and ABA-dependent pathways interact to regulate the expression of certain genes in response to osmotic stress. Interestingly, proline accumulates in a tomato *flacca* mutant that does not contain elevated levels of ABA even after osmotic stress (Stewart and Voetberg 1987), and an addition of the ABA biosynthesis inhibitor, fluridone, to wilted barley leaves did not influence proline accumulation (Stewart and Voetberg 1987). Possibly the accumulation of proline in Napoletano was caused by an activation of an ABA-independent pathway or alternatively by inhibition of its catabolism (Maggio et al., 1997). More interestingly, such accumulation confirmed the role of proline as a stress indicator, since an increased level of this metabolite was associated to a higher stress perceived by Napoletano compared to Genovese plants.

2.4.7. Genetic control of water relations: from Basil to Arabidopsis

In our experiments we observed two alternative pathways of salt stress response in the two cultivars, which are based on different mechanisms that may control cellular hydration in stressful environments. Simplistically, these two mechanisms can be conducted to 1) a short-term stress tolerance response (i.e. rapid stomatal closure) which would be functional to a transitory stress situation and 2) a long-term stress adaptation (reduced leaf stomatal density) which would be consistent with an increased ability to compromise between water loss and CO_2 uptake in non-transitory stress situation. The latter is, in other words, an improved water use efficiency (WUE), a functional trait that is very important in water-limiting environments. The genetic basis of WUE has been obscure for many years and it certainly is a very complex to study (Martin *et al.*, 1989; Thumma *et al.*, 2001). Nevertheless, Masle *et al.* (2005) have recently demonstrated that the

ERECTA gene is responsible of plant transpiration efficiency in *Arabidopsis*. The function of ERECTA is rather complicated and is involved in the regulation of stomatal density, epidermal cell expansion, mesophyll cell proliferation and cell–cell contact (Masle *et al.*, 2005). It was demonstrated that the WUE efficiency could be enhanced by reducing the stomatal density in Arabidopsis plants. These results, in some respect, are consistent with the pronounced stress tolerance of Genovese plants, which was also associated to a reduced stomatal density.

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TABLES

	Leaf Area	Yield	Dry Matter	Stomatal conductance	Stomatal size	Stomatal density
	$cm^2\ plant^{-1}$	$g\ plant^{-1}$	%	$cm\ s^{-1}$	µm	$n\ mm^{-2}$
Cv						
GEN	89.5	6.44	10.22	0.69	18.9	70.2
NAP	79.0	5.49	9.55	0.58	14.4	178.6
Salt						
0	199.7	12.38	8.30	1.24	15.7	175.0
50	87.9	6.59	8.89	0.65	17.1	131.3
100	52.2	3.99	10.04	0.55	16.9	95.5
200	22.1	2.81	11.17	0.34	16.5	89.4
300	17.0	2.30	11.14	0.32	18.9	70.2
Significance						
Cv	ns	** (0.82)	* (0.53)	* (0.08)	** (1.18)	** (13.7)
Salt	** (19.8 [1])	** (1.17)	** (1.07)	** (0.16)	ns	** (19.4)
Cv x Salt	* (19.7)	** (1.65)	ns	** (0.22)	ns	** (27.4)

Tab. 1. Results of the ANOVA. Effects of NaCl treatments on main morphological indicators (Leaf area, Yield, Dry matter, Stomatal Conductance, Stomatal size and Stomatal density). (Mean values; ns = not significant; * = significant at P≤0.05; ** = significant at P≤0.01) [[1] lsd].

	Ψ_t	Ψ_π	Ψ_p	RWC	OA
	(MPa)	(MPa)	(MPa)	(%)	
Cv					
GEN	-0.846	-1.33	0.48	81.3	0.62
NAP	-0.823	-1.33	0.51	76.8	0.71
Salt					
0	-0.34	-0.63	0.28	84.0	-
50	-0.74	-1.17	0.43	82.7	0.44
100	-0.91	-1.58	0.66	78.8	0.71
200	-1.34	-1.94	0.60	70.6	0.84
300	-1.85	-2.69	0.84	64.2	1.47
Significance					
Cv	ns	ns	ns	ns	ns
Salt	** (0.18 [1])	** (0.17)	** (0.20)	** (8.20)	** (0.19)
Cv x Salt	ns	* (0.17)	ns	* (7.96)	ns

Tab. 2. Influence of salt stress (0, 50, 100 and 200 mM NaCl) on plant water status in two cultivars of sweet basil (Genovese and Napoletano). Water potential (Ψ_t), osmotic potential (Ψ_π), pressure potential (Ψ_p), relative water content (RWC), and leaf osmotic adjustment (OA). (Mean values; ns = not significant; * = significant at P≤0.05; ** = significant at P≤0.01) [[1] lsd].

FIGURES

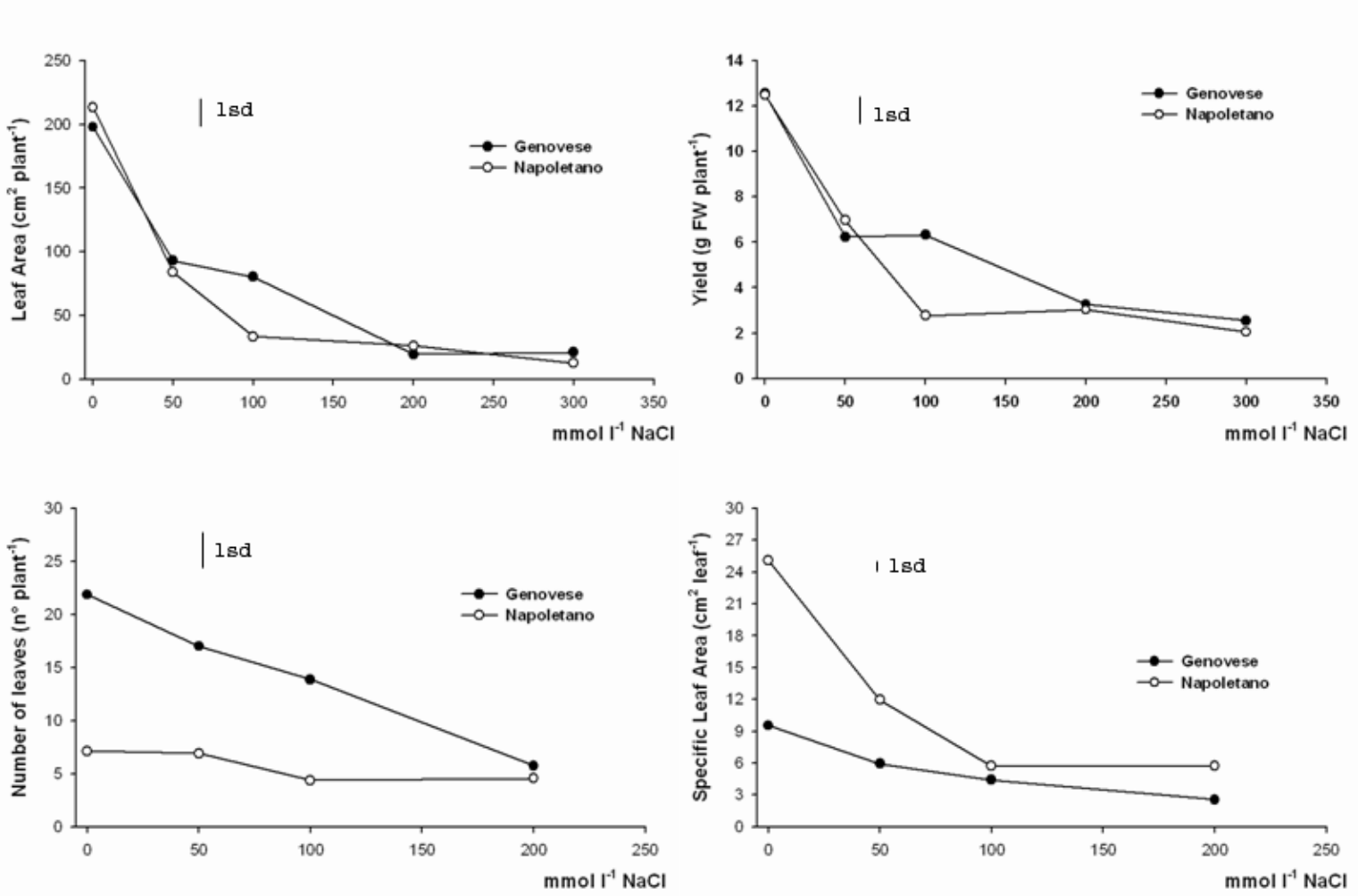

Fig. 1. Influence of salt stress (0, 50, 100 and 200 mM NaCl) on biometric indexes (leaf area, yield, number of leaves and specific leaf area) in two cultivars of sweet basil.

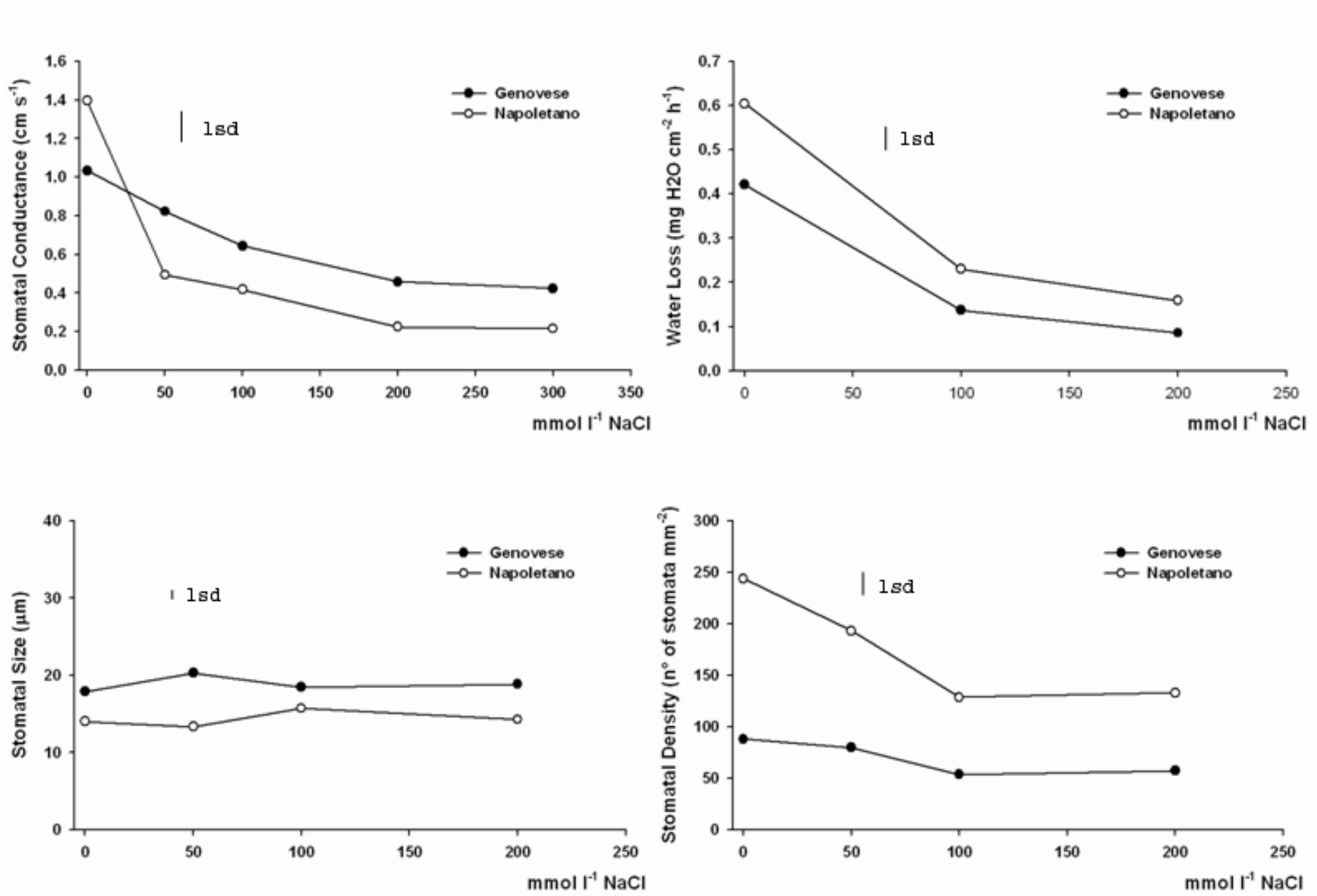

Fig. 2. Influence of salt stress (0, 50, 100 and 200 mM NaCl) on leaf transpiration (stomatal conductance, water loss, stomatal size and stomatal density) in two cultivars of sweet basil.

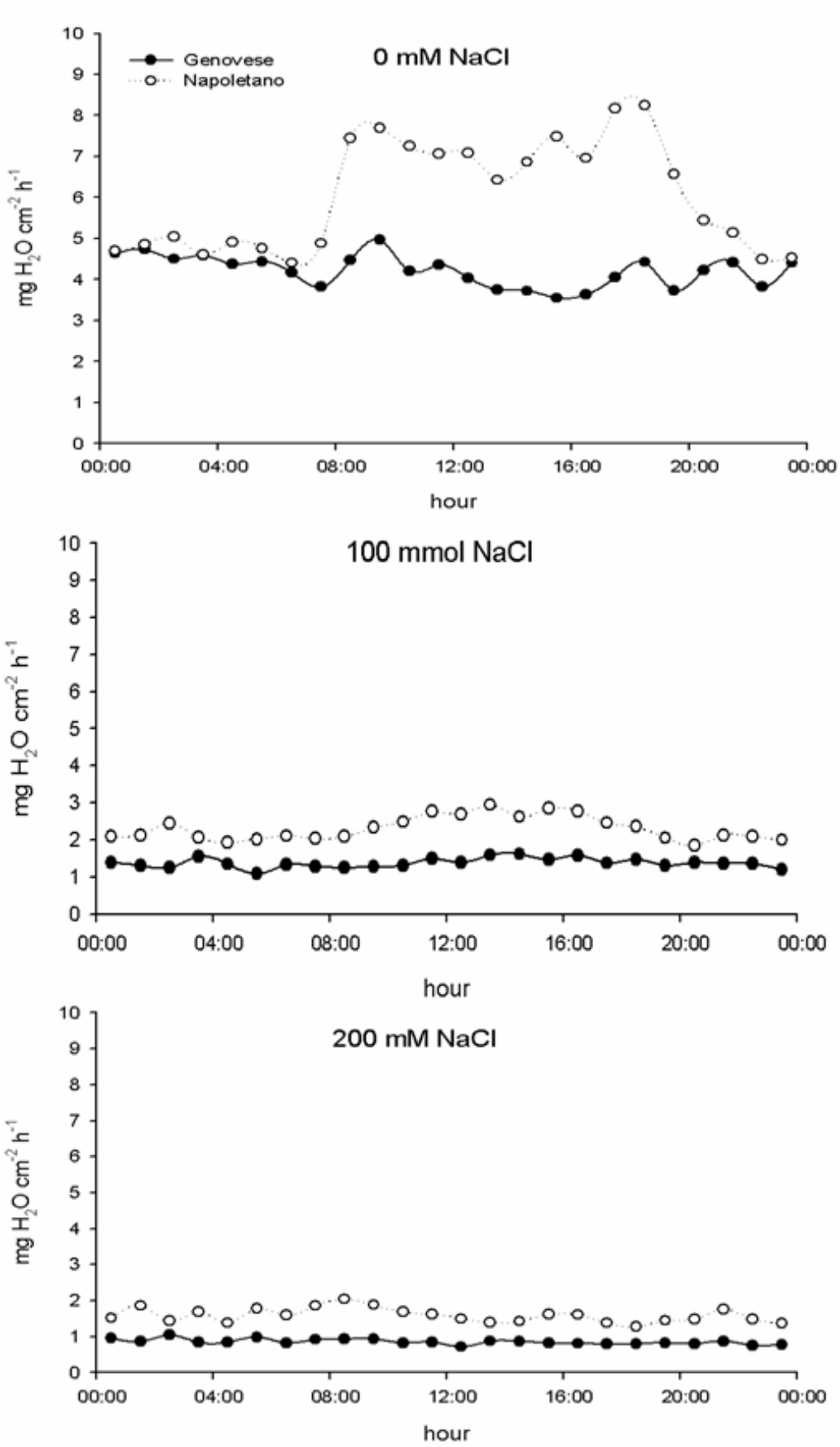

Fig. 3. Influence of salt stress (0, 100 and 200 mM NaCl) on leaf transpiration in two cultivars of sweet basil (Genovese, black, and Napoletano, white).

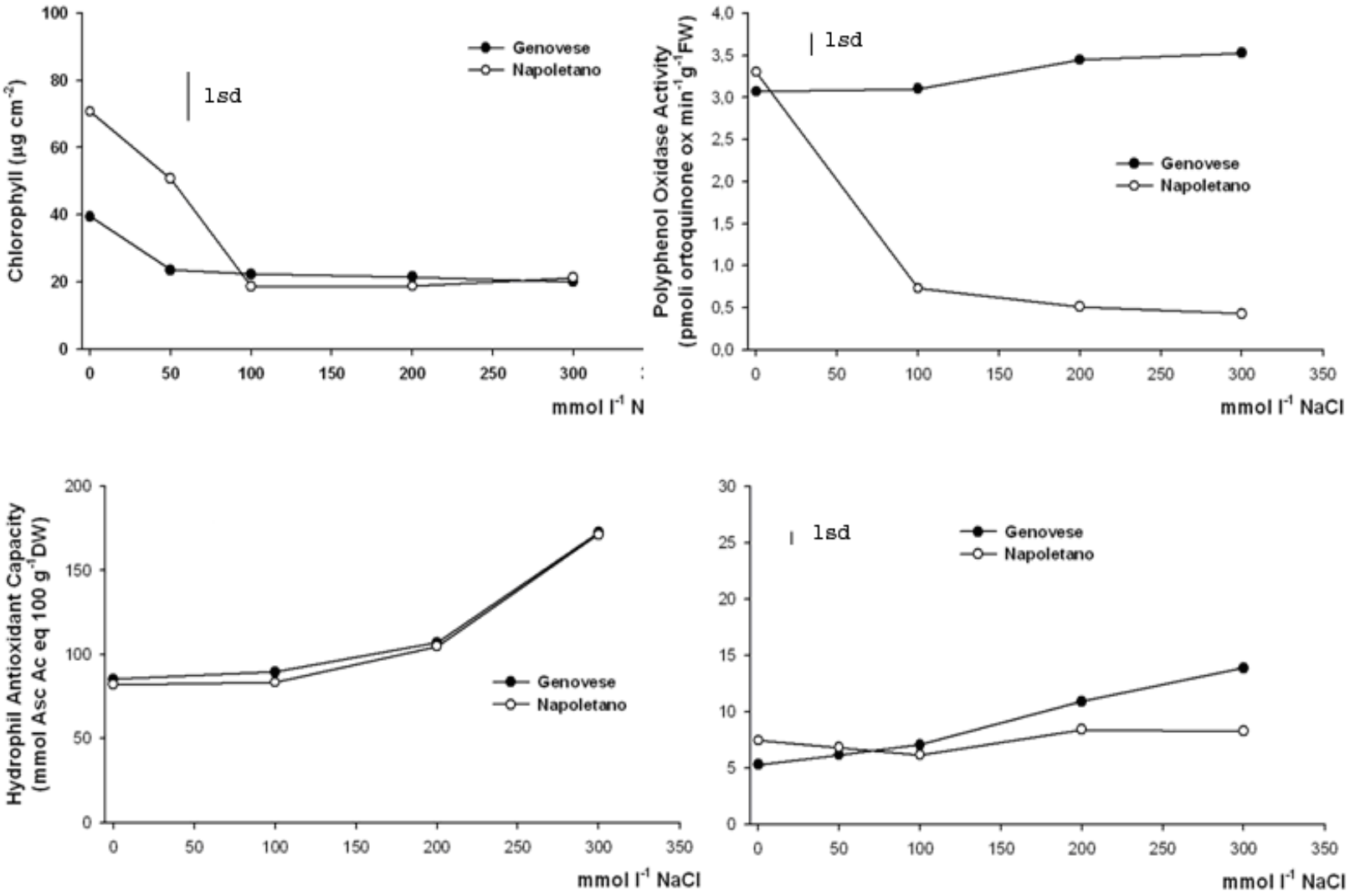

Fig. 4. Influence of salt stress (0, 50, 100 and 200 mM NaCl) on leaf composition (chlorophyll content, polyphenol-oxidase activity, hydrophilic antioxidant capacity and lipophilic antioxidant capacity) in two cultivars of sweet basil. Vertical bars mean least significant difference (lsd).

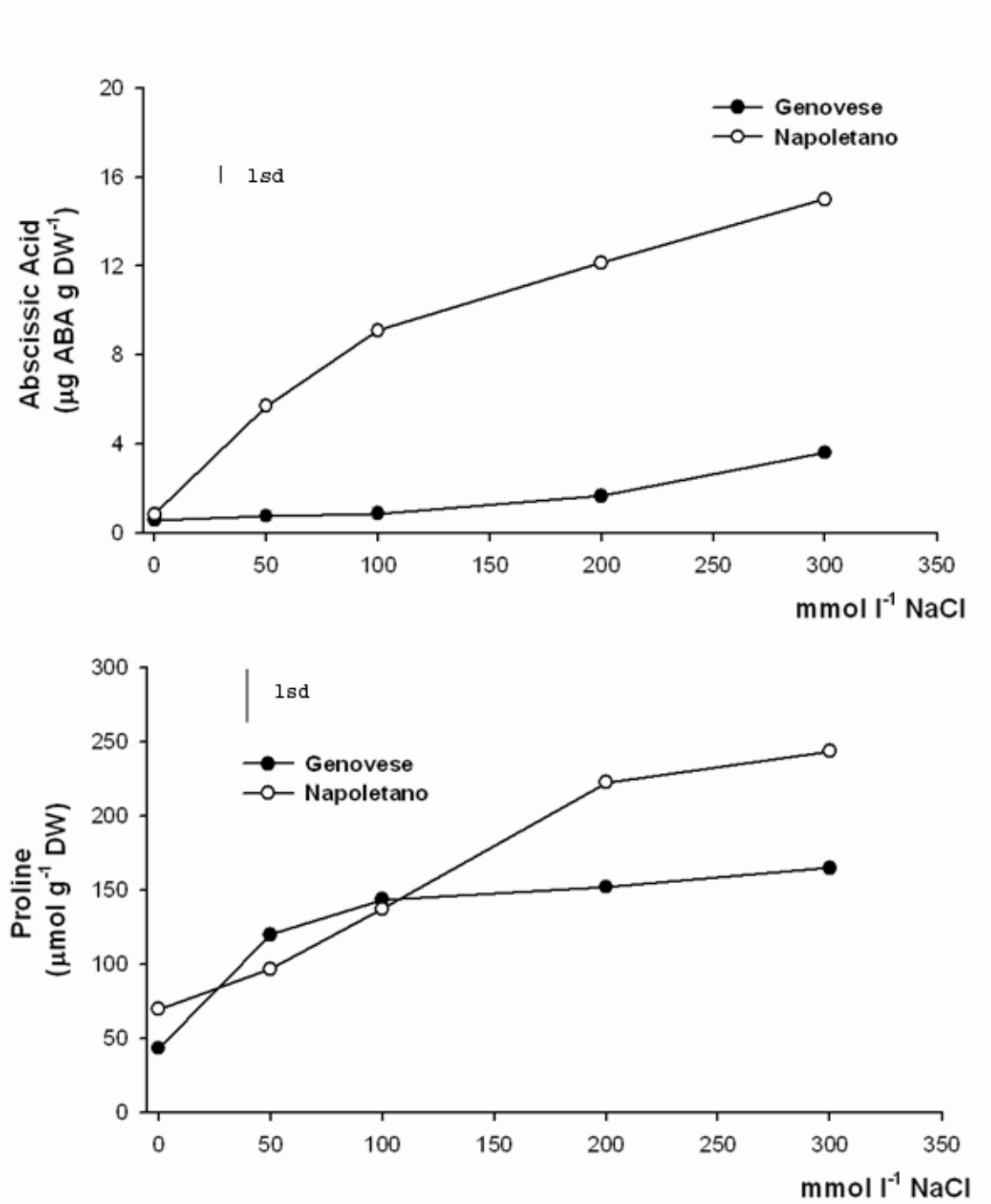

Fig. 5. Influence of salt stress (0, 50, 100 and 200 mM NaCl) on ABA and proline accumulation in leaves of two cultivars of sweet basil.

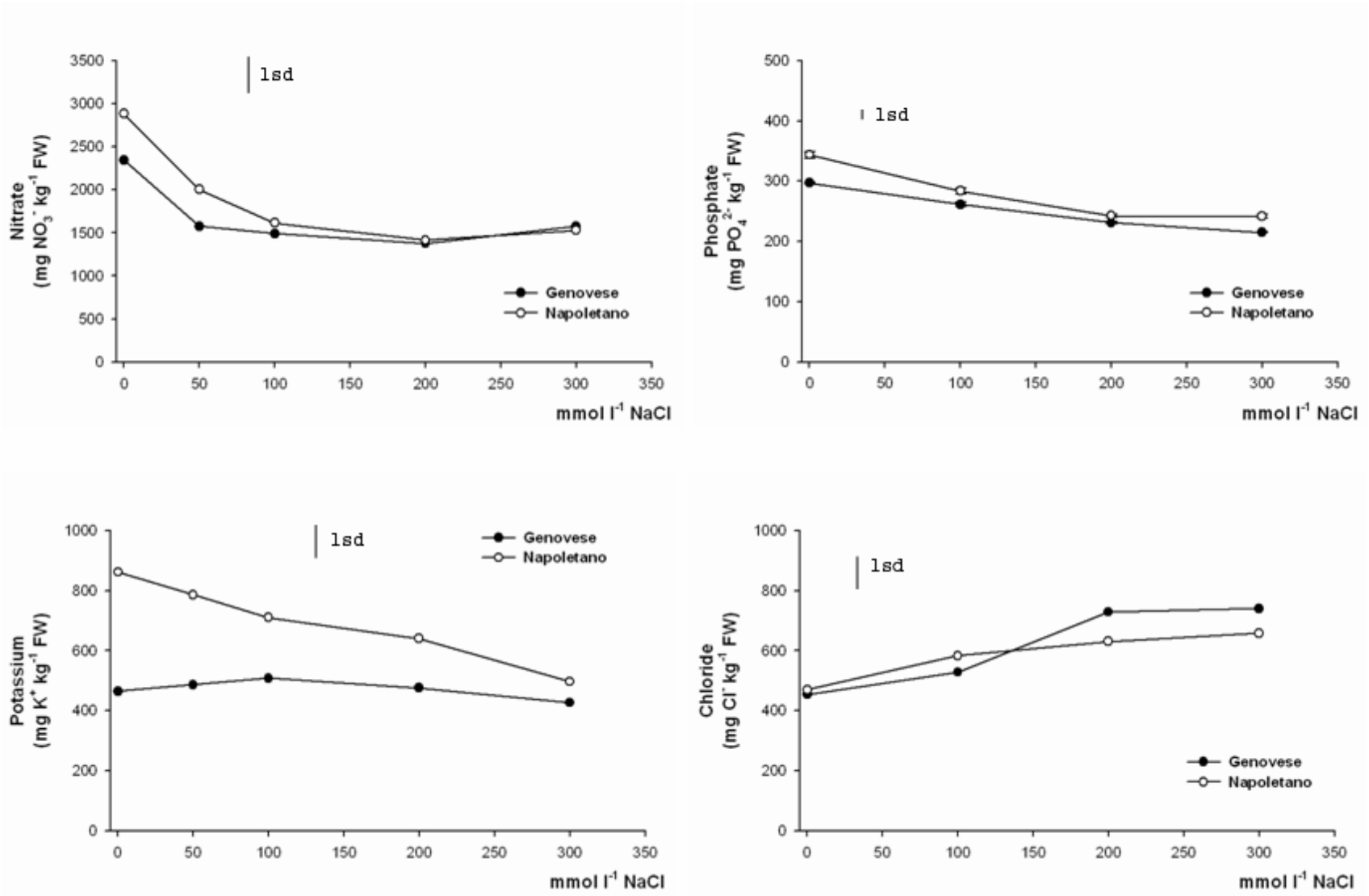

Fig. 6. Influence of salt stress (0, 50, 100 and 200 mM NaCl) on leaf composition (nitrate, phosphate, potassium and chloride contents) in two cultivars of sweet basil.

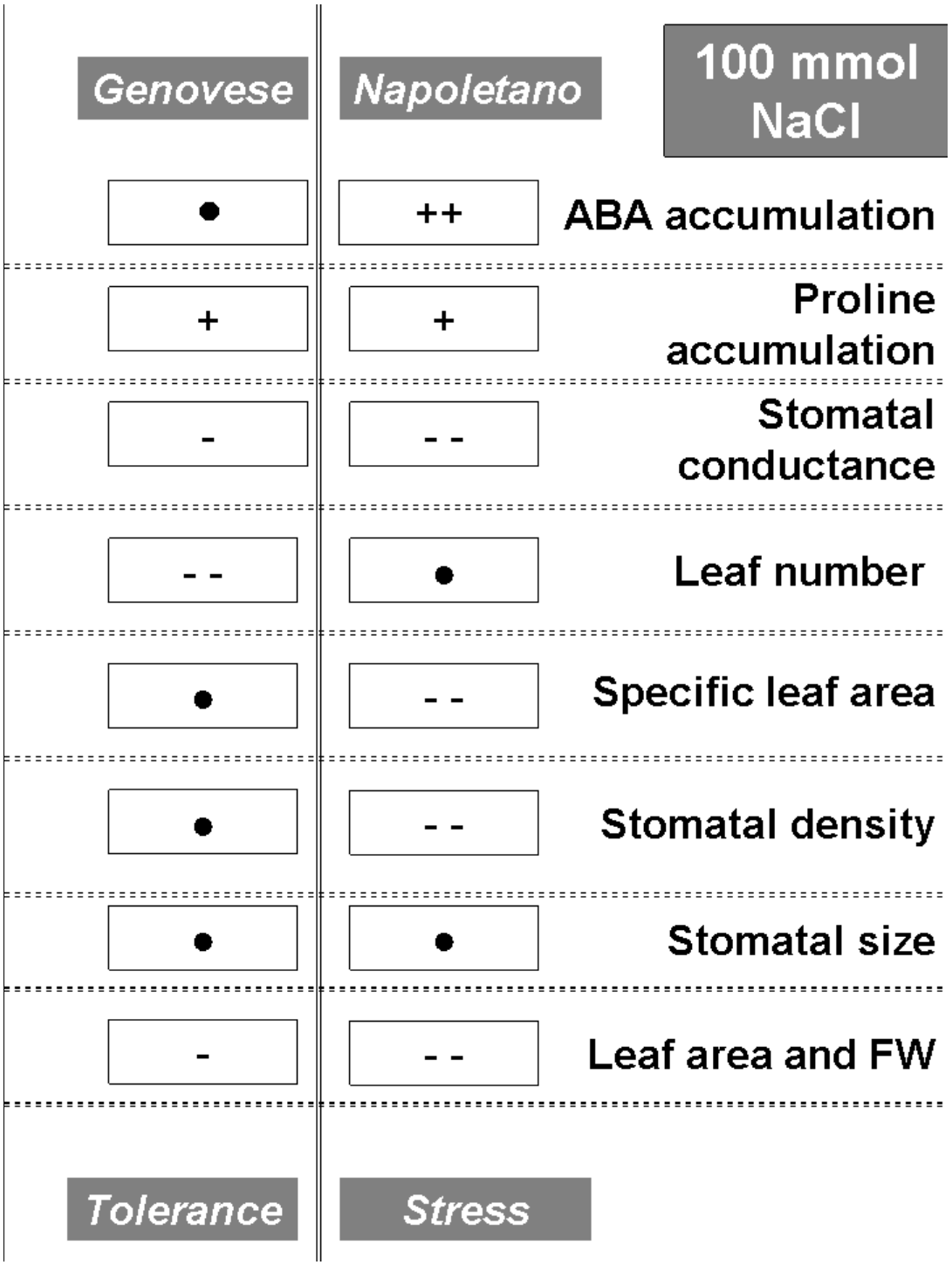

Fig. 7. Diagram summarizing the effects of 100 mmol NaCl salt stress on Genovese and Napoletano cultivars of sweet basil (*Ocimum basilicum* L.). Variation from control conditions are presented as follows: • = no differences; - - : high reduction; - reduction; + increase; + + high increase.

CHAPTER III.

ELEMENTS OF CROSS-TOLERANCE TO BIOTIC AND ABIOTIC STRESSES. RESPONSE TO SALINITY OF TOMATO (*LYCOPERSICON ESCULENTUM*) PLANTS OVER-EXPRESSING GENES ACTIVATED BY WOUNDING.

3.1. INTRODUCTION

In all multi-cellular organisms the interaction between cells is fundamental. For many years it has been believed that five hormones (auxin, cytokinin, ethylene, gibberellin and abscisic acid) were responsible for virtually all mechanisms related to intercellular signal transport in higher plants (Guern, 1987). Recently short peptides with hormone-like function have been proved to be involved in plant growth and development, response to biotic stresses, cellular division and auto-incompatibility of pollen (Lindsey *et al.*, 2002). Tomato plants and other *Solanaceae* respond to chewing insects and mechanical wounding by releasing a highly mobile peptide called systemin (Bergey *et al.*, 1996; Schilmiller and Howe, 2005). In tomato, this 18-amino acid (aa) molecule is synthesized as a 200-aa precursor protein named prosystemin (McGurl and Ryan, 1992). After proteolytic cleavage, the peptide binds a plasma membrane-bound receptor kinase (Scheer and Ryan, 2002). Subsequently, it is assumed that the signalling pathway proceeds via the activation of a phospholipase, with the release of linolenic acid and the formation of jasmonic acid (JA) and its derivative, methyl jasmonate (Bergey *et al.*, 1996). Eventually, the transcriptional activation of a

number of genes, not fully identified, leads to an increase of metabolites (i.e., proteinase inhibitors and polyphenoloxidase) that are directly toxic to phytophagous insects (Bergey *et al.*, 1996). Currently, evidence gained through mutational analysis supports the hypothesis that long-distance defence signalling is transmitted mainly by JA as a wave travelling via a positive amplification loop that involves systemin release (Schilmiller and Howe, 2005).

Jasmonic acid and its methyl-ester are both involved in plant growth regulation (Parthier, 1990) and their role in stress response has been documented in many studies (Creelman *et al.*, 1992; Farmer and Ryan, 1990; Sembdner and Parthier, 1993). Moreover, higher levels of these molecules have been found in plants undergoing to water-, osmotic-, and wound-stress (Reindbothe *et al.*, 1992; Creelman *et al.*, 1992). Interaction between JA and stress hormones has also been demonstrated. Staswick *et al.* (1992) reported that exogenous application of jasmonic acid to seeds of *Arabidopsis*, increased the inhibitory effect of the ABA on seedling germination. Dombrowski (2003) investigated the effects of salt stress on the wound response signal cascade in tomato plants. Salt stress alone was found to induce wound related genes and this gene activation was mediated via the octadecanoid pathway. The salt-dependent activation of the octadecanoid pathway was found to be independent of the wound pro-hormone prosystemin, yet prosystemin (PS) activity was necessary to achieve maximal accumulation of proteinase inhibitors. In addition, salt stress was found to strongly enhance plant's ability to cope with wounding.

Under conditions of water and salt stress, roots of many angiosperms synthesize ABA and transport it into the shoots (Jia *et al.*, 2002). ABA is therefore an essential mediator of plant adaptation to adverse environmental stimuli. This is known to occur in a number of crop plants including rice (Henson, 1984), barley (Stewart and Voetberg, 1985), soybean (Benson *et al.*, 1988), tomato (Bray, 1988), cotton (Hartung *et al.*, 1988), and alfalfa (Luo, 1992). Increased ABA levels limit water loss by reducing stomatal opening. Recent evidences suggest that under water stress the ABA hormonal signal may interact with an hydraulic signal to reduce leaf expansion rates during periods of high transpirational demand (Salah and Tardieu, 1997; Munns *et al.*, 2000). Maggio *et al.*

(2007) have found in salt-stressed tomato, a reduction of leaf area, an increase of root/shoot ratio, an increase of leaf turgor and ABA content, which were all correlated to stomatal closure. The ABA biosynthesis is also followed by the accumulation of osmolytes such as proline, which have the role of reducing the osmotic effect of salt stress. This mechanism is usually called "osmotic adjustment", and it consists of a decrease of the cellular osmotic potential which will facilitate water uptake in hyperosmotic environment (Maggio *et al.*, 2002). The magnitude of proline accumulation under abiotic stress conditions such as salinity stress and drought depends on the species and the extent of stress (Delauney and Verma, 1993; Bohnert and Jensen, 1996) and may vary from 10x to 100x in *Arabidopsis* and tomato, respectively (Liu and Zhu, 1997; Fujita *et al.*, 1998).

Based on a documented evidence of an hormone mediated cross-talk between biotic- and abiotic-stress adaptation mechanisms, we hypothesised that a constitutive overproduction of systemin would enhance tomato tolerance to salinity stress.

3.2. Material and Methods

Three experiments were carried out at the University of Naples Federico II (40°49' N, 14° 15' E, 30 m.a.s.l.) in a cold glasshouse, in 2006 and 2007.

Transgenic tomato plants over-expressing the prosystemin cDNA under the control of the 35S RNA CaMVpromoter (BBS) and their relative control (cv. Better Boy, BB) were obtained by the Dept. of *Sciences of the soil, plant, environment and animal production* of the University of Napoli and obtained as described by Mc Gurl *et al.* (1994). Plants were grown in sterilized soil and maintained in controlled environmental chambers for 3 weeks from sowing at 25±1°C with a photoperiod of 14/10 hr light/dark. Experiments *A* and *B* were carried out in summer 2006, experiment *C* in summer 2007.

Two hydroponic systems were used. For experiment *A*, plants were grown on grodan slabs, while for experiments *B* and *C*, plants were grown in plastic pots (∅ 20 cm) filled with perlite.

For all three experiments, we used a closed irrigation system, with a complete recycling of the nutrient solution. The re-circulating nutrient solution was automatically pumped in five daily irrigation events, of 3 minutes at 2 litres per minute. The composition of the standard nutrient solution used in all experiments was N = 18.25 mM (N-NO3- = 17.00 mM; N-NH4+ = 1.25 mM); P2O5 = 1.40 mM; K2O = 8.75 mM; S = 5.00 mM; Ca = 7.00 mM; Mg = 3.50 mM; Cl = 10.00 mM; Na = 10.00 mM; Fe = 15.00 μM; Cu = 0.75 μM; Zn = 7.00 μM; B = 50.00 μM; Mn = 10.00 μM; Mo = 5.00 μM. Plant stress was imposed as follows:

- Experiment *A*: starting from 40 Days After Sowing (DAS), plants were irrigated with a nutrient solution containing 0, 20 and 40 mM NaCl. The experimental design, which factorially combined 2 lines (BB and BBS) and three salinisation levels (0, 20 and 40 mM NaCl) was a randomized block with three replications.
- Experiment *B*: starting from 40 DAS, plants were irrigated with a nutrient solution containing 0, 20 and 40 mM NaCl. Moreover, two treatments with L-proline were applied,

respectively 0 (plain nutrient solution) and 5 mM, dissolved in the nutrient solution on 40 DAS. The experimental design was split-plot (replicated 4 times) with the salinization levels (0, 20 and 40 mM NaCl) assigned to the main plots, the +/- proline treatment (0 and 5 mM) assigned to the elementary plots and the "cultivar" (BB and BBS) assigned to the sub-plot.

- Experiment *C*: this was a stress-recovery experiment. Starting from 40 DAS, plants were salinised with a 40 mM NaCl nutrient solution for 15 days. Afterward, half of the plants were irrigated with regular nutrient solution (control), while the other half was irrigated with a 40 mM NaCl solution. The experimental design which included 2 lines (BB and BBS) and two salinisation (0 and 40 mM NaCl after an initial 40 mM NaCl stress) was a randomized block, with three replications.

Leaf area measurements were done with a Li-Cor 3000 area meter (Li-Cor, Lincoln, NE, USA). Fresh and dry (60 °C) yield were both measured separately on plant and fruits. Stomatal conductance was measured on the abaxial surface of the youngest fully expanded leaves with a diffusion porometer (AP-4, Delta-T Devices, Cambridge). The size and density of stomata was recorded with a microscope. The leaf water potentials (Ψ_t) were determined using a dew-point psychrometer (WP4, Decagon Devices, Washington). The Osmotic Potential (Ψ_π) was estimated on frozen/thawed leaf samples and the Pressure Potential (Ψ_p) as the difference between Ψ_t and Ψ_π, assuming a matric potential equal to 0. Leaf osmotic adjustment (OA) was determined as the difference $\Psi_{\pi o}\, V_o - \Psi_\pi\, V$, where $\Psi_{\pi o}\, V_o$ is the product of (osmotic potential)x(osmotic volume) of unstressed plants and $\Psi_{\pi o}\, V_o$ is the product of (osmotic potential)x(osmotic volume) of leaves from salinized plants. For each measurement, the osmotic volume was approximated by the corresponding RWC value calculated as: RWC = (leaf fresh weight - leaf dry weight)/(leaf saturated weight – leaf dry weight) (Morgan, 1984). ABA determinations were performed on crude extracts of the youngest fully expanded leaves using an immunoassays kit (Hormondetek-ISCI Research Institute for Industrial Crops, Bologna, Italy) (Quarrie *et al.*, 1988). Proline was

determined on leaves of plants stressed (0, 20 and 40 mM NaCl), according to Claussen *et al.* (2005). Data were analyzed with ANOVA and means were compared with the LSD test. For gene expression analysis total leaf RNA was isolated from young fully expanded leaves as described in Corrado *et al.*, 2007. All primers for candidate genes were designed as reported in Corrado *et al.*, 2007.

3.3. RESULTS

3.3.1 Plant growth and yield

When plants were grown on a standard nutrient solution (without NaCl), we did not observe substantial differences in plant fresh and dry weight (mean values of respectively 138 g FW $plant^{-1}$ and 12.4 g DW $plant^{-1}$, Tab. 1), although the leaf area was moderately yet significantly larger in BB plants (2550 vs. 2120 cm^2 $plant^{-1}$) (Fig. 1). No differences were recorded on yield, which was 1863±177 and 1787±150 g $plant^{-1}$ of commercial fruits for BBS and BB respectively (Fig. 1). No differences were detected respect to dry matter (9.6±0.2 and 8.6±0.5 % for BBS and BB respectively). Salt treatments caused a progressive reduction of leaf area (Fig. 1). However, while at 0 and 20 mM NaCl, BB and BBS had quite similar leaf areas, significant difference were noticed at 40 mM NaCl. Although 40 mM NaCl reduced the leaf area in both lines, a smaller area development was observed in BB plants, which had a total leaf area smaller than 1000 cm^2 $plant^{-1}$. In relative terms, BBS plants treated with 40 mM NaCl had a 25% reduction of the leaf area respect to the non salinized control, whereas a 66% reduction was observed in BB plants.

Plant height was also inhibited by salinity. In absence of salt, BBS plants were generally higher than BB (147 ± 7 and 107 ± 4 cm, respectively at 120 DAS, data not shown), but the plant height was reduced in both lines already at 20 mM NaCl (115 ± 6 and 53.5 ± 2.5 cm for BBS and BB respectively) and even more at 40 mM NaCl (65.5 ± 2.5 cm and 65.5 ± 2.5 cm for BBS and BB respectively).

Salt stress decreased yield. Both lines performed similarly at 0 and 20 mM NaCl, while BB yield was significantly lower than BBS at 40 mM NaCl. The plant dry mass content was linearly reduced by salt treatments, while the plant dry matter percentage was enhanced under salt stress.

3.3.3. Leaf gas exchanges and *osmo-treatments*

Measurements of stomatal conductance are reported in Fig. 1. In absence of stress, stomatal conductance was slightly higher in BB plants compared to BBS (1.18±0.02 vs 0.88±0.02 cm s^{-1}, respectively). In addition, stomata of BB plants were smaller compared to BBS, but their average aperture was bigger (Tab. 2).

Salt treatments reduced stomatal conductance in both lines, with some differences. In absence of salt stress, the stomatal conductance of BB was higher than BBS (1.18±0.02 vs 0.88±0.02 cm s^{-1}, respectively), while these differences disappeared at 20 mM NaCl. Moreover, at 40 mM NaCl treatment, the stomatal conductance of BBS plants was higher than BB (0.54±0.02 vs 0.23±0.02 cm s^{-1}, respectively). The salinity-induced reduction of stomatal conductance was confirmed in experiment *B*, where a 40 mM NaCl stress caused a 27±2 and 71±13% inhibition of stomatal conductance in BBS and BB, respectively.

The response to proline applications was remarkable. In absence of proline, the stomatal conductance of BB plants was higher than BBS at 0 mM NaCl, while these differences disappeared at 20 mM NaCl. Once again, the stomatal conductance of BBS was relativey higher than BB at 40 mM NaCl, thus confirming the results of experiment *A*. As a consequence of proline application, the stomatal conductance was significantly reduced in BB plants whose values were always lower than BBS. The results of stomatal responses to the stress-recovery experiment are displayed in Figure 3. Once again, before salt application, BB plants had a higher stomatal conductance compared to BBS. In response to salinization, this response was reverted and BBS plants maintained a higher stomatal conductance, respect to BB. Upon stress recovery, all plants that were irrigated with the standard nutrient solution partially restored the initial conductance value. However, the recovery rate was higher in BBS compared to BB plants (Fig. 3).

3.3.4. Plant water status

Salinity decreased the total water potential in both BB and BBS plants (Tab. 3 and 4). At 20 mM NaCl, the total water potential of BB plants was more negative than BBS, and this difference was enhanced at 40 mM NaCl. Similarly, the osmotic potential decreased linearly upon salinisation, and it was in generally more negative in BB than BBS. Therefore, the osmotic adjustment appeared higher in BB than BBS. The Relative Water Content (RWC) (i.e. the water retained in the plant cells, compared to the amount of water at optimal hydration) was reduced upon NaCl treatments. The lowest values were observed in BB plants at 40 mM NaCl.

3.3.5. Biosynthesis of stress metabolites

The ABA content (Fig. 3) was always lower in BBS compared to BB plants. Differences in ABA content were already visible at 20 mM NaCl with higher value in BB plants respect to BBS. In general, salt stress caused an increased production of ABA, whose accumulation may have triggered other adaptive responses. Consistently, we noticed higher levels of proline in both BB and BBS plants (Fig. 3). In particular, highest amounts of proline were recorded at 20 and 40 mM NaCl treatments in BB plants, whereas these were generally lower in BBS plants, where we measured values lower than 20 μmol g DW^{-1} in correspondence of 20 mM NaCl treatment.

3.3.6. Expression of genes involved in salt stress response

In collaboration with the *Department of Sciences of the soil, the plant, the environment and animal production* of the University of Napoli, we assessed the expression of some genes that could have been involved in the activation of the salt stress response in the two lines. The gene CAT1A is involved in the protection from oxidative stress (Giles *et al.*, 2006). The gene JERF3 is usually induced by ethylene, jasmonic acid and salt stress (Hui *et al.*, 2004). The gene SAM (S-adenosyl-L-methionine synthetase enzyme) and TomPro2 are both involved in the biosynthesis of

osmoprotective compounds (Sanchez-Aguayo *et al.*, 2004; Fujita *et al.*, 1998; Maggio *et al.*, 2002). The gene TAS14 is usually induced by salt stress and ABA, but not by wound (Del Mar Parra *et al.*, 1996), and finally the gene TFT1 activates the biosynthesis of a protein considered to play an important role on salt-stress response (Xu and Shi, 2006). Systemin overexpressing plants constitutively expressed less SAM (43% of BB), TFT1 (69% of BB) and TomPro2 (53% of BB) (Fig. 4).

3.4. DISCUSSION

3.4.1. Effects of salt stress on plant morphology and physiology

The exposure to salt stress affected more strikingly BB plants compared to BBS (Figure 1 and 2; Table 1). Numerous studies report on the consequences of salt stress on plant architecture (Chen *et al.*, 2007; Zhu, 2001), ion partitioning (Ashraf and Bashir, 2003, Maggio *et al.*, 2007), leaf area expansion (Salah and Tardieu, 1997; Munns *et al.*, 2000) and yield (Botia *et al.*, 2005; Maggio *et al.*, 2004). Salt stress tolerance is usually described as the ability to overcome salt stress. However, the complexity of salt stress responses in plants throughout their growth cycle depends on several interacting variables, including the cropping environment, the plant phenological stage and the magnitude (salt concentration and time of exposure) of the stress experienced over time (Munns, 2002). Different mechanisms of salt tolerance, including short and long term adaptive responses, contribute to stress adaptation. In this respect, recent reports indicate that the physiological basis for short (24 h) and long-term (entire growth season) osmotic adjustment may respond to different biological and environmental cues, since physiological mechanisms that contribute to short-term adaptation are not necessarily the same that are involved in long-term stress responses (Maggio *et al.*, 2004). Stomatal closure is a short term stress adaptation that is functional to the control of water fluxes and tissue dehydration. However, this mechanism is generally followed by other modifications that should allow the recovery of normal physiological functions to re-start growth. Our results indicate a faster and tighter stomatal closure in BB plants compared to BBS, which led to a reduced leaf area development and yield. Possibly, after the initial stress, recovering mechanisms were more efficient in BBS plants, which were able to re-open the stomata and restart their biological functions.

3.4.2. Effects of salt stress on plant water status

Plant response to water and salt stress share common features mostly associated to the control of water relations. In this respect, the osmotic pressure of salty water acts against root water uptake generating conditions that are comparable to drought stress (Romero-Aranda *et al.*, 2001). In our experiments, salinization increased plant dry matter and reduced the leaf relative water content (RWC) in both lines. Although, in absence of salt, BB plants appeared more hydrated than BBS, the RWC of BB plants decreased more than BBS in response to salinity. Variation in leaf water potentials were observed, concurrently to the dehydration pattern. Leaf water potential (Ψ_t) and osmotic potential (Ψ_π) values became more negative at increasing levels of salt stress. However, while a NaCl increase of the nutrient solution caused a significant reduction of both total and osmotic potentials, a similar response was observed in BBS plants only under severe salt stress. It is known that salinization of the root zone can lead to an osmotic adjustment that is considered as a fundamental mechanism of adaptation to salinity (Shannon *et al.*, 1997; Alarcòn *et al.*, 1994; Guerrier, 1996). A reduction of the cellular Ψ_π would contribute to re-establish a positive water flux that had been impaired upon salinization. However, if on one side a higher osmotic adjustment may be associated to an enhanced salt tolerance, on the other side plants that have a moderate osmotic adjustment may have activated other stress/tolerance mechanisms and/or may have not *perceived* an external stressful environment.

Based on all the above and considering the modifications observed in terms of plant architecture under salt stress (Figure 1; Table 1), BBS plants were likely sensing a lower stressful environment than BB plants. This was possibly associated to a constitutive activation of stress response mechanisms that may have induced a pre-adaptation state of BBS plants.

3.4.3. Effects of salt stress on ABA and proline accumulation

ABA controls many stress adaptive features, including stomatal closure, activation of genes involved in osmotic adjustment, ion compartmentalization, regulation of shoot-to-root ratio and

modifications of root hydraulic conductivity properties (Ruggiero *et al.*, 2004; Verslues and Zhu, 2005). ABA concentration in leaves is inversely related to their stomatal conductance (Mulholland *et al.*, 2003). Moreover, some authors (Salah and Tardieu, 1997; Munns *et al.*, 2000) suggested that, under water stress conditions, the ABA may interact with an hydraulic signal to reduce leaf expansion rates particularly during periods of high transpirational demand. Therefore, the mechanism based on which the ABA regulation occurs is still not completely understood. We observed that salt stress increased the ABA content in leaves and this would lead both to stomatal closure and to a reduced leaf area expansion. However, ABA content was much higher in leaves of BB plants, compared to the BBS. Mechanistically, higher ABA levels may be required for controlling short-term adaptation processes such as stomatal closure (Wilkinson and Davies, 2002). In contrast, long-term stress responses should restore basal levels of ABA content in leaves which would allow stomatal re-opening and the complete/partial recovery of plant functions.

In our experiments, proline pretreatments were performed to enhance plant tolerance to salinity and to induce a sort of synergistic effect between osmoprotection (Makela *et al.*, 1996; Heuer, 2003) and a constitutive systemin overexpression. The biosynthesis of proline appears as a common response of the plant to stressful environments (Claussen *et al.*, 2005). Proline accumulates in plant tissue under a broad range of stress conditions such as water shortage, salinity, extreme temperatures, and high light intensity (Aspinall and Paleg, 1981; Mansour, 2000) and is believed to play a major role in plants osmotic adjustment. Controversial results, however have questioned whether proline accumulation would exclusively act as a compatible solute or via other unknown/less documented mechanisms (Pérez-Alfocea *et al.*, 1993). Transgenic plants engineered to accumulate proline exhibited a partial tolerance that could not be associated to an osmotically active concentration (Kavi Kishor *et al.*, 1995; Maggio *et al.*, 1997). This raised some doubts on the actual contribution of proline to osmotic adjustment and it pointed to other functions that may be important in stress adaptation, including the control of cell division (Maggio *et al.*, 2002; Ruggiero *et al.*, 2004). According to Hare *et al.* (1999), the metabolic effects of osmolyte accumulation may

be equal or even more important than their role in osmotic adjustment, since stress-regulated changes in proline synthesis and degradation may also affect expression of other genes, ensuring that the genetic response to stress is appropriate to the prevailing environmental stress conditions. In our experiments we observed that proline accumulation was higher in control plants, compared to the systemin over-expressing, and that BB plants were adapting to the same stressful conditions less favourably than BBS plants. Enhancement of the ABA content in leaves would lead to higher proline biosynthesis and such induction was higher in BB plants. Therefore, the different proline accumulation was consistent with the hypothesis that the effect of salt stress was stronger in BB plants compared to BBS.

3.4.4. Effects of Salt Stress on gene expression

Down-regulation of stress genes was consistent with some of the physiological mechanisms described above. TFT1 gene expression has been documented to be up-regulated on tomato plants undergoing salt stress (Xu and Shi, 2006). Its expression is considered to be crucial within the salt signalling pathway, and therefore a lower relative expression is consistent with a reduced stress perception of BBS plants. Moreover, also the constitutive expression of the TomPro2 gene was reduced in BBS. TomPro2 encodes for a key enzyme of proline biosynthesis (Maggio *et al.*, 2002). Similarly to TomPro2, SAM (S-adenosyl-L-methionine synthetase enzyme) is involved in the byosinthesis of osmotically active compounds, which are usually induced by plant perception of osmotic stress (Zorb *et al.*, 2004). Consistently, BBS plants undergoing stressful conditions had a lower TomPro2 expression compared to BB plants, which mirrored the level of proline accumulation. This results confirmed the hypothesis that a pre-adaptation of BBS plants would make these more tolerant to saline stress, as demonstrated by the higher yield and the absence in BBS plants of most of those metabolic responses that are typically activated upon stress.

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TABLES

	Yield	Plant Fresh Weight	Plant Dry Weight	Plant Dry Matter
	g plant^{-1}	g plant^{-1}	g plant^{-1}	%
Line				
BBS	1491.0	87.2	9.0	10.6
BB	1127.6	81.8	8.2	11.3
Salt				
0	1845.4	134.9	12.3	9.1
20	1334.4	73.7	8.0	10.9
40	748.0	44.8	5.5	12.8
Proline				
0	1409.6	87.1	8.7	10.6
5	1008.5	79.2	8.4	11.7
Significance				
Line - L	** (141.0[1])	n.s.	** (0.71)	* (0.41)
Salt - S	** (172.7)	** (9.89)	** (0.87)	** (0.73)
Proline - Pr	** (141.0)	* (5.65)	n.s.	** (0.59)
L x S	** (244.2)	** (13.99)	** (1.23)	** (1.03)
L x Pr	** (199.4)	n.s.	n.s.	* (0.59)
S x Pr	n.s.	n.s.	n.s.	* (0.72)
L x S x Pr	n.s.	n.s.	n.s.	n.s.

Tab. 1. Results of the ANOVA. Yield, plant fresh weight, plant dry weight and plant dry matter in response to salt stress, proline application and salt stress recovering in systemin over-expressing tomato (BBS) and its control (BB). (Mean values; ns = not significant; * = significant at P≤0.05; ** = significant at P≤0.01).[[1] lsd].

	Stomatal density	stomatal length	stomatal aperture
	n° of stomata mm^{-2}	µm	µm
BBS	180.04	16.2	2.3
BB	190.52	10.4	2.8
	ns	**	**

Tab. 2. Stomatal size, aperture and density in systemin over-expressing tomato (BBS) and control plants (BB). (Mean values; ns = not significant; * = significant at P≤0.05; ** = significant at P≤0.01).

	Ψ_t	Ψ_π	Ψ_p	OA	RWC
	(MPa)	(MPa)	(MPa)		%
Line					
BBS	-0.76	-1.60	0.84	0.27	76.8
BB	-1.05	-2.01	0.96	0.60	74.2
Salt					
0	-0.47	-1.14	0.68		87.8
20	-0.92	-1.82	0.90	0.38	77.3
40	-1.33	-2.45	1.12	0.49	61.4
Proline					
0	-0.82	-1.60	0.78	0.49	79.4
5	-1.08	-2.21	1.13	0.31	67.8
Significance					
Line - L	** (0.050 [1])	** (0.080)	** (0.100)	** (0.060)	n.s.
Salt - S	** (0.061)	** (0.098)	** (0.122)	** (0.073)	** (3.93)
Proline - Pr	** (0.049)	** (0.080)	** (0.100)	** (0.060)	** (3.21)
L x S	** (0.086)	** (0.139)	** (0.173)	** (0.104)	** (5.56)
L x Pr	** (0.070)	** (0.114)	* (0.099)	** (0.085)	n.s.
S x Pr	* (0.060)	n.s.	n.s.	n.s.	n.s.
L x S x Pr	** (0.122)	** (0.197)	* (0.171)	n.s.	* (5.5)

Tab. 3. Total leaf water potential (Ψ_t), osmotic potential (Ψ_π), pressure potential (Ψ_p), osmotic adjustment (OA), and relative water content (RWC) in response to salt stress and proline application in systemin over-expressing tomato (BBS) and its control (BB). (Mean values; ns = not significant; * = significant at P≤0.05; ** = significant at P≤0.01). [[1] lsd]

	Ψ_t	Ψ_π	Ψ_p	RWC	OA
	(MPa)	(MPa)	(MPa)	%	
Line					
BBS	-0,87	-1,32	0,45	79	-0,87
BBS	-1,08	-1,77	0,68	71	-1,08
Recovery					
recovered	-0,87	-1,26	0,39	84	-0,87
not recovered	-1,08	-1,82	0,74	65	-1,08
Significance					
Line - L	** (0.077 [1])	** (0.095)	** (0.075)	** (2.64)	ns
Salt – S	** (0.094)	** (0.118)	** (0.092)	** (3.23)	-
L x S	** (0.133)	** (0.166)	ns	ns	-

Tab. 4. Total leaf water potential (Ψ_t), osmotic potential (Ψ_π), pressure potential (Ψ_p), osmotic adjustment (OA), and relative water content (RWC) in response to salt stress recovery in systemin over-expressing tomato (BBS) and its control (BB). (Mean values; ns = not significant; * = significant at P≤0.05; ** = significant at P≤0.01). [[1] lsd]

FIGURES

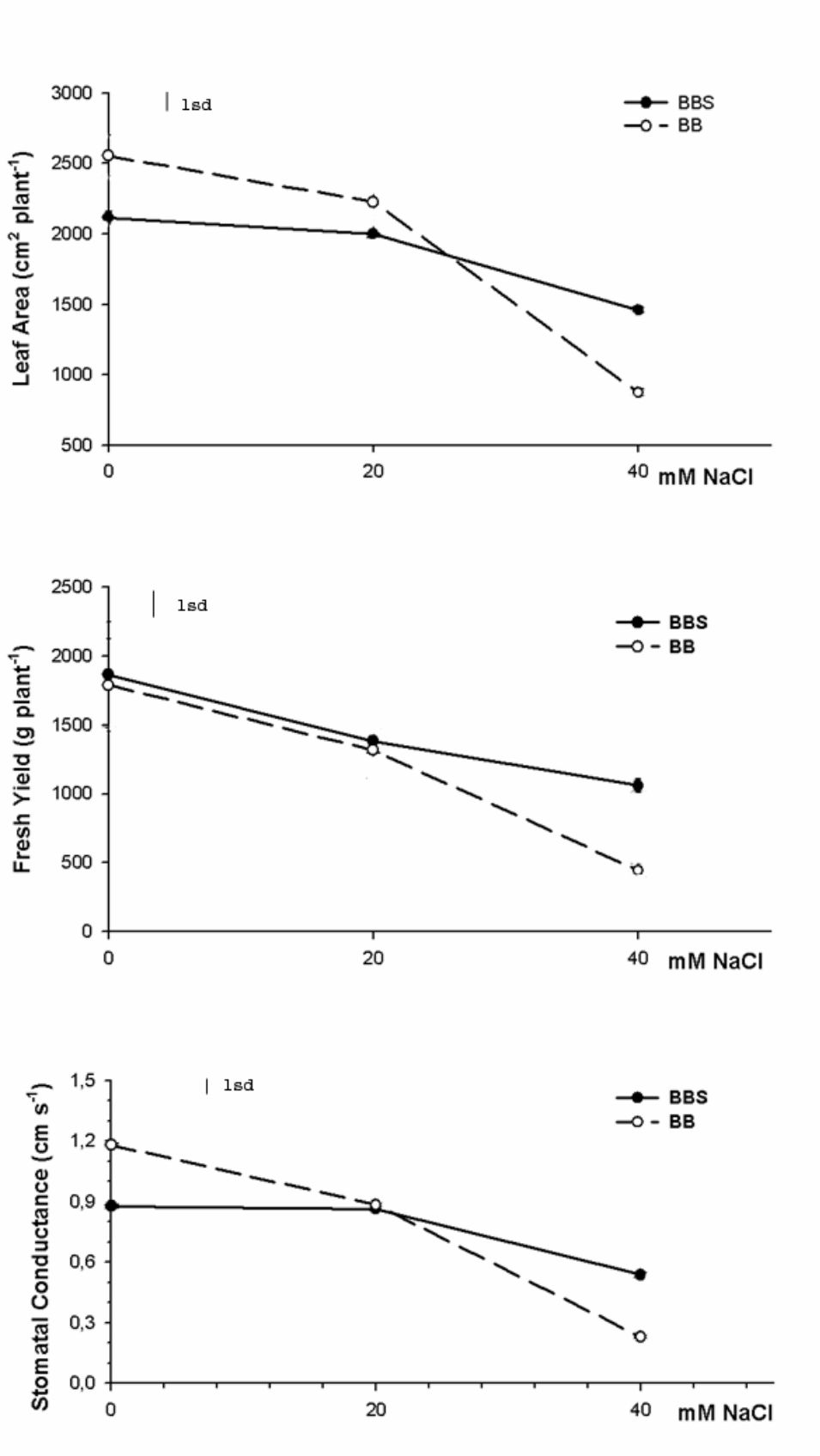

Fig. 1. Influence of salt stress on leaf area, fresh yield, stomatal conductance and relative water content in systemin over-expressing tomato (BBS) and its control (BB).

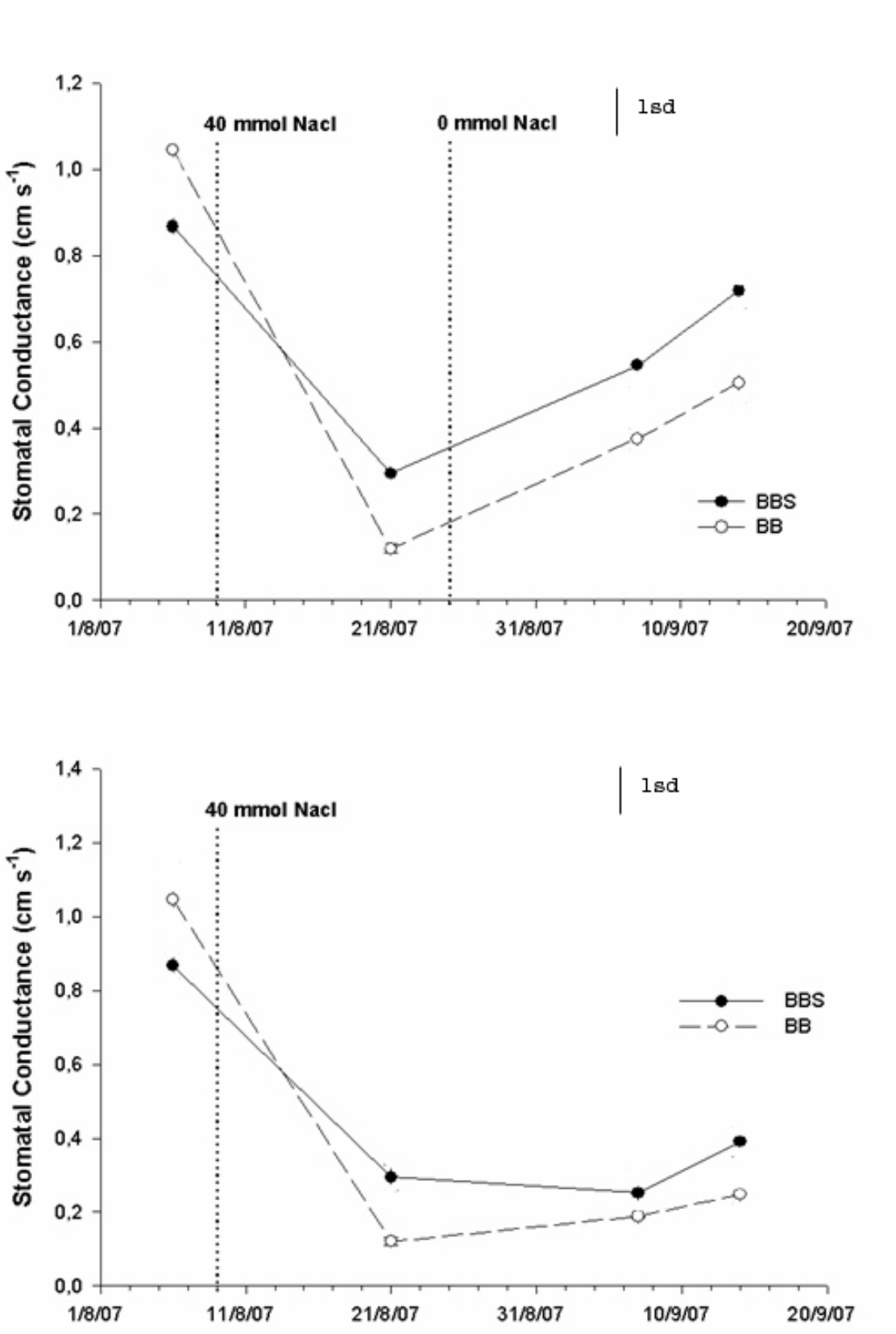

Fig. 2. Stomatal conductance in systemin over-expressing tomato (BBS) and its control (BB) as influenced by 40 mmol l^{-1} salt stress and recovery.

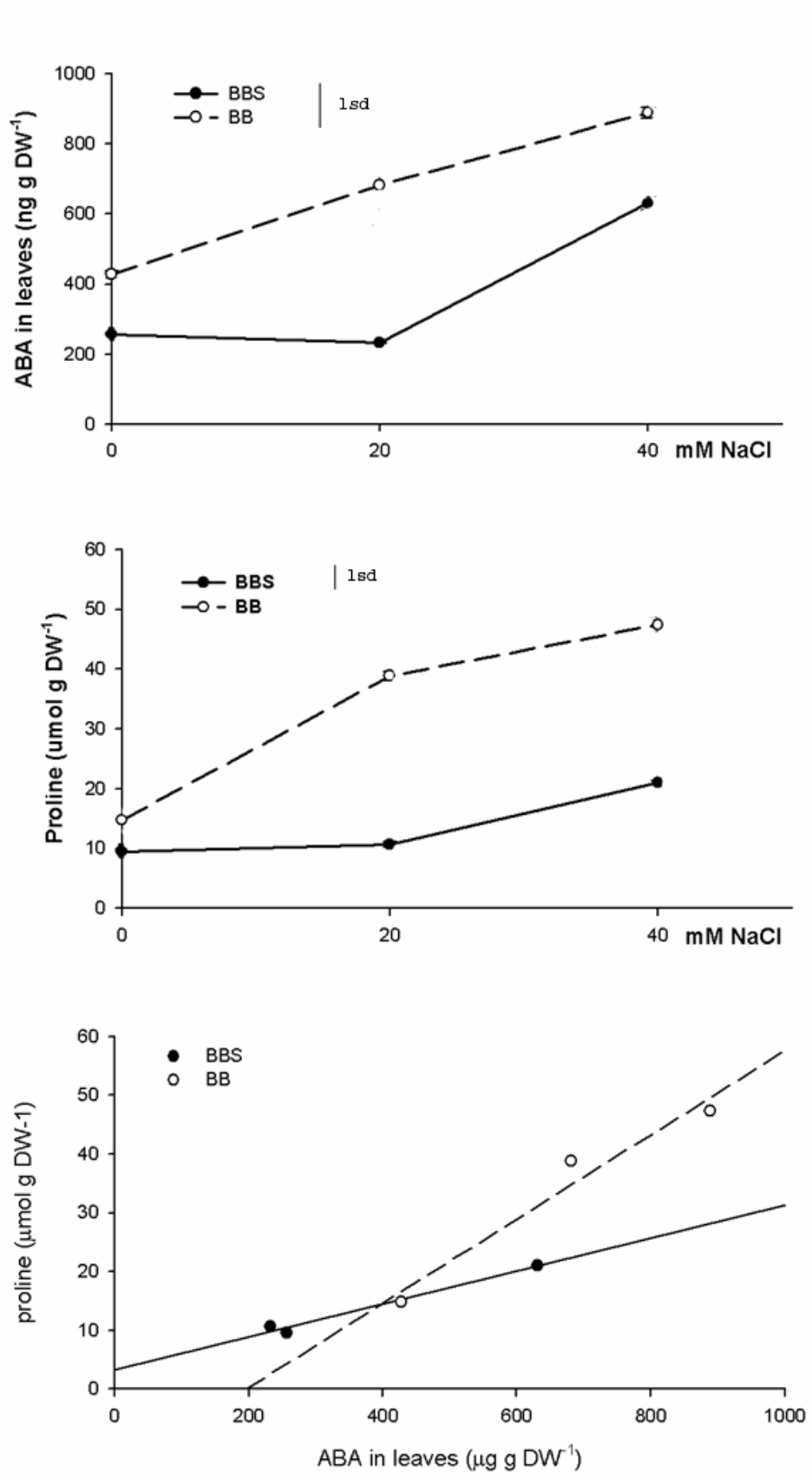

Fig. 3 ABA content, proline and proline/ABA content in systemin over-expressing tomato (BBS) and its control (BB) as influenced by salt stress.

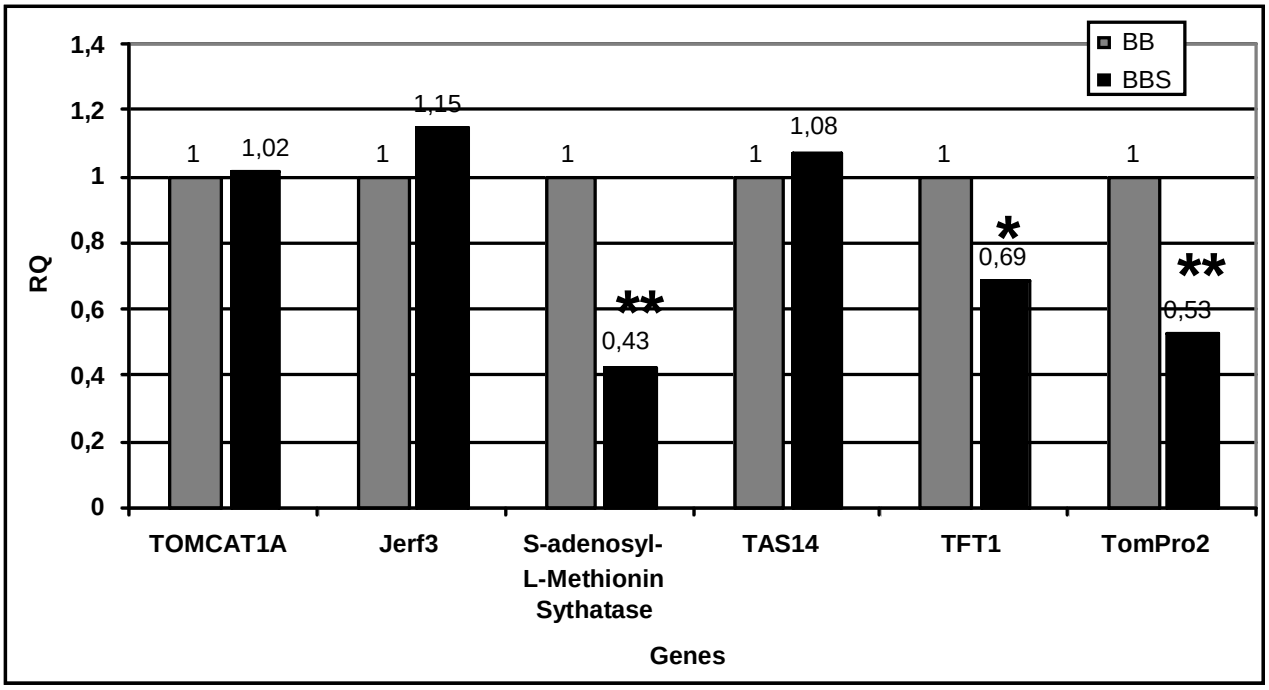

Fig. 4. Constitutive expression of genes involved in stress response in systemin-overexpressing tomato (BBS) and its control (BB) - (RQ=Relative Quantity). [* = significant at P≤0.05; ** = significant at P≤0.01].

CHAPTER IV.

CONCLUSIONS.

The control of water fluxes with respect to both growth, water uptake and thermoregulation plays a fundamental role in hyperosmotic environment. In the first chapter of this thesis we showed how extremely low transpirational fluxes were not associated with best performances under salt stress. In contrast, among halophytes, plants extremely tolerant to salt seemed to be those with a slightly higher leaf transpirational flux in absence of stress, a physiological trait that may contribute, upon salinisation, to reach over time a relatively delayed stomatal closure and consequent restriction of CO_2 uptake.

In the second chapter we confirmed, in crop species, how a reduced transpiration limits salt loading of the shoots an event that may be useful in short-term stress adaptation. More interestingly, while short term responses, typical of Napoleatano, were consistent with physiological mechanisms able to overcome a transitory stress situation, a long-term adaptation strategy based on an optimal regulation of water fluxes (increased stomata size and reduced stomata density) was present in Genovese, which was able to better cope with the stressful environment. These results were consistent with recent findings in which the genetic basis of water use efficiency was associated to several, metabolic, signalling and morphological traits, including stomatal density.

Cross-talk in stress response has been previously suggested in several studies. However, the mechanisms involved in the stress adaptation are still unclear. In the first chapter we showed how tolerance to drought would increase salt tolerance, although plants that were best performers on salt would display higher leaf transpiration. Similarly, plant salt tolerance was associated to an increased adaptation to freezing, even though plants showing extreme adjustment to high salinization would show poor tolerance to cold. Among these stresses, the role of ABA in

modulating stress responses has been repeatedly reported. However, we hypothesised that, in extremely salt tolerant species (*T. parvula* and *M. triloba*), mechanisms of salt stress adaptation would only be partially related to ABA-dependent pathway, while a strong influence could be related to an ABA-independent cascade. In line with the role of hormones and hormone-like signalling molecules involved in stress adaptation, in the third chapter, we showed how a peptide, involved in the wound-stress response in tomato, may interact with other mechanisms mediating salt stress adaptation. Through a reduced induction of other genes involved in osmotic adjustment and salt signalling, transgenic plants seemed to have a sort of pre-induced tolerance or a lower perception of stressful conditions. Transgenic tomato plants also displayed a more efficient recovery mechanisms, which would allow plants to re-open the stomata and restart their biological function. As previously indicated, higher ABA levels may be required for controlling short-term adaptation processes such as stomatal closure. In contrast, long-term stress responses should restore basal levels of ABA content in leaves which would allow stomatal re-opening and the complete/partial recovery of plant functions. Consistently, the accumulation of proline in plant tissue, which proved to be a good indicator of stress, was significantly higher in plants facing stronger stressful conditions.

From this work it emerged that most progress in salinity research it is expected by an interdisciplinary approach. Advancement in science will consent more and more to *fast move* from model plants to crop species, and viceversa. This will consent to unravel more efficiently the functional biology of salt stress adaptation and to define margins for improving salinity tolerance of agricultural crops.

Acknowledgements

Usually, these last pages of a PhD thesis end up being the most read sheets of the entire dissertation. Here is where people will leaf through, looking for something more exciting than growing a weed on salt, or trying to understand the value that they have played on the student's existence. Indeed, whether you will find your name or not in the following lines, please do not have doubts on the significance that you have had in my life! Yet consider the low level of sanity left into my brain after these last few crazy weeks, dotted by a considerable amount of *flip the bird* days. So, as I like to do, I will start from the end, walking back through the steps that brought this dissertation here. And if some common sense is still flying into my brain, I have to thank my tyre specialist Gaetano, for saving a good handful of my neurones, and the Informatica service team, that repeatedly delayed my laptop suicide.

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A controversial place in my thoughts is inhabited by the pictures of my life in Indiana, the time I spent experiencing the Midwest in its most favourable weather conditions. Thinking back to those days, I whish to send my warmest hug to my eclectic American family, Gipsy, Ben and Peppino, the way they managed to make me see things differently, visit old Indiana cemeteries at night, and understand the Pink Floyd. A great good bye to all Bressan's lab, Irina, Meena, Wanda, Bruno, Jean, Bai, and Tao, naming only some of the persons I met. And of course, Ray Bressan, his guide and the way he showed me how to think differently about experiments and research. I also will thank Pat, for supporting and helping me, and Sara, which is still working on some of the work that I have left there. And Andrea, whose words have been crucial in giving this thesis the format you see.

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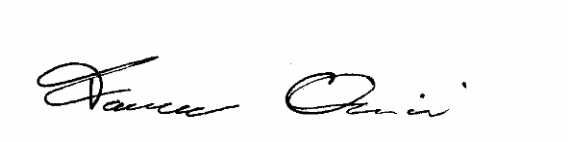

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