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Physiological characterization of the tomato landrace "Ciettaicale"

by

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Ph. D. Thesis

Agriculture, Food and Environment

Department of Agriculture, Food and Environment
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A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy in
Agriculture, Food and Environment

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Accepted by the Ph.D School
The Coordinator
Prof. Andrea Cavallini Date: 22/02/2019

Declaration

This thesis is a presentation of my original research work. Wherever contributions of others are involved, every effort is made to indicate this clearly with due reference to the literature and acknowledgement of collaborative research and discussions.
This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma.

Signature *Date*

22/02/2019

Preface

This dissertation is submitted for the degree of Doctor of Philosophy at the University of Pisa. The research described herein was conducted under the supervision of Dott. Lorenzo Guglielminetti and Prof. Piero Picciarelli in the Department of Agriculture, Food and Environment, between November 2015 and October 2018.

Part of this work has been presented in papers published or submitted or prepared to submission in peer-reviewed journals as follows:

CHAPTER 2

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CHAPTER 3

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CHAPTER 5

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Abstract

During the last century, the strong anthropic pressure and the global climate changes have exacerbated the depletion in water resources availability in term of quantity and quality. The expected scenario will be more catastrophic, especially in arid areas including the Mediterranean Basin. More frequent and severe extreme weather events will negatively affect food production, especially to keep up with the projected population growth. The development of crop plants well adapted to harsh climatic conditions such as drought and salinity will become essential to increase or even maintain the actual levels of food productivity. The identification and characterization of crop germplasm with high water use efficiency remains a hopeful sustainable strategy to provide food and valorise marginal areas. A deeper understanding of plant adaptive physiological and molecular mechanisms will be invaluable to select such germplasm. Biodiversity represents a target for scientific investigations and consequently a source for new breeding strategies. Landraces are members of that part of plant biodiversity which is often well-adapted to wild environments and extreme conditions. In the Mediterranean area, tomato (*Solanum lycopersicum* L.) is often exposed simultaneously to drought and salinity and many tomato landraces showed high adaptation capacity to these stress conditions.

The objective of this thesis was to investigate the salt and drought tolerance capacity of "Ciettacale", a Southern Italy tomato landrace, as observed by local farmers in comparison with commercial tomato cultivars at different phenological stages; with this purpose, physiological, biochemical and metabolic parameters were used.

The biochemical profile and germination rate of Ciettaicale and San Marzano were clearly altered by a moderate concentration of sodium chloride (25 mM NaCl) during 5 days-experimental time-course. Salt induced a promotion of endosperm weakening-related enzymes endo-β-mannanase and β-mannosidase activities in Ciettaicale, as well as starch mobilization, contributing both to the enhancement of total soluble sugars, and also to the scavenge oxidative capacity, as indicated by increased total antioxidant and catalase activities. Conversely, in San Marzano, we found some salinity-induced physiological changes only at the end of our experimental observations, suggesting that seeds were not dormant, but impaired by the stress to to properly achieve or complete the germination process.

Leaf gas exchange and chlorophyll *a* fluorescence measurements supported by growth and biochemical analyses were evaluated to identify salt tolerance differences between Ciettaicale and San Marzano during the vegetative stage under short-time high salinity stress (0, 300, 450 and 600 mM NaCl). After a week of salt treatments, salinity effects culminated in photoinhibition and/or photodamage events at 600 mM NaCl in San Marzano plants resulting in a source-sink imbalance. Among the salt gradient, Ciettaicale plants showed an efficient physiological and metabolic plasticity provided by improved photosynthesis efficiency and osmotic adjustment resulting in a higher energy availability to be used in root exploration. Thus, the Ciettaicale NaCl tolerance strategy allowed this landrace to survive or at least to slow down the appearance of actual damages at high NaCl concentrations.

We also combined chlorophyll *a* fluorescence measurements and metabolic determinations to investigate the performance of Ciettaicale and Moneymaker plants subjected to 20 days-drought stress. Physiological and metabolic changes, in terms of abscisic acid (ABA), indol-3-acetic acid (IAA), proline, soluble sugars and phenols contents, occurred in both Ciettaicale and Moneymaker under water deficit. Our results highlighted the ability of Ciettaicale to manage plant water status under drought in order to preserve both leaf and root activities. This strategy was achieved thanks to the preservation of the source-sink relations: a more efficient PSII photochemistry at leaf level associated with a major investment in root growth and activity in order to improve water uptake. On the contrary, drought-stressed Moneymaker plants reduced electron transport rate and enhanced starch reserve mobilization in both leaves and roots, possibly suggesting a major role of the osmotic adjustment to counteract tissue dehydration, but meantime a feedback potential disruption of source-sink relations. This hypothesis was also supported by the more pronounced redox state disequilibrium, as suggested by the higher hydrogen peroxide and malondialdehyde contents, that affected both PSII photochemistry and root activity and markedly trigged in turn the non-photochemical fluorescence quenching (NPQ) and antioxidant responses by Moneymaker plants compared to Ciettaicale.

In an autumn-winter greenhouse hydroponic experiment we evaluated yield and fruit quality of Ciettaicale under salt stress, in comparison with other two Southern Italy tomato landraces (Linosa and Corleone) and one commercial cultivar (UC-82B) Salt treatments (60 mM and 120 mM NaCl) promoted the anticipation of fruit ripening in landraces and UC-82B compared to non-salt conditions. At harvest, no

losses in marketable yield were noticed in all genotypes. Instead, fresh and dry fruit yields, as well as cation concentrations, were more affected under stress in the commercial cultivar as compared to landraces. Different trends of lycopene content and soluble sugars amount were found in the fruits among all investigated accessions. Data obtained by UPLC-MS revealed differential accumulation of glycoalkaloids, phenolic acids, flavonoids and their derivatives in the landrace fruits under stress in all genotypes. Overall, despite the non-optimal environmental conditions represented by salt stress and off-season light irradiance/temperature, our results showed a differentiation between the Italian landraces and the commercial variety UC-82B under 60 mM NaCl: in this stress condition, landraces showed a tolerable compromise between yield and quality attributes. Conversely, off-season high salinity stress (120 mM NaCl) significantly reduced the antioxidant activity both in UC-82B and in the landraces.

Our data suggested that Ciettaicale could carry interesting traits at vegetative stage such as improved root/shoot ratio, water use efficiency, osmotic regulation and maintenance of shoot-to-root relationships under high salinity and water deficit, as well as good performance in term of germination rate and fruit yield and quality under salt stress. We point to the feasible use of the tomato landrace Ciettaicale as a target to select interesting genetic traits to improve stress response and fruit functional values. Thus, deep investigations are required in order to enhance the possibility of introducing this landrace in tomato genetic improvement programs.

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Chapter 1

General introduction and thesis outline

1.1 Landraces: definitions and points of view

The term “landrace” appeared for the first time during the International Agriculture and Forestry Congress held in Vienna in 1890 (Zeven, 1998). On this occasion the importance of landraces as genetic resource was emphasized and they should be collected and safeguarded in specific conservation programmes. In fact, during the following decades several genetic accessions banks appeared worldwide. The advent of commercial hybrids era has shown the landraces as potential start for new genetic improvements to the eyes of breeders. Consequently, new concepts of “landraces” started to appear, with particular reference to the traits, such as quality, stress tolerance and low-input associated-agriculture farming, which are normally associated to these genotypes.

Zeven (1998) drew up an exhaustive review about the historical evolution of the landrace definition from 1890 to his time, proposing a concise description of a landrace as “a variety with a high capacity to tolerate biotic and abiotic stress resulting in a high yield stability and an intermediate yield level under a low input agricultural system”. In his description, Zeven took also into account the continuous evolution imposed on the landraces both by the natural selection and also by the human/farmer selection, since “the environment changes annually and the landrace could become contaminated with other genotype(s) grown in the same farming system concerned”.

Villa et al. (2005) defined landrace as “a dynamic population of a cultivated plant that has historical origin, distinct identity and lacks formal crop improvement, as well as often being genetically diverse, locally adapted and associated with traditional farming systems.” This definition pointed out the connection between the landrace and the willingly or unwillingly selection programme done by the farmers through the choose of a farming system. Moreover, in the abovementioned definition the term “variety” used by Zeven was substituted by the substantive “population”.

Genetically, it is more correct to consider landraces as population, namely a group of genotypes/individuals (each one could differ from the others), which have in common a long-time natural/artificial selection happened in a specific geographical contest. In other terms, landrace can be considered as a particular case of ecotype, that is a population of individuals within a species, in which the natural selection applied a certain force leading to a strong genetic adaptation to the associated habitat (Fig. 1.1). In the case of landrace the main selection force is the human (farmer) selection (domestication) applied by plant breeding and farming techniques.

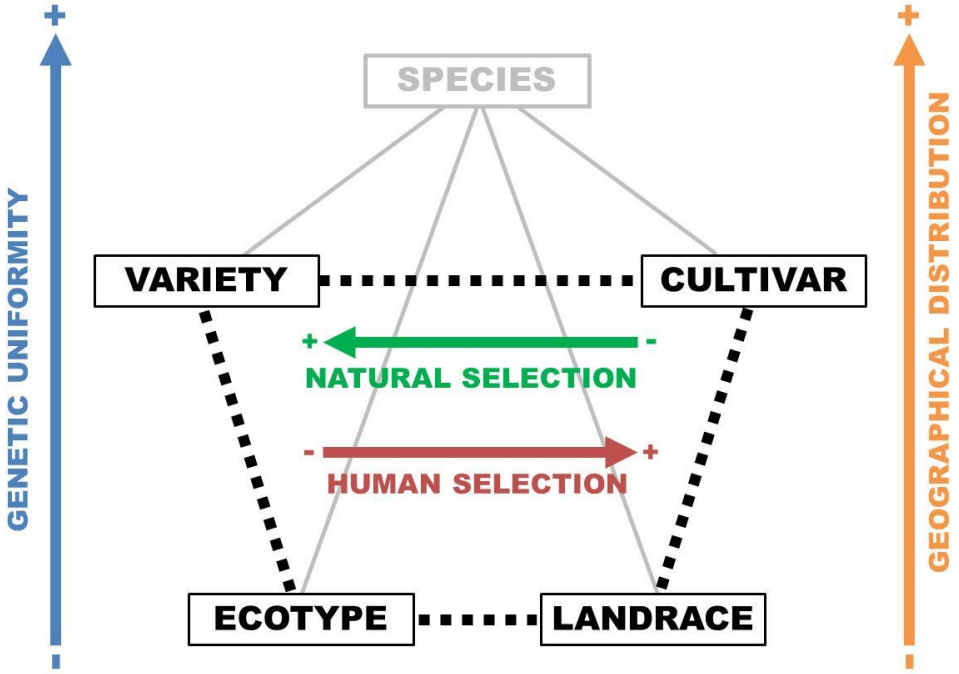

Figure 1.1. Schematic representation of variety, cultivar, ecotype and landrace categories placement within a species, depending both on the genetic uniformity and the geographical distribution (vertical axis), and also on the contribution in the selection process by the environment and/or by anthropogenic activity (horizontal axis).

In addition, landrace cannot be legally considered as "variety" because, to be introduced in the official register of varieties, it needs to satisfy the "distinct, uniform and stable" (DUS) criteria, that is excluded by the definition of genetic population. Moreover, landraces cannot be even recognized as cultivated variety (cultivar, cv.), whose term, not botanically correct, identifies a variety with a particular agricultural/horticultural relevance. However, the term "landraces" are often synonymous of "traditional/farmer/local varieties" (Negri, 2003). A list of additional synonymous, that in turn underline different facets of the concept of "landrace", is reported in Table 1.1.

Table 1.1. Different translations of the term "landrace" used in official national legislative texts (adapted from Bocci, 2009).

NATION	TRANSLATION OF 'LANDRACES'	MEANING IN ENGLISH	POINT OF VIEW EXPRESSED
France	Races primitives	Primitive, original races	Historical, social or biological
Germany	Landsorten	Landraces	
Italy	Ecotipi	ecotypes	Ecological
Spain	Variedades	varieties	Biological
Romania	Soiurilor locale	Local variety	Geographical
Portugal	Variedades autoctones	Autochthonous varieties	Geographical and social
Hungary	Honos fajok	home variety	Sociological

According to Negri et al. (2009), "a landrace of a seed-propagated crop is a variable population, which is identifiable and usually has a local name. It lacks 'formal' crop improvement, is characterized by a specific adaptation to the environmental conditions of the area of cultivation (tolerant to the biotic and abiotic stresses of that area) and is closely associated with the uses, knowledge, habits, dialects, and celebrations of the people who developed and continue to grow it". Comparing to the previous definitions, Negri et al. (2009)'s description clearly revealed the intimate association between the landraces and their own socio-historical background: thus, landraces are intrinsically carriers of a cultural treasure. Due to this association, landraces are known by local people with terms often related to phenotypic or transformation traits, but it is frequent that different genotypes have the same local name and/or landraces with diverse names derived in reality from the same population.

According to Casañas et al. (2017), landraces are "plant materials consisting of cultivated varieties that have evolved and may continue evolving, using conventional or modern breeding techniques, in traditional or new agricultural environments within a defined eco-geographical area and under the influence of local human culture". This new definition of landraces included some of the previous orthodox conceptualizations (see the adjectives "conventional" and "traditional"), but also dealt with the on-going Agriculture Era (see the adjectives "modern" and "new"). In fact, the authors supported that the current progress in agronomic and genetic/biotechnological fields should be introduced to improve not only the modern varieties, but also the landraces themselves, as evidenced in some recent works (Bitocchi et al., 2009; Causse et al., 2013; Bitocchi et al., 2015).

1.2 Landraces: agrobiodiversity to be safeguarded

In order to summarize the previous paragraph, landraces are a part of the agro-biodiversity of a certain place that can include a large number of genotypes; thus, landraces are lack of genetic uniformity, according to the definition of genetic population, but share a common historical, cultural and socio-economic background.

Crop landraces often carry desirable traits like tolerance to biotic/abiotic stresses, high fruit quality, adaptability to low-input or sustainable agriculture, allowing them to occupy a niche position in the market (Negri 2003; Sanchez et al. 2008; Mazzucato et al. 2008). However, the relative income from landrace cultivation is often lower than that derived from high-yield commercial varieties and, consequently, the effort to use landraces seems to be not diffused among farmers (Nikolaou and Maxted, 2009). The main target of breeding programmes was (and still is) the constitution of stable lines (or at least good hybrids) that optimize crop yield, especially when the cultivation is associated with high-input agro-techniques. Once the high- and uniform-yield genotypes appeared on the market, often receiving a huge promotion from the commercial breeder companies, farmers have generally preferred to safeguard own income by substituting the local varieties in favour of a market-oriented monoculture cultivation system. In this way, landrace biodiversity started to be affected by a drastic and speedy erosion process (Hammer et al., 1996, Negri, 2003). Landraces have always promoted and are still supporting the live-hood of the agriculture-based rural family and community (Jarvis et al. 2008). In fact, the genetic heterogeneity within a landrace population allows to its members to response differently to the variable conditions of the agro-ecosystem where are cultivated, also guaranteeing a production in challenging seasons. However, the exodus from the rural countries to the cities, the evolution of the production system, the increasing of average age of the farmer-maintainers and the interruption of knowledge heredity transmission from the old generation to the next one are additional reasons that are contributing to the loss of landraces biodiversity (Negri et al., 2009).

Landraces represent a genetic back-up (in term of alleles or combination of alleles) for future use in breeding programmes. Thus, the first action should be to preserve this biodiversity. Therefore, the creation of national landrace inventories is critical and mandatory in the short time.. At the moment, the European landraces database is not accurate and totally comprensive (Maxted et al., 2008). Two complementary conservation strategies could be applied to safe landrace

biodiversity: the ex situ conservation (the population is moved in a new environmental contest) and in situ conservation (preservation of the population in the original habitat). Negri et al. (2009) supported the importance of preserving the landraces pool through the concept of the "on-farm conservation", namely "the management of genetic diversity of locally developed crop varieties (landraces) by farmers within their own agricultural, horticultural or agri-silvicultural systems". Few notable projects have dwelt the importance of on-farm landrace conservation (Negri et al., 2000; Scholten et al., 2008), showing a cooperative network among farmers, scientists and members of the community. Fortunately, these reports showed that landrace cultivation still persists around Europe.

The "on-farm conservation" strategy preserves the entire landrace populations on their own agro-ecosystem, allowing them to evolve and acquire new potential suitable traits in contrast to the stable modern cultivars. For these reasons, landrace should be maintained as faithfully similar as possible to the type according to the Union for the Protection of New Varieties of Plants (UPOV) rules (Casañas et al., 2017). In addition, landraces represent a promising target, in comparison with crop wild relatives, because they are characterized by diversity without having the associated disadvantages of undesirable traits (Hammer and Diederichsen, 2009).

Despite the DUS criteria for the registration of a variety in the official list is a normative "against the nature of landraces which are supposed to evolve in dynamic interaction with the environment" (Marum and Daugstad, 2009), European legislation started to allocate financial support to encourage the cultivation of landraces and the increase of the commercial value of landrace products. With this aim, for example, the labelling system PDO (Protected Designation of Origin), PGI (Protected Geographical Indication) and TSG (Traditional Speciality Guaranteed) have been introduced to identify the association between crop variety/product with cultural or biological heritage within a limited geographical area (Veteläinen et al., 2009).

While genetic uniformity is a desirable attribute in a short-term agro-ecosystem evolution prevision, it can be considered a vulnerable trait in a long-term period due to the unpredictable and mutable global climate changes with novel biotic/abiotic stress and multi-stress conditions. Thus, the preservation of the genetic potentiality of the landraces should be a prerogative for scientists and breeders in order to mitigate the impact and the scale of the incoming changes (Negri, 2005).

1.3 The tomato landrace "Ramellet": an inspirer case study

The modern tomato germplasm is the result of a series of genetic bottlenecks during a long process of domestication to which limited accessions were subjected (Koenig et al., 2013; Sacco et al., 2015). This selection process already started in South and Middle America where the several microclimates and habitats of the Andean regions (also including the Galapagos Archipelago) certainly influenced the high variability of the wild tomato relatives, such as *Solanum pimpinellifolium*, *Solanum pennelli* and *Solanum chilense*. Recently, this pool obtained great attention from scientists and have been exhumed to be used in genetic improvement of the commercial varieties. In the XVI century, the Spanish *conquistadores* imported limited number of tomato accessions in Europe, including the *Solanum lycopersicum* var. cerasiforme which is considered the ancestor of the modern cultivars (Ranc et al., 2008), originated in turn from a long domestication of *Solanum pimpinellifolium* (Blanc et al., 2012). Then, through the colonization commercial routes, tomato cultivation spread worldwide, finding new domestication centres where the selection contributed not only to the morphological diversification of the modern tomato (Tanksley, 2004), but also to the constitution of several heterogeneous populations, including landraces (Sacco et al., 2015). The Mediterranean Basin, especially Spain and Italy, represents the ideal area for the development of an extensive array of tomato landraces with interesting genetic profiles that differ from the commercial varieties (Garcia-Martinez et al., 2006; Garcia-Martinez et al., 2013; Corrado et al., 2014; Sacco et al., 2015).

The investigation for the variation of Quantitative Traits Loci (QTLs), which control for example fruit shape/size and fruit metabolic profile traits, has made great progress, mainly thanks to the so-called Genome Wide Association Strategy (GWAS), a powerful technique that, through a large number of molecular markers, allow to screen/genotype a wide number of populations. In the case of tomato, the release of its genome sequence (Tomato Genome Consortium, 2012) and the progress in the array technology (Sim et al., 2012) enormously facilitated the identification of new molecular markers and their association with QTLs. Simple Sequence Repeats (SRR) and Single Nucleotide Polymorphisms (SNPs) markers have been used for the molecular characterization of tomato germplasm collections, including landraces. The association of these molecular markers with the QTLs variation revealed not only new insights into the evolution of tomato populations structure, but also offered a practical tool to use in breeding improvements of traits

like fruit shape/size (Mazzucato et al., 2008; Sacco et al., 2015) and fruit quality (Sauvage et al., 2014; Zhang et al., 2015).

Among interesting traits within landrace population, extended fruit shelf life is one of the most attractive from a market-oriented point of view. In the North-East Spain and in the Balearic Archipelago the "Ramellet" and the "Penjar" (from Spanish, "tomato for hanging") are two tomato landraces widely cultivated characterized by a great heterogeneity in fruit morphology (Casals et al., 2011; Bota et al., 2014). The fruits are normally harvested in summer, then tied in bunches and stored hanging in the house-farming until the next spring. During this period, the fruit maintains almost the same consistence, allowing the farmers to consume them fresh, usually rubbed on bread, or as basis for sauces during the winter. This phenotypic trait have been scientifically investigated. These landrace populations carry the *alcobaça* (*alc*) mutation, that is allelic with the *non-ripening* (*nor*) gene, which is involved in the ethylene production and, consequently, in the fruit ripening; the mutation causes a structural change in a NAC domain in the *nor* gene, affecting ethylene biosynthesis. Tomato with *alc* mutation has less than 25% of the ethylene production comparing to the wild-type; this trait increases not only the fruit firmness, but also the resistance to bacterial disease (Mutschler, 1984). However, these positive traits were more marked when the *alc* mutation is combined with small fruit size (Casanals et al., 2012). These Spanish accessions showed a similar phenotype of the *Delayed Fruit Deterioration* (*DFD*) mutant (Saladié et al., 2007), another landrace population from Southern Europe with extended shelf life. The *DFD* fruits remain firm for at least 6 months after achieving a fully ripe stage, exhibiting minimal water loss thanks to peculiar properties of the fruit cuticle ultrastructure and its ripening-related metabolism, that also confers resistance to infection by opportunistic pathogens.

Interestingly, the Ramellet landrace is also well-adapted to the drought characterizing the summer season of the calcareous soils in the Balearic region. Galmes et al. (2011, 2013) investigated the drought tolerance trait observed in the Spanish tomato population through an eco-physiological approach. In fact, comparing to commercial varieties, Ramellet showed an increased Water Use Efficiency (WUE) under drought. This capacity was achieved by stress-induced changes in mesophyll structure and ultrastructure: the higher leaf thickness/porosity, as well as the enriched chloroplast distribution facing the intercellular airspaces,

resulted in rearrangements in stomatal and mesophyll CO_2 conductances, that guarantee in turn improved photosynthetic performances and plant water management under stress. The observation that Ramellet landrace population represents a useful genetic source related to traits both for adaptation to the Mediterranean drought conditions, such as enhanced WUE, and for marketing, such as extended shelf life, have been consolidated by the creation of an official germplasm collection bank (Bota et al., 2014).

However, similar investigations for drought/salinity traits have been carried out on different tomato landraces within Mediterranean Basin and neighbour regions, like Southern Italy (Patanè et al., 2016), Jordania (Bsoul et al., 2016) and Pakistan (Amjad et al., 2014). As the landrace Ramellet, these populations also exhibited peculiar physiological and yield differences in response to stress comparing to commercial varieties.

1.4 The tomato landrace "Ciettaicale"

"Ciettaicale" is a tomato landrace cultivated in Basilicata region in Southern Italy. The cultivation of this landrace, known as "all'assich" (in local dialect, "grow without water"), was originally conducted on open field using the only water derived from rainfall, since both the hilly landscape of most of Basilicata territory and the poor economic conditions of the local farmers did not provide the possibility to have access to the groundwater by a sufficient irrigation system (Fig. 1.2A). Basilicata, as other regions in South Italy, is normally invested by intense drought fluctuations, especially during the summer season, that are recurrently cause of agriculture yield losses. Despite this harsh scenario, the cultivation of the tomato landrace Ciettaicale guaranteed to obtain adequate yields (Fig. 1.2B).

Fig. 1.2. [A] Aerial view of the hilly landscape of Basilicata region in Southern Italy (adapted from http://www.aptbasilicata.it). [B] Open-field cultivation of the tomato landrace "Ciettaicale" (photo by courtesy of Giovanni Infantino).

During the World Wars and in the following decades, the cultivation of many landraces, including Ciettaicale, disappeared due to the exodus of farmers from the countryside to the industrialized cities. However, the livelihood in the countryside was mostly based on cereal agriculture and animal husbandry. During this period, Lucia Aicale, kindly called "Ciettaicale" among townsfolk of Tolve (Fig. 1.3), continued the propagation and the selection of seeds of this tomato landrace in the fields of Tolve, a small rural town closed to the regional capital Potenza, becoming unwittingly a guardian of a buried treasure which today brings her name.

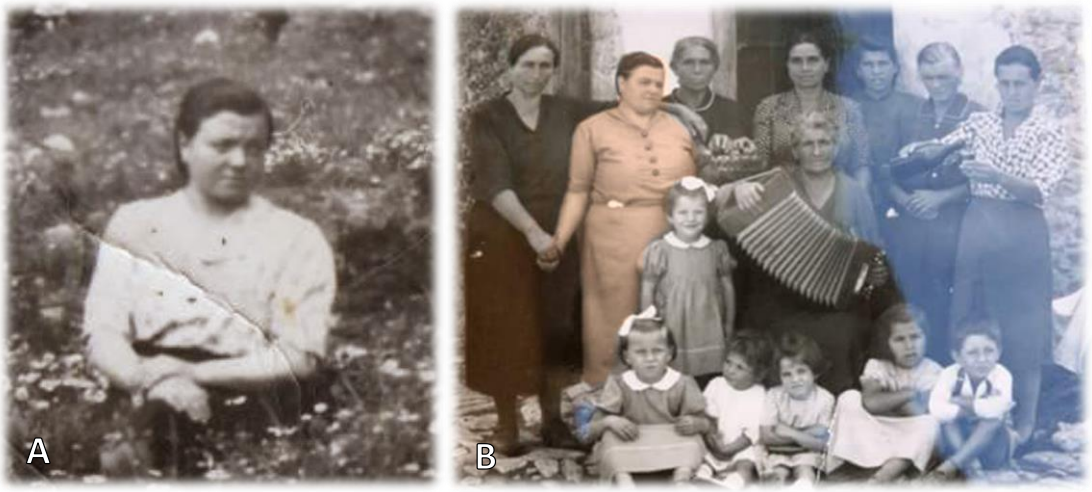

Fig. 1.3. Old pictures showing "Ciettaicale", short for "Lucia Aicale", the farmer-guardian of the namesake tomato landrace. [A] Lucia Aicale sitting in the field at a young age, and [B] (indicated by sepia colour) with some townsfolk women and kids of Tolve (photos by courtesy of Giovanni Infantino).

The heritage of Lucia Aicale is now in the hands of her daughter Antonia De Angelis and her grandson Giovanni Infantino. Antonia accidently found a metal box with inside some Ciettaicale tomato seeds and from that moment her family is committed to promote the Ciettaicale story in events and initiatives at regional and national level, in order to arouse public opinion to the importance to protect and conserve Italian biodiversity. The labelling mark of Ciettaicale as "Prodotto Agroalimentare Tradizionale" (PAT, is an official approval for traditional Italian regional food products by the Ministry of Agricultural, Food and Forestry Policies similar to the PGI one granted by the European Union) is one of many successes achieved thanks to the collaboration with the Coldiretti Basilicata, the University of Basilicata and the Agency for Development and Innovation in Agriculture of Basilicata (ALSIA). The PAT mark is applied on the product derivate from the traditional transformation of the Ciettaicale fruits: the red-orange berry is cut in half

(Fig. 1.4 A and B), drowned with salt and basil and then dried directly under the sun. The exsiccated product is finally stored in olive oil (Fig. 1.4D). Ciettaicale fruits are also used to prepare fresh tomato sauce (Fig. 1.4C).

The first physiological characterizations of the Ciettaicale under drought and salt stress have been conducted at the University of Pisa (Moles et al., 2016; Moles et al., 2018). New investigations, including the characterization of the Ciettaicale genetic background and the evaluation of its photosynthetic and physiological performances under drought/salt stress conditions through phenotyping platforms, are now going on at the Institute of Biosciences and Bioresources Research (IBBR) - Research Center Metapontum Agrobios.

Fig. 1.4. Fruits of the tomato landrace Ciettaicale [A] on the plant, [B] after harvesting, [C] used to prepare fresh sauce, and [D] drowned with salt and basil before drying under the sun. The tomato "Ciettaicale" is registered as "Traditional Speciality Guaranteed", as indicated by the yellow-blue circle labelling mark in [D] (photos by courtesy of Giovanni Infantino).

1.5 Thesis outline

During the last century, the strong anthropic pressure and the global climate changes have exacerbated the depletion in water resources availability in term of quantity and quality (Turner and Meyer, 2011). The expected scenario will be more catastrophic, especially in arid areas including the Mediterranean Basin. More frequent and severe extreme weather events will negatively affect food production (Wheeler and Braun, 2013), especially to keep up with the projected population growth (Gerland et al., 2014). The development of crop plants well adapted to the adverse climatic conditions, such as drought and salinity, will be essential to increase or even maintain food productivity. Developing crop germplasm with higher water use efficiency remains a hopeful sustainable strategy to valorise marginal lands and to mitigate the incoming unpredictable changes (Geerts and Raes, 2009). A deeper understanding of the physiological and molecular mechanisms adopted by plants to cope with environmental stress is mandatory to develop such germplasm. However, these mechanisms may be different inter-/intra- species and depend on plant growth stage and organ/tissue (Verslues et al., 2006). Natural biodiversity represents a target for scientific investigation and consequently a source for the application of new breeding strategy. Landraces are member of that biodiversity which is often characterized by interesting traits, like tolerance to abiotic/biotic stresses and adaptability to low-input agriculture, acquired during their evolution under the selection forces driven by the farmers of a certain agro-ecosystem and the environment itself (Negri et al., 2009).

Tomato is one of the most important agricultural crop worldwide thanks to its high nutritional values, in terms of vitamins, antioxidants and minerals, as well as a model species for research studies. In the Mediterranean region, tomato is often simultaneously exposed to drought and salinity events and many tomato genotypes (cultivars and landraces) showed high adaptation to these stress conditions. It is widely accepted that salinity and drought affect the cell osmotic potential resulting in a similar effects on plant physiology (Mahajan and Tuteja 2005; Kautz et al., 2014).

The objective of this thesis was to evaluate the drought tolerance observed by local farmers in the Southern Italy tomato landrace Ciettaicale. We also investigated Ciettaicale response under short-term salinity stress, according to the abovementioned considerations. We compared Ciettaicale with other commercial tomato cultivars grown under environmental controlled conditions: we chose San

Marzano, which shared with Ciettaicale a well adaptation to the Mediterranean climate (**Chapters 2 and 3**), and Moneymaker, which is a worldwide standard cultivar widely used in research studies (**Chapter 4**). We used a physiological/biochemical approach to screen the effects of salt and drought stresses on two plant phenological stages: germination and vegetative stages, respectively. Finally, in an autumn-winter greenhouse hydroponic experiment we evaluated yield and fruit quality of Ciettaicale under salt stress, in comparison with other two Southern Italy tomato landraces (Linosa and Corleone) and one commercial cultivar (UC82-B) (**Chapter 5**).

Chapter 2 focused on the effects of different sodium chloride (NaCl) concentrations-containing media (0, 25, 50 and 100 mM NaCl) on germination rate of Ciettaicale and San Marzano. Then, we monitored the biochemical dynamics in the germination and post-germination stages of Ciettaicale and San Marzano seeds for 5 days under 25 mM NaCl, a moderate salt concentration that induced clear differentiation in the biochemical profile, and then in the germination rate, of Ciettaicale and San Marzano germinating seeds. In particular, we evaluated the dynamics of some endosperm weakening enzymes, namely endo-β-mannanase, β-mannosidase and α-galactosidase, as well as starch and total soluble sugars contents. Finally, analyses of some indicators of redox state such as hydrogen peroxide (H_2O_2) content, catalase activity and total antioxidant capacity were also performed in order to evaluate the salt-induced differences between Ciettaicale and San Marzano at germination and post-germination stages.

The screening genetic populations at germination level seems to be a simple and useful strategy to identify salt tolerance in tomato, despite the QTLs involved in salt resistance during germination can be differently localized comparing to the vegetative stage (Foolad, 1999). According to these findings, in **Chapters 3** we explored the response of Ciettaicale and San Marzano at vegetative stage to strong NaCl concentrations (0, 300, 450 and 600 mM) in irrigation water for a short-term (one week) exposure to exacerbate differences on stress response between the two genotypes, mimicking supplemental irrigation in field conditions in marginal Mediterranean coastal areas during drought period. We correlated the results provided by chlorophyll *a* fluorescence and gas exchange analyses with the content of photosynthetic pigments and the distribution of Na^+, K^+, sugars and total antioxidant activity between source and sink organs.

Moreover, in order to investigate the effects of 20-days drought stress in Ciettaicale and Moneymaker plants, we evaluated *in vivo* chlorophyll *a* fluorescence and leaf water potential, together with the analyses of non-structural carbohydrates, hormonal and antioxidant responses in source and sink organs (**Chapter 4**).

We assessed the effect of salt treatments (60 mM and 120 mM NaCl) on fruit yield and quality parameters of different genotypes (Ciettaicale, Corleone, Linosa and UC-82B) grown from September 2016 to February 2017 in the greenhouse of the Department of Agriculture, Food and Environment - University of Pisa (**Chapter 5**). We evaluated cation and sugars contents as well as functional compounds, such as lycopene and polyphenols. Part of these metabolic determinations was carried out at the Department of Plant and Microbial Biology – University of Zürich, where I spent my third PhD year.

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Chapter 2

Differential effects of sodium chloride on germination and post-germination stages of two tomato genotypes

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Abstract

Salinity induces inhibition and/or delay of seed germination. The imbibition phase re-actives enzymes involved in seed reserve mobilization in order to sustain embryo growth, but also implies reactive oxygen species (ROS) production. A balance between ROS content and antioxidant scavenging activity is necessary to maintain cellular redox state and signalling. Tomato seed endosperm contains large amounts of galacto(gluco)mannans, which are hydrolysed by the synergic action of cell wall enzymes, including endo-β-mannanase, β-mannosidase and α-galactosidase, leading to the completion of germination. The effect of different sodium chloride (NaCl) concentrations-containing media (0, 25, 50 and 100 mM NaCl) on germination rate of two Italian tomato genotypes (Ciettaicale and San Marzano) was evaluated. Further biochemical analyses were performed during seed germination under 25 mM NaCl for 5 days. Salt induced a promotion of endo-β-mannanase and β-mannosidase in Ciettaicale, as well as starch mobilization contributing the increment of total soluble sugars and providing more energy to maintain organogenesis and scavenge oxidative stress. In fact, a boosted total antioxidant activity and catalase activity coped with high hydrogen peroxide content in Ciettaicale under stress. Conversely, in San Marzano, we found some salinity-induced physiological changes only at the end of our observation, suggesting that seeds were not dormant, but impaired by the stress to conclude properly the germination process.

Keywords

Salt tolerance; Germination; Tomato; Redox status; Cell-wall enzymes; Soluble sugars; Starch

2.1 Introduction

Salt stress causes injury in seed germination both due to osmotic stress, which compromises water uptake required for the reactivation of quiescent seed metabolism, and due to ion toxicity that could damage directly seed tissues and the embryo (Huang and Redmann, 1995). Improper irrigation practices, as well as the absence of significant rainfall and the strong evaporation in warm region, often contribute to the accumulation of salt precipitates on the soil superficial layers where seed germination occurs, resulting in the inhibition and/or delay of seed germination and deterioration (Li et al., 2014b).

During germination, a strong increase in the respiratory activity typically occurs, as well as reactive oxygen species (ROS) overproduction (Bailly, 2004). This unstable cellular condition is boosted due to osmotic stress, like salinity (Kranner and Seal, 2013). ROS may lead to oxidative stress, but also could participate as signalling molecules governing seed physiology. Detoxifying enzymes and antioxidant molecules play an important role to maintain ROS levels under control in order to avoid oxidative burst and thereby ensuring the signalling function maintenance (Bailly, 2004; Kranner and Seal, 2013).

For most seeds, germination starts with the uptake of water by imbibition, followed by the embryo expansion, and is completed with the radicle emergence (Finch-Savage and Leubner-Metzger, 2006). Tomato seed embryo is surrounded by two covering tissues: the endosperm and the testa, which act as mechanical obstacles for the radicle protrusion. Thus, in tomato the occurrence of endosperm weakening and testa degradation are also required for the completion of germination (Groot et al., 1988).

Tomato endosperm cell wall contains large amounts of galacto(gluco)mannans. Endo-β-mannanase (EC 3.2.1.78), β-mannosidase (EC 3.2.1.25) and α-galactosidase (EC 3.2.1.22) are key enzymes involved in the complete hydrolysis of galactomannans. These enzymes act on endosperm polymer chains releasing oligo- and mono-saccharides, which are used as fuel for the growing embryo (Groot et al., 1988; Nonogaki and Morohashi, 1996). In particular, endo-β-mannanase activity differs among tomato seed tissues and pre- and post- germination events; moreover, its activity has been generally recognized as marker for tomato germination (Nonogaki et al., 1998; Nonogaki and Morohashi, 1999). Other cell-wall degrading

enzymes, like polygalacturonase, chitinases and glucanases, are also involved in the weakening of the endosperm during and after germination (Sitrit et al., 1999; Petruzzelli et al., 2003).

Most commercial tomato cultivars are sensitive to salinity during seed germination and at early seedling growth stages (Jones, 1986). Germination rate of tomato seeds is reduced at relative low salt concentrations. At high salt concentrations only few genotypes are able to germinate, but with low percentages and/or lengthening the time needed to complete germination. Indeed, at moderate salt concentrations, the capacity to germinate is variable among tomato genotypes (Cuartero and Fernandez-Munoz, 1999). Overall, environmental and genetic factors mediate tomato dry seed metabolic profile which is in turn associated with germination vigor (Rosental et al., 2016).

Previously, we found a clear differentiation between two Italian tomato genotypes, the landrace Ciettaicale and the well-known commercial cultivar San Marzano, in response to high salinity levels at vegetative stage (Moles et al., 2016). Here, we investigated the effect of sodium chloride (NaCl) on the germination process of these two tomato genotypes. Seeds were sown in different NaCl concentrations-containing media (0, 25, 50 and 100 mM) and the germination rate was monitored. Further analyses of the dynamics of some hydrolytic enzymes involved in endosperm weakening, as well as starch and total soluble sugars contents in seeds sown at 25 mM NaCl were evaluated. Finally, analyses of some indicators of redox state such as hydrogen peroxide (H_2O_2) content, catalase activity and total antioxidant capacity were also performed in order to evaluate the salt tolerance differences between Ciettaicale and San Marzano at germination and post-germination stages.

2.2 Materials and methods

2.2.1 Plant material and growth condition

Ciettaicale seeds (De Angelis S.r.l., Tolve, Italy) and San Marzano seeds (Blumen, Milan, Italy) were sterilized with diluted bleach as previously described (Huarancca Reyes et al., 2015). Sterilized seeds were sown on square plates containing 10 g/L sucrose, 4.33 g/L Murashige-Skoog (MS) medium, 0.8% agarose, pH 5.7 plus 0 mM, 25 mM, 50 mM or 100 mM NaCl. Each plate was divided in two parts containing 15

seeds per tomato genotype. Plates were covered with aluminium foil and placed at 4°C for 48 h. After vernalization, plates were transferred in a growth chamber at 25°C for 48 h under dark, and then were exposed under light (100 μmol m-2 s-1; 12/12 h photoperiod) at 25°C. Germination rates were monitored in a time course within 15 days after vernalization (0, 1, 2, 3, 4, 5, 10 and 15 d) using four square plates for each salt treatment. For biochemical analyses, 25 mM NaCl-containing MS media was selected according to the germination rate results. New set of plates were prepared and samples were collected in a time course within 5 d after vernalization (0, 12, 24, 36, 48, 72, 96 and 120 hours). Collected samples were ground in liquid nitrogen and stored at -80°C for the biochemical analyses.

2.2.2 Cell-wall enzymes activities

Endo-β-mannanase, β-mannosidase and α-galactosidase were assayed following the procedures described in Lara-Núñez et al. (2009). Frozen seeds powder was homogenized in 0.1M HEPES-KOH, pH 8. After centrifugation, the supernatant was used to assay endo-β-mannanase and α-galactosidase activities, while the pellet was processed to assay β-mannosidase. Endo-β-mannanase: an aliquot of the supernatant, opportunely diluted, was put on 0.8% (w/v) agarose plates containing 0.05% (w/v) locust bean galactomannan and incubate for 24 h at 25°C. After incubation, the agarose gel plates were stained with 0.5% (w/v) Congo red dye. The hydrolyzed areas were visible as clear circles on a dark background. The diameter of the hydrolyzed area is logarithmically related to the enzyme activity and was quantified by comparison with endo-β-mannanase standards. β-mannosidase: the pellet was resuspended in 0.1M HEPES-KOH, pH 8 containing 0.5M NaCl. After appropriate dilutions, an aliquot of this solution was incubate in McIlvaine buffer (0.1M citric acid, 0.2M Na_2HPO_4, pH 5) containing 2mM *p*-nitrophenyl-β-D-mannopyranoside for 2 h at 37°C. Then, 0.2M Na_2CO_3 solution was added to stop the reaction and absorbance was measured at 405 nm (ε =18400 M^{-1} cm^{-1}). α-galactosidase: an aliquot of the supernatant was first mixed with a solution of 2% polyethylenamine and then incubated in McIluvaine buffer (0.1M citric acid, 0.2M Na_2HPO_4, pH 4.5) containing 10mM *p*-nitrophenyl-α-D-galactopyranoside for 15 min at 37°C. Finally, 0.2M Na_2CO_3 solution was added and absorbance was measured at 405 nm (ε =18400 M^{-1} cm^{-1}).

2.2.3 Total soluble sugars and starch quantifications

Frozen seeds powder was extracted in perchloric acid as described in Tobias et al. (1992). After centrifugation, neutralized supernatant was assayed for glucose, fructose and sucrose contents (pooled as total soluble sugars, TSS) by coupled enzymatic assay methods and measuring the increase in A_{340}, as in Huarancca Reyes et al. (2018). A known amount of glucose was used as an external standard. Incubations of samples and standards were carried out for 30 min at 37°C. The reaction mixtures were included: 100mM Tris-HCl, pH 7.6; 3mM $MgCl_2$; 2mM ATP; 0.6mM NAD; 1 unit glucose-6-phosphate dehydrogenase and 1 unit hexokinase. The fructose was assayed as described for glucose plus the addition of 2 units of phosphoglucose isomerase and measuring the increase in A_{340} as above. Sucrose was first broken down into glucose and fructose using 85 units of invertase (in 15mM Na-acetate, pH 4.6) and was assayed through its resulting glucose as described above.

Starch in the insoluble pellet was determined according to the K_2-KI method adapted from Pompeiano and Guglielminetti (2016). The pellet was resuspended in 10mM KOH, boiled for 1 min and finally neutralized with HCl. The starch standard solution was prepared using potato soluble starch processed as above. To both samples and standards, freshly prepared iodine solution (0.13% K_2 and 0.3% KI, dissolved in dH_2O) was added and the absorbance was measured at A_{595}.

2.2.4 H_2O_2 content, catalase activity and total antioxidant capacity

H_2O_2 was measured spectrophotometrically in frozen seeds powder according to the potassium iodide method used in Zou et al. (2015). Frozen seeds powder was homogenized in 0.1% trichloroacetic acid (TCA) on ice and centrifuged at 12,000×g for 15 min at 4°C. Concentrations of H_2O_2 in the assay mixture (supernatant, 10mM potassium phosphate buffer, pH 7.0, and 1M KI, 1:1:2) were measured at 390 nm after incubation of 1 h in darkness. The amount of hydrogen peroxide was calculated using a standard curve prepared with known concentrations of H_2O_2.

Catalase (EC 1.11.1.6) assay protocol was adapted from Pistelli et al. (2017). Frozen seeds powder was homogenized in the extraction buffer, consisting of 50mM sodium phosphate buffer, pH 7.0, 1mM EDTA, 1mM phenylmethylsulfonyl fluoride (PMSF), and 2% (w/v) insoluble polyvinylpolypyrrolidone (PVPP). The homogenate

was centrifuged at 20000×g for 30 min at 4 °C and the supernatant was used to assay catalase activity according to Aebi (1974), by monitoring the decomposition of H_2O_2. The enzyme activity was calculated by measuring the decrease of absorbance at 240 nm in 1 min ($\varepsilon = 0.04\ mM^{-1}\ cm^{-1}$).

Total antioxidant capacity (TAC) was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay according to (Wong et al., 2006) with some modifications. Frozen seeds powder was homogenized in dH_2O. The mixture was allowed to stand at room temperature for 1 h in darkness, with occasional agitation. The aqueous extract was obtained after centrifugation at 20000×g for 10 min. Briefly, a 0.1 mM solution of DPPH in methanol was prepared to obtain a stable absorbance of 1 ± 0.02 units at 515 nm. An aliquot of the aqueous extract (with appropriate dilution, if necessary) was added to 1 mL of methanolic DPPH solution. The change in absorbance at 515 nm was measured after 30 min of incubation in darkness. The antioxidant capacity based on the DPPH free radical scavenging ability of the extract was expressed as μmol Trolox equivalents per of plant material on fresh basis.

2.2.5 Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) and the mean values were compared using Tukey's test ($P<0.05$) to check the significant differences. Statistical analysis was performed with Statgraphics Centurion XVII (Statpoint Technologies, Inc. Warrenton, Virginia, U.S.A.) software.

2.3 Results and discussion

Our study reported the effects of salt stress on the germination of two Southern Italian tomato genotypes. After an initial screening of different [NaCl]-containing MS media (0-100 mM NaCl), we monitored the biochemical dynamics in the germination of Ciettaicale and San Marzano seeds under 25 mM NaCl for 5 d.

We observed different patterns of seed germination rate in both tomato genotypes (Fig. 2.1). Clearly, Ciettaicale and San Marzano differed into the achievement of germination already from control condition (0 mM NaCl). In absence of salt, Ciettaicale reached around full germination percentage after 5 d, while

approximately 75% of the San Marzano seeds germinated. Nevertheless, both tomato accessions reached 100% germination after 10 d under control condition.

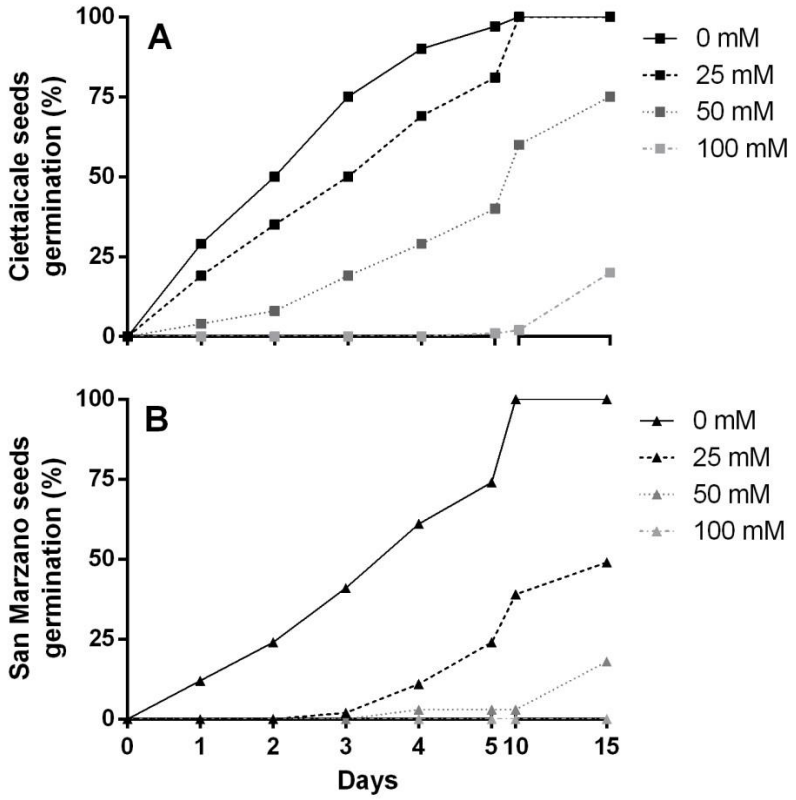

Figure 2.1. Seed germination rate percentage (%) of [A] Ciettaicale and [B] San Marzano tomato genotypes grown under different sodium chloride (NaCl) concentrations (0, 25, 50, 100 mM)-containing MS media recorded in 15 d after vernalization.

In tomato, the speed of germination under osmotic condition depends on the genetic background (Foolad et al., 2007), but also on the temperature, water potential and exogenous hormonal concentrations used in the investigation assays (Dahal et al., 1997). Interestingly, 25 mM NaCl revealed clear differences between both tomato genotypes in germination rate. Whereas Ciettaicale showed a slight reduction in its germination rate (approx. 75%) during the first 5 d comparing to control condition (approx. 100%), the radicle protrusion in San Marzano was drastically delayed and its germination rate was reduced to approximately 25%. Under 50 mM NaCl, 40% of Ciettaicale seeds were germinated after 5 d, while we recorded only 3% germination in San Marzano. Moreover, 100 mM NaCl inhibited the protrusion of radicle in both tomato genotypes in the short term. However, 20% of Ciettaicale

seeds were germinated after 15 d, while no germination was observed in San Marzano.

Weakening of endosperm is considered an indispensable event to complete the germination in tomato (Groot et al., 1988). Dahal et al. (1997) suggested that anatomical differences in the endosperm region, like the presence of free space that could help the management of water uptake during imbibition, may contribute to the activity of the hydrolytic enzymes involved in the endosperm cell-wall degradation. Moreover, it has been hypothesized that these structural differences could support the variance of germination rates and consequently the salt tolerance observed among tomato genotypes (Fooland and Jones, 1991). Endo-β-mannanase was originally considered the rate-limiting enzyme involved in the endosperm weakening and, in turn, in the radicle emergence; however, many works demonstrated that other enzymes are also likely to be involved in this complex process and their temporal and space synergisms govern the pre- and post-germination events (Toorop et al., 1996; Dahal et al., 1997; Still and Bradford, 1997; Pinto et al., 2007). In absence of salt, Ciettaicale and San Marzano differed in endo-β-mannanase activity between 24 and 48 h (Fig. 2.2A). This result was in accordance with Nomaguchi et al. (1995), who observed higher mannanase activity in faster germinating tomato line than in a slowly germinating line. Ciettaicale showed a promotion in endo-β-mannanase activity in the first 24 h under 25 mM NaCl comparing to control condition. Instead, San Marzano seeds under 25 mM NaCl showed a slight increase of mannanase activity after 72 h, which was lower than the respective control condition.

Nonogaki et al. (1992) showed that the mannanase activity could also increase in osmotically treated tomato seeds, despite no radicle protrusion was observed when seeds remain in the osmotic media. In this context, we cannot exclude the involvement of abscisic acid (ABA) in the different response induced by salt in Ciettaicale and San Marzano. In fact, ABA effect on tomato seed germination has been deeply investigated leading to the conclusion of its inhibitory effect on mannanase activity (Toorop et al., 1999; Toorop et al., 2000). Analysis of β-mannosidase activity in Ciettaicale showed a gradual increase during the time course under control condition, while the activity level was significantly higher between 36 and 72 h when seeds were grown in 25 mM NaCl (Fig. 2.2B).

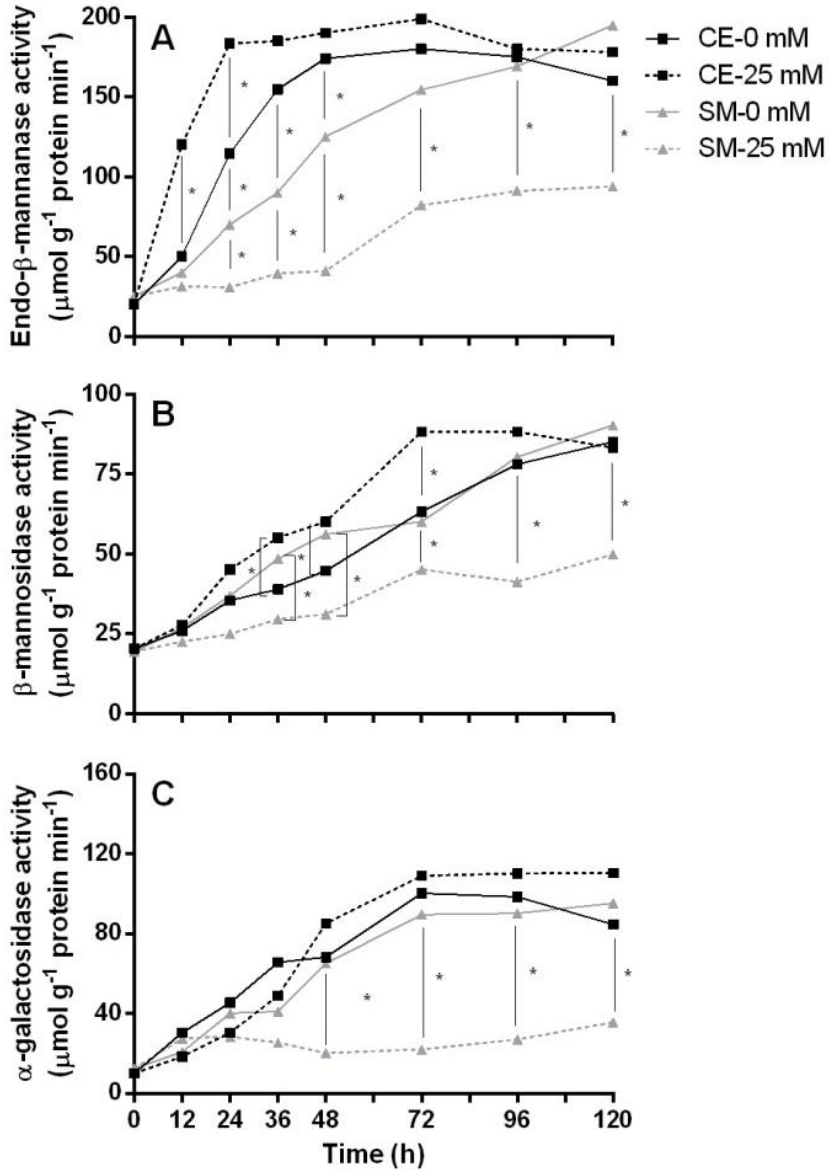

Figure 2.2. Enzymatic activity of [A] endo-β-mannanase, [B] β-mannosidase and [C] α-galactosidase in germinating Ciettaicale (CE) and San Marzano (SM) tomato genotypes. Seeds were grown in control MS media (0 mM) or containing 25 mM sodium chloride (NaCl). Enzymatic activities were recorded within 120 h after vernalization. Asterisks (*) indicated statistical differences using Tukey's test ($P < 0.05$).

The activity of this exo-enzyme is often associated with the action of endo-β-mannanase, but this relation is not always required (Mo and Bewley, 2003). The promotion of β-mannosidase could derive from the high level of its available substrate represented by mannose residues from the mannan backbone released by endo-β-mannanase. According to this hypothesis, our results showed that San

Marzano grown in 25 mM NaCl exhibited lower mannosidase activity in comparison to the control, since lower mannanase-released substrate was accessible (Fig. 2.2A and B). The activity of α-galactosidase in Ciettaicale did not show changes when compared control and 25 mM NaCl-containing MS media conditions during the time course (Fig. 2.2C). Similar activity pattern to Ciettaicale was observed in San Marzano when grown in control condition. However, the α-galactosidase activity of San Marzano grown in 25 mM NaCl was drastically lower than its respective control. Feurtado et al. (2001) reported no changes in the amount of α-galactosidase activity in tomato cv. Glamour seeds subjected to both PEG and ABA treatments comparing to control.

The study of Mounet et al. (2007) reported a well-detailed list of metabolites present in mature tomato dry seeds, including high amount of fatty acids, such as linoleic and oleic acids, which represent the main energy reserve for an early germination. Among other seed reserves, starch is also present but in small concentration. Overall, dry tomato seeds contain sugars like mannose, sucrose, fructose, glucose and planteose, where the latest one is the major tomato seed galactooligosaccharide with the function to prevent cellular structure damages during seed desiccation and imbibition (Downie, 2003).

Stressed germinating seeds normally required more energy than control seeds. Thus, high concentration of sugars could help the maintenance of energy-expensive organogenesis process providing carbon fuel and/or osmotic adjustment (Monerri et al., 1986). Up-regulation of enzymes activity involved in carbon metabolism are associated with the increased level of sugars that sustain the vigour of primed seeds and seedlings (Gurusinghe and Bradford, 2001; Kaur et al., 2005; Zheng et al., 2016; Farooq et al., 2017).

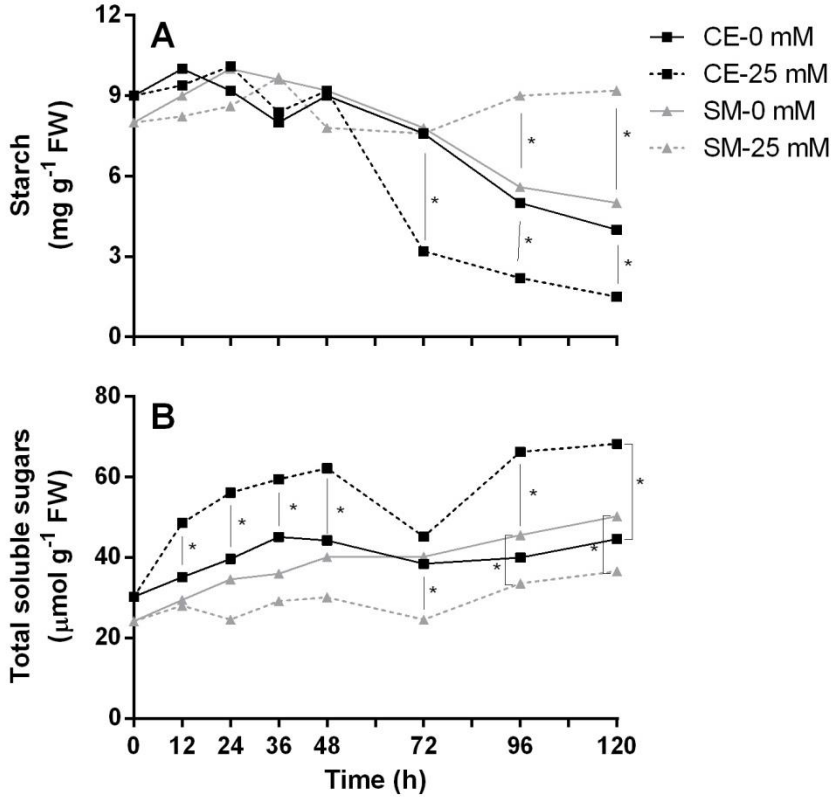

Fig. 2.3. Contents of [A] starch and [B] total soluble sugars in germinating Ciettaicale (CE) and San Marzano (SM) tomato genotypes. Seeds were grown in control MS media (0 mM) or containing 25 mM sodium chloride (NaCl). Carbohydrates content were recorded within 120 h after vernalization. Asterisks (*) indicated statistical differences using Tukey's test ($P < 0.05$).

After exposure of seeds to the light, we observed a general decreasing trend in starch content in Ciettaicale grown under control and salinity conditions; this trend was also observed in San Marzano under control growth condition (Fig. 2.3A). Interestingly, treated Ciettaicale germinating seeds showed an increase in the starch turnover in comparison to control after 72 h. Conversely, no changes in starch amount were found in San Marzano grown in salt-containing media during the time course. Salt applied during germination could have contradictory effects on the activity of amylases, one of the main enzymatic classes involved in starch degradation, depending on the salt concentration and the genotype (Sidari et al., 2008; Adda et al., 2014; Chiraz et al., 2014). In addition, San Marzano grown in salt condition had lower TSS content comparing to its control, and this difference was statistically pronounced after 72 h. Differently, germinating Ciettaicale seeds mostly accumulated more TSS when grown in salt condition than its control (Fig. 2.3B).

Higher content of sugars is associated with a promoted amylase activity in tomato primed seeds comparing to unprimed ones (Nawaz et al., 2011; Amooaghaie and Nikzad, 2013), as we can also hypothesized in Ciettaicale seeds under salt stress. However, since fatty acids constitute the main reserve in tomato seeds, we cannot exclude that Ciettaicale salt-induced TSS accumulation is related to gluconeogenesis, which usually occurs during tomato seed germination (Eckardt, 2005).

During imbibition, the reactivation of various processes, such as mitochondrial electron transport and lipid/protein catabolism, supports energy and intermediates production, but also triggers the release of superoxide anion ($O^{2-\cdot}$), H_2O_2 and hydroxyl radical ($OH^{\cdot}$) (Puntarulo et al., 1991). Here, we found a conventional pattern of H_2O_2 content in Ciettaicale and San Marzano under control conditions (Fig. 2.4A), in accordance with previous report (Li et al., 2014a). However, tomato seeds germinated in 25 mM NaCl-containing media showed different H_2O_2 pattern depending on the genotypes. H_2O_2 values were higher in Ciettaicale seeds grown in salt condition comparing to its control, reaching a peak at 36 h. On the other hand, the content of H_2O_2 in San Marzano seeds grown in salt condition was maintained constant during the first 48 h, followed by a dramatic increase in the rest of the time course. During the early phases of germination, H_2O_2 is a regulatory modulator of the feedback loop which governs the balance between ROS production and scavenging (Lariguet et al., 2013). Detoxification of H2O2 is mediated by catalase, but ascorbate peroxidase (APX) and guaiacol peroxidase (POD) also participate in the H_2O_2 catabolism (Chen and Arora, 2010). Catalase remains active in both quiescent and germinating seeds probably because no energy is required for its activity, whereas APX activity seems to have a germination-induced stimulation and its activity is inserted in the ascorbate-glutathione cycle, which in turn requires other enzymatic and non-enzymatic participants (De Tullio and Arrigoni, 2003). Our results showed similar catalase activity in Ciettaicale and San Marzano under control condition except between 36 and 48 h in which San Marzano presented significantly lower activity than Ciettaicale (Fig. 2.4B). Under 25 mM NaCl, Ciettaicale showed higher catalase activity than its control after 48 h, while San Marzano showed lower activity than its control after 72 h.

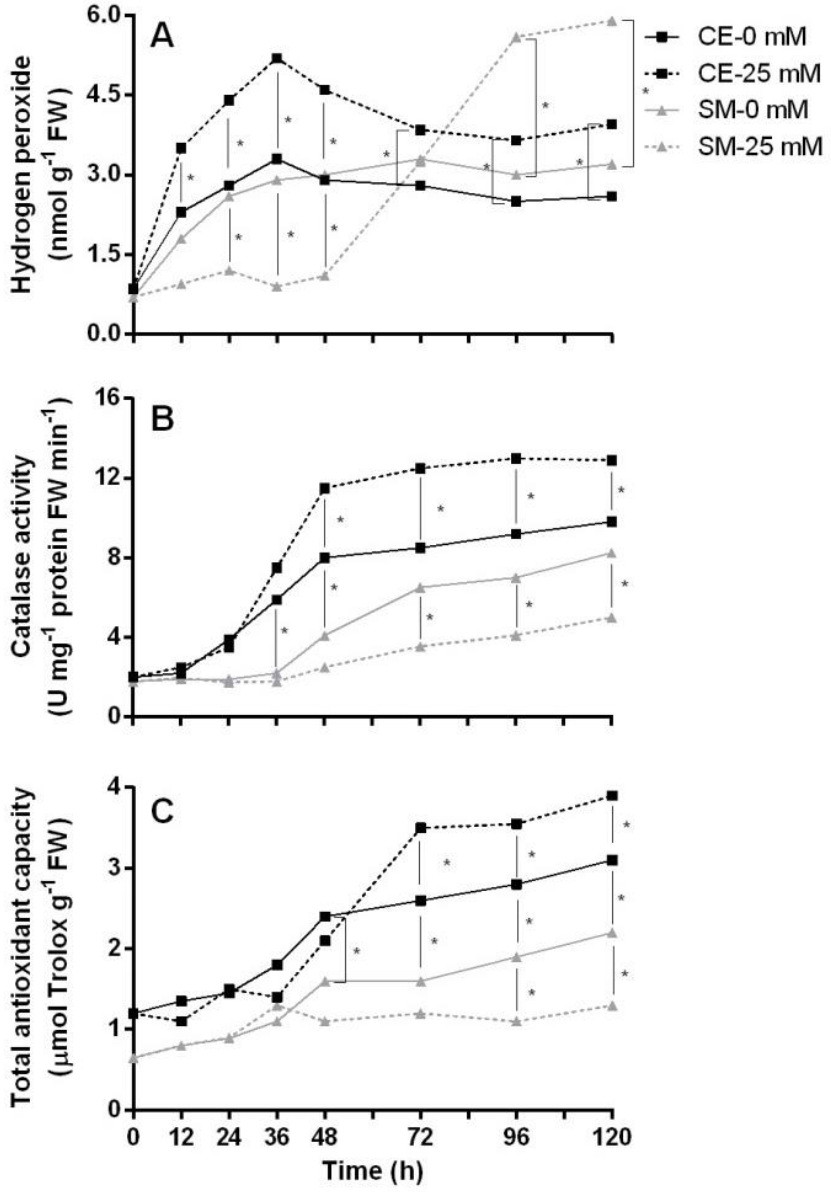

Fig. 2.4. [A] Hydrogen peroxide concentration, [B] catalase activity and [C] total antioxidant capacity in germinating Ciettaicale (CE) and San Marzano (SM) tomato genotypes. Seeds were grown in control MS media (0 mM) or containing 25 mM sodium chloride (NaCl). Results were recorded within 120 h after vernalization. Asterisks (*) indicated statistical differences using Tukey's test ($P < 0.05$).

The excessive accumulation of H_2O_2 is considered one of the factors causing seed germination inhibition when the antioxidant machinery cannot control the oxidative burst (Bailly, 2004). Here, the increment in H_2O_2 content observed after 72 h in San Marzano under salt condition indicated that seeds were non-dormant and it probably represents a starting point of seed physiology changes, for example acting on the

ABA metabolism (Ishibashi et al., 2017) and also on endosperm enzymatic weakening (Fig. 2.2A and B). In fact, ROS might mediate cell wall loosening during seed germination and seedling growth (Schweikert et al., 2002; Muller et al., 2009), as well as endosperm degradation (Morohashi, 2002), allowing cell expansion.

To scavenge the imbibition-induced ROS production, seeds arrange their own metabolism producing antioxidant molecules, such as α-tocopherol, flavonoids and phenols (Bailly, 2004). In particular, the ascorbate system is a crucial component in the antioxidant machinery during the early germination phases (De Tullio and Arrigoni, 2003). Accordingly, we recorded an increasing trend in TAC content in both Ciettaicale and San Marzano control seeds. Interestingly, the two genotypes differed in TAC after 48 h, suggesting constitutively differences in the various antioxidant compounds metabolism (Fig. 2.4C). Under 25 mM NaCl, TAC content was higher in Ciettaicale comparing to its control after 72 h, while in San Marzano was lower after 96 h comparing to its control. Our findings were in line with Aloisi et al. (2016), who reported that salinity in quinoa germinating seeds induced high levels of bioactive molecules and then activated radical scavenging capacity in a genotype-dependent manner.

2.4 Conclusion

In conclusion, our results showed that moderate NaCl concentration (25 mM) induced clear differentiation in the biochemical profile and then in the germination rate of Ciettaicale and San Marzano germinating seeds. Salt induced a promotion of endo-β-mannanase and β-mannosidase in Ciettaicale, as well as starch mobilization contributing the increment of total soluble sugars and providing more energy to maintain organogenesis and scavenge oxidative stress. In fact, a boosted total antioxidant activity and catalase activity coped with high hydrogen peroxide (H_2O_2) content in Ciettaicale under stress. Conversely, in San Marzano, we found some salinity-induced physiological changes only at the end of our observations, suggesting that seeds were not dormant, but impaired by the stress to conclude properly the germination process. Our future perspective will be to study more deeply the performances of Ciettaicale and San Marzano during germination and post-germination stages looking at hormonal profile and using different osmotic media.

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Chapter 3

The efficient physiological strategy of a tomato landrace in response to short-term salinity stress

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Abstract

Landraces represent an important part of the biodiversity well-adapted under limiting environmental conditions. We investigated the response of two Southern Italy tomato landraces, the well-known San Marzano (our commercial standard) and a local accession called "Ciettaicale", to different levels of sodium chloride in water irrigation (from 0 up to 600 mM) for a short-time exposure (one week). The combination of the chlorophyll *a* fluorescence and gas exchange analyses suggested that Ciettaicale maintained a higher efficiency of photosystem II (PSII) photochemistry and CO_2 utilization at high salinity concentrations than San Marzano. Stomatal and non-stomatal limitations occurred in San Marzano according to the reduction of maximum efficiency of PSII photochemistry and the increase of intercellular CO_2 concentration. Higher Na^+/K^+ ratio and higher concentration of total soluble sugars contributed to non-stomatal limitations in San Marzano leaves. These effects were significantly less evident in Ciettaicale. We also observed changes in total antioxidant capacity and leaf pigment content that emphasized the occurrence of modifications in the photosynthetic apparatus according to salt gradient. The more efficient assimilates supply and an integrated root protection system provided by sugars and antioxidants can explain the significantly higher root/shoot ratio in Ciettaicale. Overall, our results suggest that a comprehensive assessment of salinity tolerance in a genotypes comparison could be also obtained evaluating plant response to high salinity levels at early vegetative stage. In addition, further studies will be carried out in order to evaluate the possibility of using Ciettaicale in tomato improvement programs.

Keywords

Salt tolerance, tomato landrace, chlorophyll *a* fluorescence, gas exchange, soluble sugars, antioxidants

3.1 Introduction

Soil salinization is a growing problem in Mediterranean basin resulting from seawater intrusion into freshwater aquifers and irrigation with brackish water (Carillo et al., 2011). Salinity affects not only crop production but also several aspects of the plant‘s physiology and biochemistry. Thus, high levels of ions in the soil solution induce quickly osmotic effects reducing the water uptake by roots (physiological drought), and the increasing excess uptake of ions starts to produce cytotoxicity and pH/nutrient imbalance interfering with physiological and cellular processes (Hunsche et al., 2010; Munns and Tester, 2008).

Plants regulate cell biophysics promoting cellular turgor decrease and leaf stomatal closure to avoid water losses due to evapotranspiration, as well as a large root system to reach and absorb accessible water conserving the inner water balance (Rigano et al., 2014). Consequently, a reduction of stomatal conductance limits CO_2 uptake and CO_2 availability as substrate for photosynthesis (Chaves et al., 2009). Under salinity the chloroplast is exposed to excessive light excitation energy that triggers the formation of reactive oxygen species (ROS) (Apel and Hirt, 2004). The resulting oxidative burst can affect plant photosynthesis that is associated with the change of source-sink demand and the inhibition of growth. Photosystem II (PSII) is a crucial point in the chloroplast energy management and the estimation of its activity in combination with CO_2 fixation can reflect plant health state during stress events (Krall and Edwards, 1992). In fact, excessive radiation destabilizes PSII electron transport activity promoting photoinhibition and photodamage events (Degl'Innocenti et al., 2009). However, despite ROS generation participates to detrimental effects of salt stress, new evidences are emerging about the involvement of ROS production in salt stress signalling (Apel and Hirt, 2004; Miller et al., 2010; Xiong et al., 2002). Crops show a gradient of salt tolerance that is the result of a combination of anatomical, physiological and molecular strategies more or less efficient to minimize the detrimental effects of salinity. The main salt tolerance target is keeping the ionic excess away from the metabolic active tissues to preserve leaf photosynthesis and meristematic activity. This aim is obtained generally thanks to the regulation of membrane ionic channels and pumps at different structural levels (Deinlein et al., 2014; Munns and Tester, 2008). Evidences supported the important role of ion transporters in salt tolerance (Apse et al., 1999; Shi et al., 2000; Zhang

and Blumwald, 2001). In addition, the biosynthesis of chaperones and osmolytes such as proline, betaine, polyalcohol compounds and sugars can improve osmotic adjustment and, at the same time, act as signals part of an intense cross-talking (Munns, 2002; Cardi et al., 2015; Mittal et al., 2016). However, salt tolerance is a complex trait that is strictly related to salt concentration, stress exposure, genotype and plant growth stage (Flowers, 2004).

Harsh environmental conditions, such as an endurable soil salt contamination, have certainly contributed to the selection of genetic or physiological traits in individuals among the sessile plant biodiversity. During evolution these traits have ensured a high fitness to tolerate and survive under stress (Rigano et al., 2014). However, natural selection has not been in agreement with crop improvement programs, mostly driven by market force to meet the consumers. The history of tomato (*Solanum lycopersicum* L.) breeding programs is an example of this controversy. The commercial tomato germplasm is the result of a series of genetic bottlenecks during domestication to which limited accessions were subjected (Koenig et al., 2013; Sacco et al., 2015). Yield, shelf-life, fruit size and organoleptic quality were the main targets of tomato breeders at the expense of some traits such as stress tolerance/resistance (Bai and Lindhout, 2007). Given its importance as crop characterized by its economic and nutritional relevance, tomato is a well characterized model system and the availability of its genome is useful for biological investigation (Tomato Genome Consortium, 2012). Most commercial tomato cultivars are classified as moderately sensitive to salinity (Foolad, 2007). Fortunately, potential drought/salt tolerance traits have been identified within ancestral tomato related germplasm (Liu et al., 2015; Mittova et al., 2002; Orellana et al., 2010) and landraces (Galmes et al., 2013; Rigano et al., 2014; Assimakopoulou et al., 2015). Landraces are also an important part of the cultural heritage (Hagenblad et al., 2012). Local accessions are often predisposed to low-input or organic farming lowering the farmer capital investments thanks to their high adaptability to the territory (Negri, 2003). Thus, the conservation of landrace germplasm appears as a sustainable strategy for scientists and breeders to obtain plants able to counteract the future climate change (Halford and Foyer, 2015). Ciettaicale is a landrace cultivated in Basilicata region (Southern Italy) where is affected by an intense drought fluctuation during summer and by salt-contaminated water from aquifers. However,

Ciettaicale shows an interesting adaptation capacity in these harsh environmental conditions.

The objective of this work was to study salt tolerance observed in Ciettaicale ecotype. We selected San Marzano as standard industrial cultivar which is well adapted to the Mediterranean climate. In a growth chamber experiment we subjected plants at vegetative stage to strong sodium chloride concentrations (the highest NaCl concentration was 600 mM) in irrigation water for a short-term exposure to exacerbate differences on stress response between the two entries mimicking supplemental irrigation in field conditions in marginal Mediterranean coastal areas during drought period. We correlated the results provided by chlorophyll *a* fluorescence and gas exchange analyses with the content of photosynthetic pigments and the distribution of Na^+, K^+, sugars and total antioxidant activity between source and sink organs.

3.2 Materials and methods

3.2.1. Plant material and growth conditions

Ciettaicale seeds (De Angelis S.r.l., Tolve, Italy) and San Marzano seeds (Blumen, Milan, Italy) were germinated in rockwool plugs (Grodan, Roermond, the Netherlands) in growth chamber (temperature 22 ± 1°C, 16 h photoperiod, irradiation intensity 100 μmol photons m^{-2} s^{-1}). After two weeks, germinated seedlings were transplanted in plastic pots containing a mixture of soil and perlite (3:1, v/v) and placed for other eight weeks in the same growth conditions. Plants received distilled water three times a week and nutrient solution (NPK 5-5-5) once a week. For each species, uniform plants were divided into four groups, each with a different NaCl concentration in irrigation water (0, 300, 450 and 600 mM). Distilled water for the control group and salt solutions were applied once a day to the top of the pots.

3.2.2. Biometric analysis and cations content

After one week of treatment, plants were separated into leaves, stems and roots and then weighed (fresh weight, FW). Root tissues were previously washed with water to remove soil and perlite. The dry weights (DW) of plant organs were recorded after drying at 60°C for 72 h. Additional set of plants not used for testing biometric traits, were collected and immediately processed or ground in liquid nitrogen, then stored at

–80°C for further biochemical analyses. For determination of cations content, powdered dry leaf (youngest fully expanded one) or root samples were digested in a solution of HNO_3:H_2O_2 (2:1, v/v). Na^+ and K^+ were determined using atomic absorption spectrometry (AAnalyst-300, HGA-800, Perkin Elmer).

3.2.3. Leaf gas exchange and chlorophyll a fluorescence measurements

Leaf gas exchange and chlorophyll fluorescence parameters were measured simultaneously using an open-type portable measurement system (Li-6400, Li-Cor Inc., NE, USA) equipped with an integrated fluorescence chamber head (Li-6400-40 leaf chamber fluorometer, Li-Cor Inc.). Analysis setting was maintained at 25°C and at growth chamber light condition. Cuvette CO_2 concentration was set at 400 ppm CO_2 and the relative humidity was maintained between 45 and 55%. The measurements were carried out after one week of treatment for all plants of the four compared experimental treatments on youngest fully expanded leaves with similar exposition to radiation. Instantaneous values of net CO_2 assimilation rate (A), transpiration rate (E), stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) were determined. Intrinsic water-use efficiency (A/g_s) and instantaneous water-use efficiency (WUE) were calculated as the A to g_s and A to E ratios, respectively.

The potential efficiency of PSII photochemistry (F_v/F_m) was calculated on dark-adapted leaves as $F_v/F_m = (F_m - F_o)/F_m$, where F_o and F_m are the minimum and maximum fluorescence yield emitted by the leaves in the dark-adapted state respectively. The actual photochemical efficiency of photosystem II in the light (Φ_{PSII}) was determined as $\Phi_{PSII} = (F_m'-F')/F_m'$ (Genty et al., 1989) at steady state, where F_m' is the maximum fluorescence yield with all PSII reaction centres in the reduced state obtained superimposing a saturating light flash during exposition to actinic light and F' is the fluorescence at the actual state of PSII reaction centres during actinic illumination. Non-photochemical fluorescence quenching (NPQ) was determined according to the Stern-Volmer equation as $NPQ = (F_m/F_m')-1$ (Bilger and Bjorkman, 1990).

3.2.4. Analysis of pigments

Pigments were extracted and analysed as previously reported (Pompeiano et al., 2015). Pigments were extracted by incubating tissues (50-100 mg) in 1.5 mL 80%

acetone for 1 week at 4°C in darkness. The absorbance of extracts was measured spectrophotometrically at 470.0, 663.2 and 646.8 nm. These absorbance value swere used for calculation of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and total carotenoids (Car) contents by means of formulae suggested by Lichtenthaler (1987):

$$[\text{Chl } a]\ \mu g\ mL^{-1} = (12.25 \times A_{663.2}) - (2.79 \times A_{646.8})$$

$$[\text{Chl } b]\ \mu g\ mL^{-1} = (21.50 \times A_{646.8}) - (5.10 \times A_{663.2})$$

$$[\text{Car}]\ \mu g\ mL^{-1} = [(1000 \times A_{470.0}) - (1.82 \times [\text{Chl } a]) - (85.02 \times [\text{Chl } b])]/198$$

3.2.5. Total antioxidant capacity

The total antioxidant capacity (TAC) was determined by the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay reported in Chapter 2.

3.2.6. Total soluble sugars quantification

Leaves (youngest fully expanded one) and roots samples (0.5 g FW) were rapidly frozen in liquid nitrogen, ground to a powder, and then extracted and assayed for glucose, fructose and sucrose contents through coupled enzymatic assay methods reported in Chapter 2.

3.2.7. Statistical analysis

Pots were arranged in a randomized complete-block design with ten replicates for each time point. Following Bartlett's test for homogeneity of variance, data were subjected to analysis of variance (ANOVA), and means separation of treatment effects were accomplished using a least significant difference (LSD) test. To identify relationships between the different treatments based on biometric and physiological data recorded at the end of the experiment, multiple factorial analysis (MFA) was carried out (Escofier and Pagès, 2008). MFA was performed in two steps. First a principal component analysis (PCA) was computed for each data set, which was then "normalized" by dividing all its elements by the square root of the first eigenvalue obtained from the PCA. Then, the normalized data sets were merged to form a single matrix, and a global PCA was performed on this matrix. The individual data sets were then projected onto the global analysis to analyze communalities and discrepancies. Each treatment had seven partial points corresponding to the trait classes (biometric, leaf gas exchange, chlorophyll *a* fluorescence, pigments,

antioxidant activity, sugars, and cations). Trait classes that significantly contributed to MFA dimensions were used to explain differences between treatments ($\alpha = 0.05$). The length and the direction of the vectors were directly correlated to their significance within each treatment. A hierarchical clustering on principal components (HCPC) was performed to confirm the product groups observed graphically. All computations were performed with R 3.2.5 (R Core Team, 2016), and the R packages *agricolae* (De Mendiburu, 2015) and *FactoMineR* (Husson et al., 2016) were used.

3.3 Results

3.3.1. Biometric analysis and cations determination

Ciettaicale and San Marzano plants were differently affected by increasing salt concentration. ANOVA indicated that salt treatments significantly affected leaf, stem and epigeal biomass FW as well as leaf DW with no interactions salinity *versus* entries (Table 3.1). Complementary, in Fig. 3.1 are reported all the biometric parameters significantly affected by the interaction between entries and salt treatment as indicated by ANOVA.

Table 3.1. Mean effects of salinity stress on biometric traits one week after treatment.

Factor: salinity treatment*	Leaves FW (g plant^{-1})	Stems FW (g plant^{-1})	Leaves DW (g plant^{-1})	Epigeal FW (g plant^{-1})
0	60.92 a	28.72 a	6.15 b	89.63 a
300	72.60 a	27.15 ab	7.59 a	99.75 a
450	41.50 b	23.38 bc	4.54 c	64.88 b
600	36.13 b	21.70 c	4.85 bc	57.83 b
LSD (0.05)†	11.81	3.85	1.41	13.90

* mM NaCl in irrigation water.

† Means within a column followed by the same letter are not significantly different based on LSD (0.05).

It was observed that 300 mM NaCl induced a promotion in the epigeal organs biomass DW in both entries, while 450 mM and 600 mM negatively affected these parameters (Fig. 3.1B). The main contribution in the highest epigeal biomass observed in Ciettaicale derived from a major investment in stem biomass (Fig. 3.1A). Ciettaicale showed higher root FW and DW along the salt gradient compared to San Marzano, rising to maximum values at 300 mM NaCl (Fig. 3.1C and D). In fact, San Marzano root biomass remained roughly close to the values of the control condition (0 mM NaCl) (Fig. 3.1C and D).

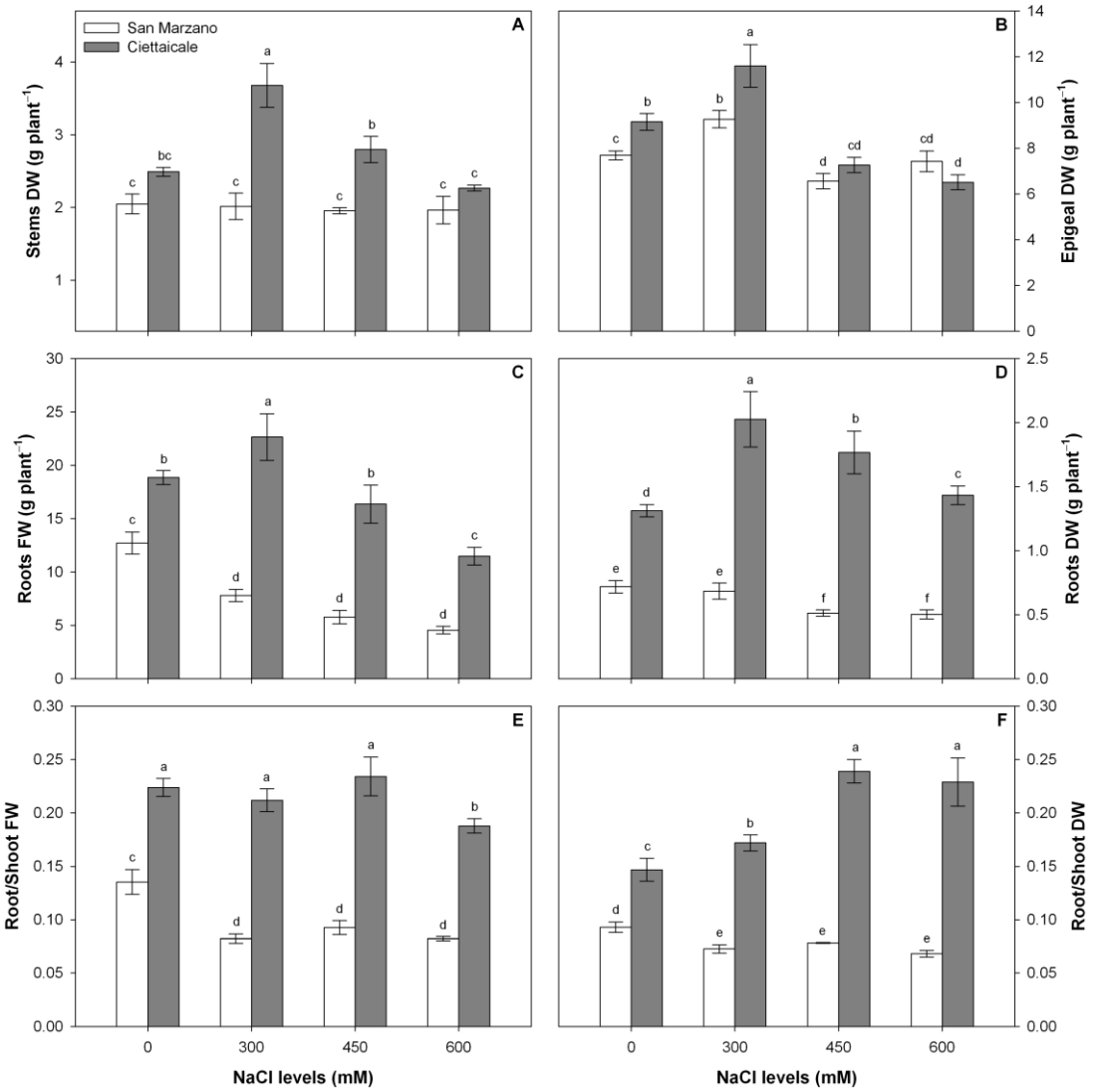

Fig. 3.1. Biometric traits of San Marzano and Ciettaicale tomato plants subjected to different NaCl concentrations after one week of salt stress. [A] stem biomass DW, [B] epigeal biomass DW, [C] root biomass FW, [D] root biomass DW, [E] root/shoot ratio FW and [F] root/shoot ratio DW. Error bars represent the standard error of the mean (n = 10). Bars with same letters are not statistically different from one another according to Fisher's protected LSD (α = 0.05).

Results of ratio root/shoot FW showed that the values in Ciettaicale were higher than San Marzano without differences between 0 mM NaCl and salt treatment, while San Marzano showed a significant decrease at 300 mM NaCl in comparison to 0 mM NaCl and then the ratio was maintained constant up to 600 mM NaCl (Fig. 3.1E).

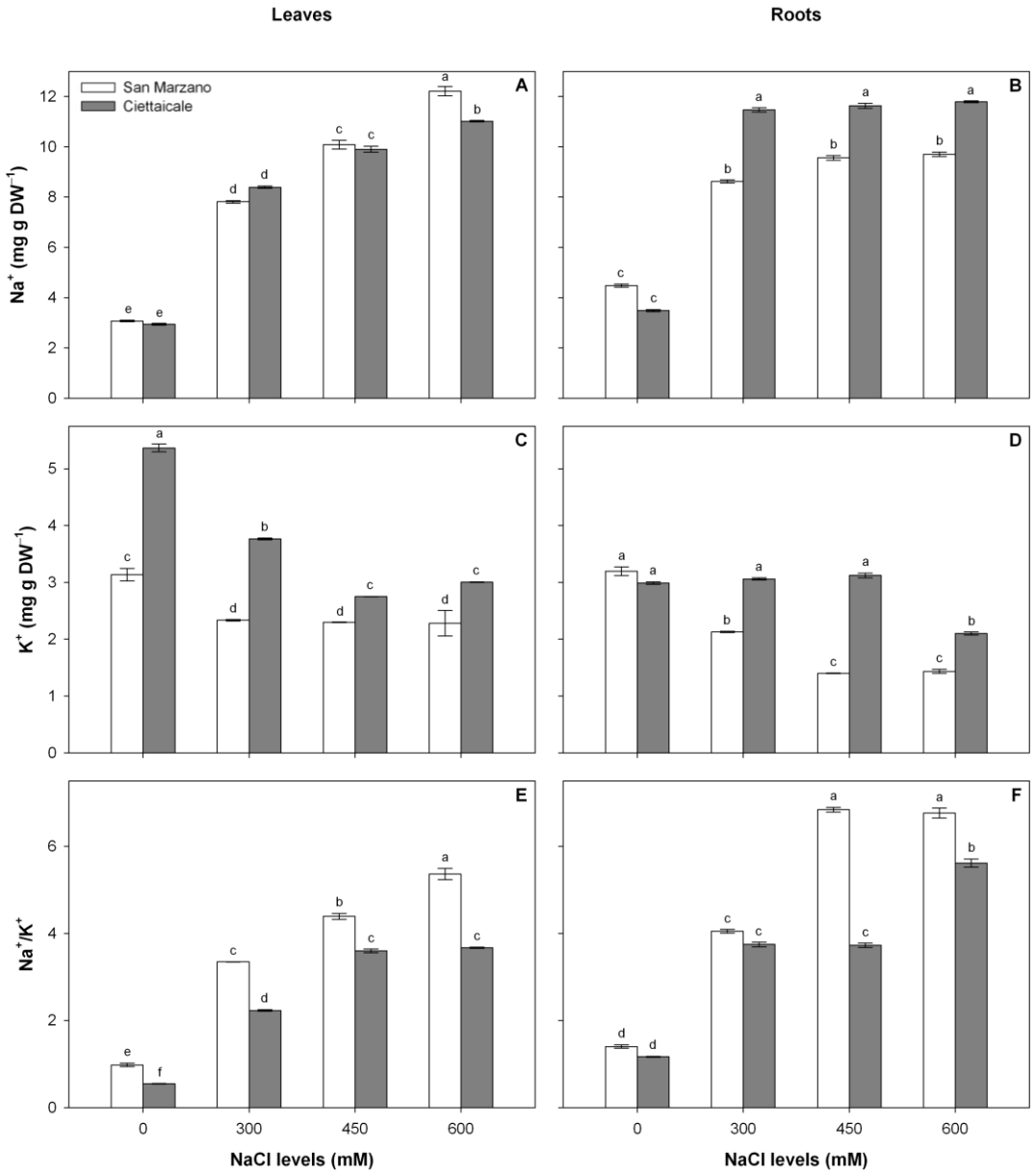

Fig. 3.2. Cations content in San Marzano and Ciettaicale tomato subjected to different NaCl concentrations after one week of salt stress. [A] leaf Na^+ content, [B] root Na^+ content, [C]leaf K^+ content, [D] root K^+ content, [E] Na^+/K^+ ratio in leaves and [F] Na^+/K^+ ratio in roots. Error bars represent the standard error of the mean (n = 10). Bars with same letters are not statistically different from one another according to Fisher's protected LSD (α = 0.05).

Interestingly, the root/shoot DW significantly increased in Ciettaicale under salinity treatment, especially at 450 mM NaCl, while stable values of root/shoot ratio were observed in San Marzano among salt treatments (Fig. 3.1F). Cations content results showed that both entries accumulated Na^+ in leaf and root tissues when salinity increased, which was generally accompanied by a decrease in K^+ content compared to control conditions (Fig. 3.2A-D). Overall, the trend of Na^+/K^+ ratio clearly depicted the existence of differences on cations uptake and root-shoot translocation between the two landraces (Fig. 3.2E and F). In San Marzano leaves Na^+/K^+ ratio increased linearly with salt gradient reaching the highest value at 600 mM NaCl, while in Ciettaicale leaves, the maximum ratio of Na^+/K^+ was obtained at 450 mM NaCl which was the same levels observed in San Marzano at 300 mM NaCl (Fig. 3.2E). Evaluation of Na^+/K^+ ratio in roots was also determined; while San Marzano showed the highest value of Na^+/K^+ ratio at 450 mM NaCl, Ciettaicale maintained stable the cations ratio at intermediate NaCl levels and reached the highest value at 600 mM NaCl (Fig. 3.2F).

3.3.2. Leaf gas exchange and chlorophyll a fluorescence

According to the salt gradient, the salt-induced reduction of A was more pronounced in San Marzano than in Ciettaicale and this difference was significantly marked at 600 mM NaCl (0.67 μmol CO_2 m^{-2} s^{-1} vs. 2.51 μmol CO_2 m^{-2} s^{-1}) (Fig. 3.3A). Salt treatments had a strong impact on g_s and E (Fig. 3.3B and C). In control plants, San Marzano showed g_s values twice than the Ciettaicale one (Fig. 3.3B), but these differences did not affect A (Fig. 3.3A). Hence, control plants of Ciettaicale showed a higher A/g_s and WUE than San Marzano (Fig. 3.3E and F). At 300 mM of NaCl, g_s was reduced on average by 91.7% in San Marzano and 78.9% in Ciettaicale, compared to control (Fig. 3.3B). The drop in A and g_s observed at intermediate salt levels in both landraces compared to control was reflected in a decrease of C_i and a strong increase of A/g_s and WUE (Fig. 3.3D and F). At 600 mM, the low value of A in San Marzano was associated with the high value of C_i (353.7 μmol CO_2 mol^{-1}) compared to that of Ciettaicale (286.2 μmol CO_2 mol^{-1}) (Fig. 3.3D). According to the salt gradient, Ciettaicale showed higher values WUE than San Marzano at the highest salt treatment with an evident difference at 600 mM NaCl, where San Marzano and

Ciettaicale showed values of WUE around 1.69 μmol mmol^{-1} and 4.59 μmol mmol^{-1}, respectively (Fig. 3.3E and F).

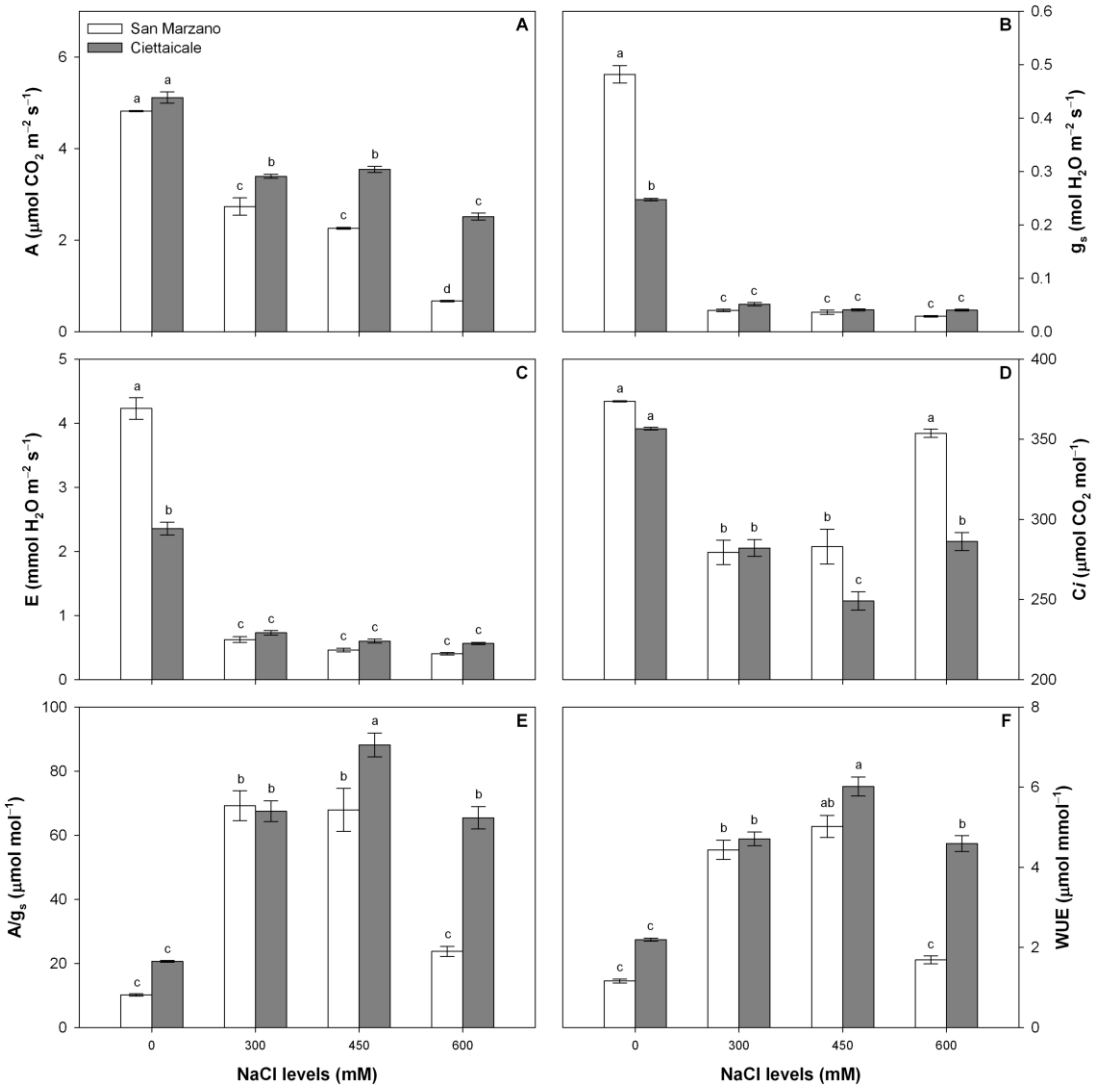

Fig. 3.3. Leaf gas exchange of San Marzano and Ciettaicale tomato subjected to different NaCl concentrations after one week of salt stress at growing light conditions. [A] net CO_2 assimilation rate assimilation (A), [B] stomatal conductance (g_s), [C] transpiration rate (E), [D] intercellular CO_2 concentration (C_i), [E] intrinsic water use efficiency (A/g_s) and [F] instantaneous water use efficiency (WUE). Error bars represent the standard error of the mean (n = 10). Bars with same letters are not statistically different from one another according to Fisher's protected LSD (α = 0.05).

Compared to controls, F_v/F_m of Ciettaicale was not significantly affected by salinity treatments, while in San Marzano F_v/F_m significantly decreased only at 600 mM NaCl (Fig. 3.4A). In both entries, Φ_{PSII} was not significantly affected by 300 mM treatment respect to control condition (0 mM NaCl) (Fig. 3.4B).

On the other hand, Φ_{PSII} decreased significantly in San Marzano plants at higher NaCl concentrations, getting the lowest value at 600 mM NaCl (66.6% less than control) (Fig. 3.4B). In Ciettaicale plants Φ_{PSII} values at intermediate salinity were similar to control condition, and then reduced at 600 mM NaCl (27.6% less than control) (Fig. 3.4B).

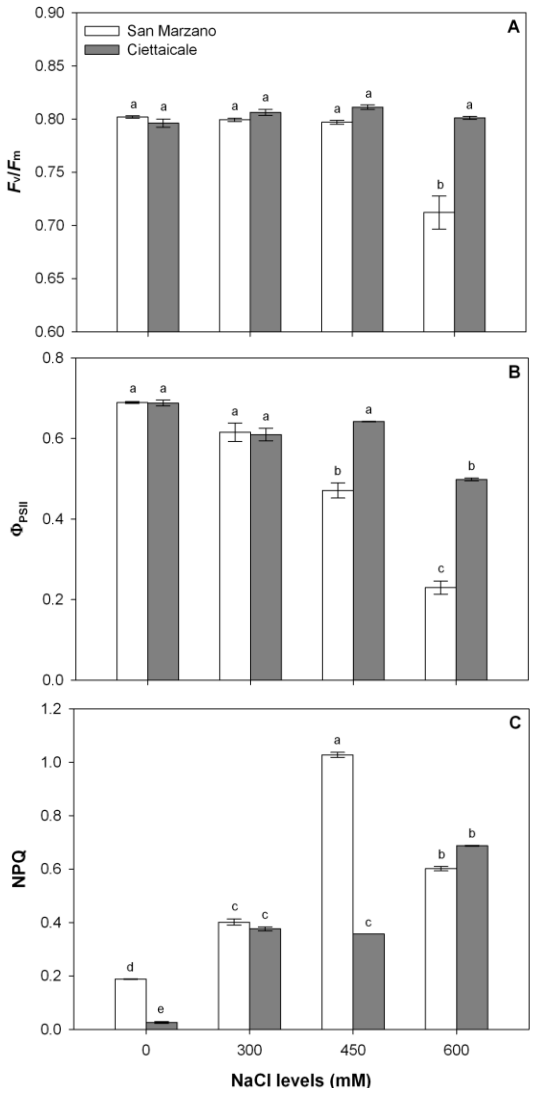

Fig. 3.4. Leaf fluorescence parameters of San Marzano and Ciettaicale tomato subjected to different NaCl concentrations after one week of salt stress stress at growing light conditions. [A] maximum photochemical efficiency of PSII (F_v/F_m), [B] actual photochemical efficiency of PSII in the light (Φ_{PSII}) and [C] non-photochemical fluorescence quenching coefficient (NPQ). Error bars represent the standard error of the mean (n = 10). Bars with same letters are not statistically different from one another according to Fisher's protected LSD (α = 0.05).

Salt stress also affected the non-radiative energy dissipation capacity of both landraces, as shown by the increasing NPQ (Fig. 3.4C). In San Marzano NPQ reached the highest value at 450 mM NaCl (5 fold more than control) and then reduced at 600 mM NaCl (0.60), while Ciettaicale maintained NPQ values in the range of 0.36 and 0.38 at intermediate level and getting the highest value at 600 mM NaCl (0.69).

3.3.3. Leaf photosynthetic pigments

Changes in leaf photosynthetic pigments were observed in both cultivars (Fig. 3.5). Ciettaicale showed a reducing trend of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and total carotenoids (Car) according to salt gradient, which was less pronounced than San Marzano one (data not shown). Overall, the profiles of Chl *a/b* and Car/Chl$_{(a+b)}$ ratios emphasized the previous finding where Ciettaicale showed relatively stable Chl *a/b* ratio values at intermediate salt treatments followed by a significant reduction at 600 mM NaCl, while in San Marzano leaves the Chl *a/b* ratio decreased with increasing the salt concentration and reaching the lowest level at 600 mM NaCl (1.90 comparing to 2.06 at control condition) (Fig. 3.5A). Evaluation of Car/Chl$_{(a+b)}$ ratio showed that San Marzano increased the ratio with increasing stress until intermediate salinity level (0.18 at 450 mM NaCl), although a pronounced decline was observed at 600 mM NaCl (Fig. 3.5B). Ciettaicale Car/Chl$_{(a+b)}$ ratio slightly increased when the salinity level increased (from 0.12 to 0.14) reaching a relatively stable value between 450 and 600 mM NaCl (Fig. 3.5B).

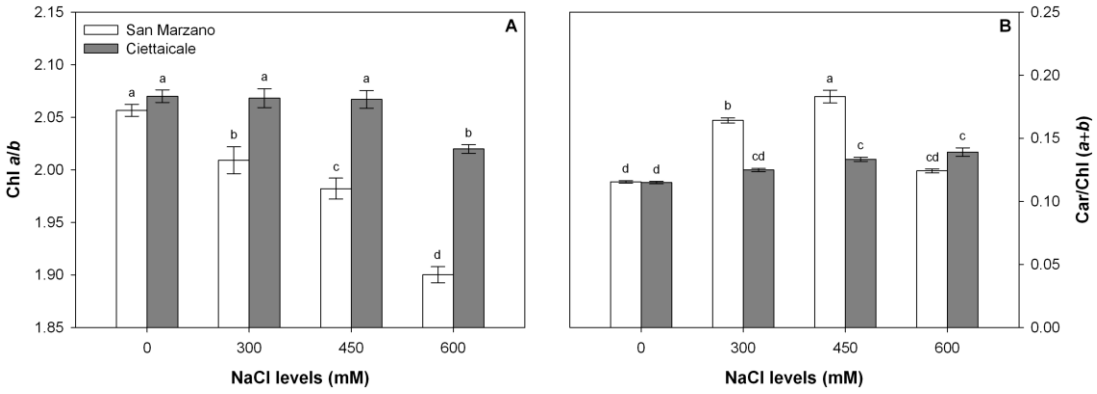

Fig. 3.5. Photosynthetic pigments in leaves of San Marzano and Ciettaicale tomato subjected to different NaCl concentrations after one week of salt stress. [A] Chl$_a$/Chl$_b$ ratio and [B] Car/Chl$_{(a+b)}$ ratio. Error bars represent the standard error of the mean (n = 10). Bars with same letters are not statistically different from one another according to Fisher's protected LSD (α = 0.05).

3.3.4. Total antioxidant capacity

No significant changes of TAC in leaves were observed in both entries until intermediate salt treatments compared to that of the control, while at 600 mM NaCl, San Marzano and Ciettaicale showed a significant increase in TAC respect to control plants (53% and 28%, respectively) (Fig. 3.6A). Interestingly, a different trend of TAC was observed in roots, a gradual decrease in Ciettaicale was recorded when the salt concentration was increased, while in San Marzano all salt treatments caused a complete inhibition of this capacity respect to control conditions (Fig. 3.6B).

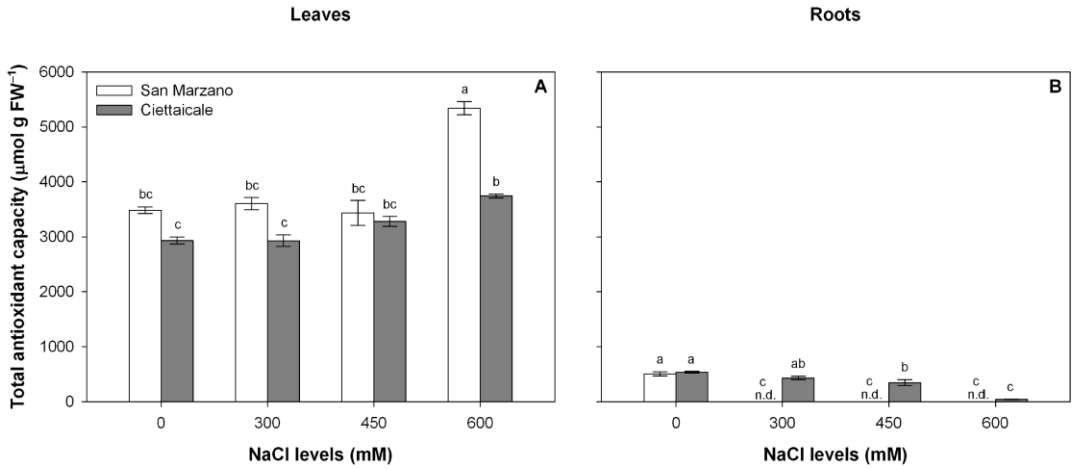

Fig. 3.6. Total antioxidant capacity in San Marzano and Ciettaicale tomato subjected to different NaCl concentrations after one week of salt stress. [A] TAC in leaves and [B] roots. Error bars represent the standard error of the mean (n = 10). Bars with same letters are not statistically different from one another according to Fisher's protected LSD (α = 0.05).

3.3.5. Total soluble sugars

Results showed marked differences among the entries in total soluble sugars (TSS) partitioning between source and sink tissues induced by salt treatments. Overall, a higher concentration of TSS in leaf than in root tissues was recorded for both entries (Fig. 3.7). In San Marzano leaves TSS concentration increased as salt magnitude increased, reaching the highest value at 600 mM NaCl (269.1 µmol g FW^{-1} compared to 71.02 µmol g FW^{-1} in control plants) in which the main contribution is provided by sucrose content (78.1% of TSS) (Fig. 3.7A). At the same conditions, a progressive decrease in TSS content in San Marzano roots was observed, reaching the lowest value at 600 mM NaCl (6.92 µmol g FW^{-1} compared to 43.52 µmol g FW^{-1} in control plants) (Fig. 3.7B).

In Ciettaicale, TSS leaves content increased at 300 mM NaCl (3 fold higher than the control) and then remained relatively stable among salt treatments, in which the main contribution is provided by sucrose. At 600 mM NaCl, Ciettaicale leaves showed halved values of sucrose concentration (126.3 μmol g FW^{-1}) compared to the values found in San Marzano (269.1 μmol g FW^{-1}) (Fig. 3.7A). In roots, Ciettaicale at 300 mM NaCl maintained same values of TSS as at control condition and then increased at 450 mM NaCl, in which sucrose represented the main contribution. Interestingly, at 600 mM NaCl a significant reduction of TSS in root was observed reaching to the same values of San Marzano one (Fig. 3.7B).

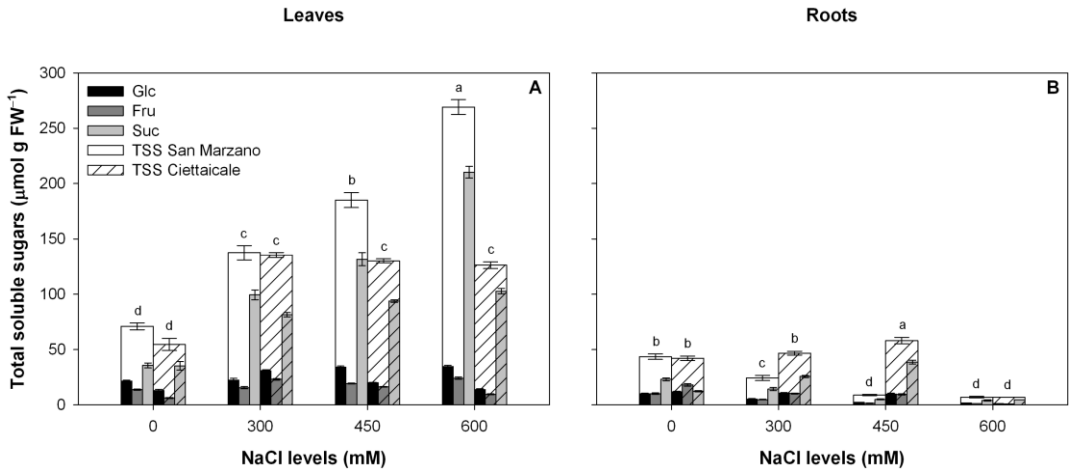

Fig. 3.7. Soluble sugars content in San Marzano and Ciettaicale tomato subjected to different NaCl concentrations after one week of salt stress. [A] TSS in leaves and [B] roots. Error bars represent the standard error of the mean (n = 10). Bars with same letters are not statistically different from one another according to Fisher's protected LSD (α = 0.05).

3.3.6. Multiple factorial analysis

MFA revealed a canonical relationship between the treatment fingerprints obtained from biometric and physiological data recorded at the end of the experiment (Fig. 3.8). The coordinates of the seven groups of variables were displayed and used to create a map of the groups "Groups representation", where the coordinates were calculated using the first two dimensions of the MFA (Dim 1 and 2 on the diagram) which resumed 77.00% of the total inertia (the inertia is the total variance of a dataset i.e. the trace of the correlation matrix) (Fig. 3.8A).

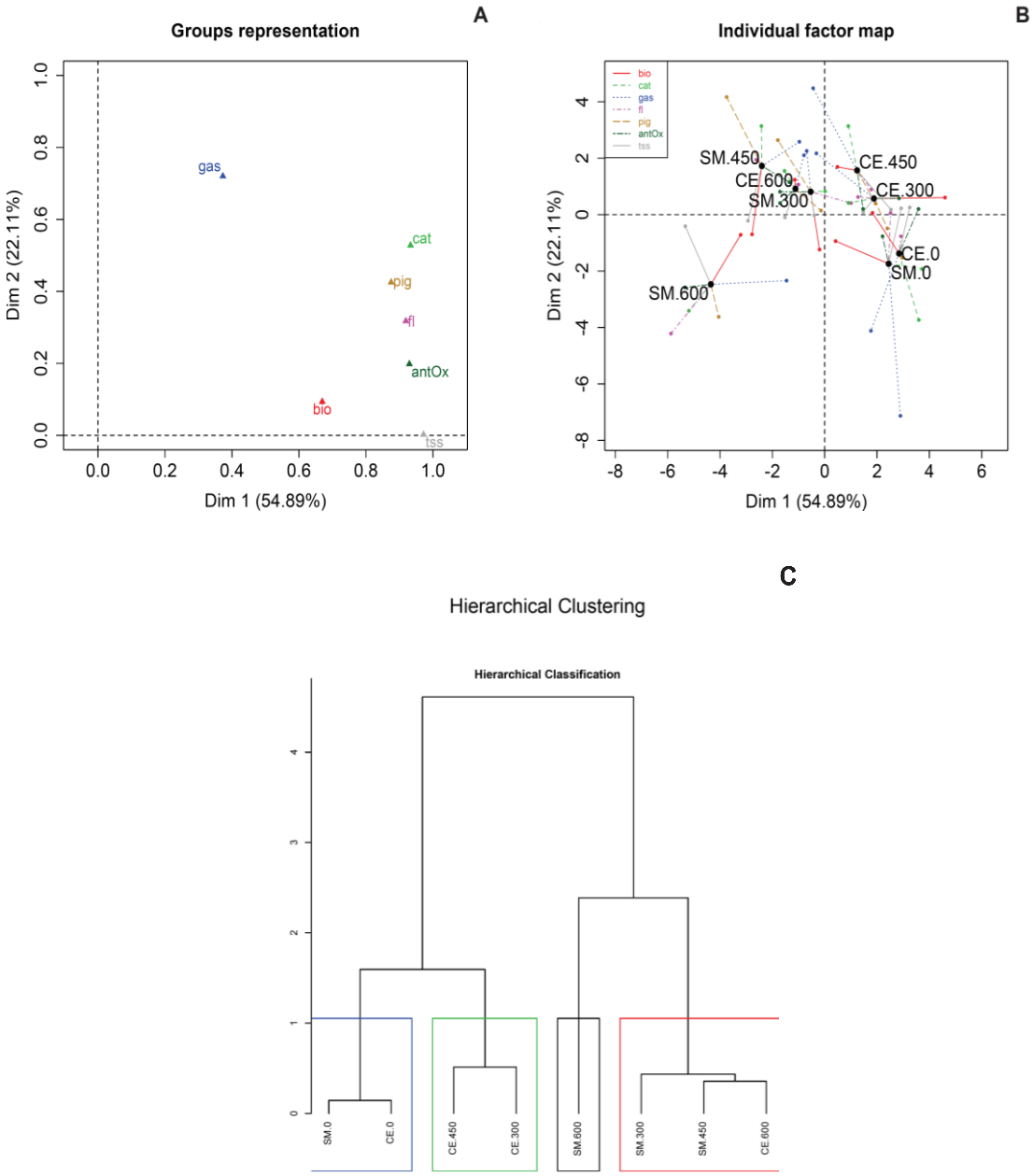

Fig. 3.8. Multiple factor analysis of biometric and physiological data in San Marzano and Ciettaicale tomato subjected to different NaCl concentrations after one week of salt stress. [A] Representation of groups of variables. "gas", leaf gas exchange, "cat", cations content in leaves and roots; "pig", photosynthetic pigments content; "fl", leaf fluorescence parameters; "antOx", total antioxidant capacity in leaves and roots; "bio", biometric traits FW-DW; "tss", total soluble sugars in leaves and roots. [B] Score plot describing the treatments and groups of variables of the two-first principal components. SM, San Marzano; CE, Ciettaicale; 0, 300, 450, 600 mM NaCl. [C] Hierarchical clustering of treatments based on their biometric and physiological traits.

As to the contribution of individual groups of variables, a general equilibrium can be observed for axis 1 in a range that varies from 15.43% (pigments) up to 17.14% (sugars), with the exception of biometric and leaf gas exchange, which accounted respectively only for 11.80 and 6.57%. Different conclusions can be drawn regarding the contribution of each group of variables to axis 2. The contribution of leaf gas exchange appears to be the most statistically significant (31.52%), which is the most useful group of variables for discriminating among the analysed treatments on the axis 2 of the MFA (Fig. 3.8A).

The representation of the treatments provided by MFA can be read as in a usual PCA (Individual factor map), where the coordinates of the descriptors correspond to the correlation coefficients between these variables (biometric and physiological traits) and the factors (treatments) (Fig. 3.8B). The length and the direction of the vectors are directly correlated to their significance within each treatment. Factorial axis 1 (54.89% of the variance) clearly separated the treatments according to the overall performance of the sugars, cations, antioxidant activity, Chl *a* fluorescence and pigments, whereas the second axis (comprising 22.11% of the variance) separated the treatments mainly according to the leaf gas exchange (Fig. 3.8B).

The hierarchical clustering provided by the MFA highlighted the overall performance of the treatments obtained through the single analysis of the biometric and physiological data (Fig. 3.8C). The three phylogenetic trees show that San Marzano at 600 mM NaCl (SM.600) clearly differentiated from the other treatments analysed. Ciettaicale at 600 mM NaCl (CE.600), San Marzano at 450 (SM.450) and 300 mM NaCl (SM.300) share more similarity among them, as well as Ciettaicale at 450 mM NaCl (CE.450) to 300 mM NaCl (CE.300), while the controls (SM.0 and CE.0) cannot be separated on the basis of their biometric and physiological traits.

4. Discussion

Our study reported an eco-physiological comparison of the effect of severe salinity stress for a short-term exposure time on a Southern Italy tomato landrace compared to an industrial standard cultivar. Leaf gas exchange and Chl *a* fluorescence supported by growth and biochemical analyses were evaluated to identify salt tolerance differences between San Marzano and Ciettaicale at vegetative stage. Growth chamber conditions and short-term experiment allowed us to test halophytic

response to sodium chloride stress in order to analyse the effects of salinity on photosynthetic apparatus. Most salt tolerant genotypes within cultivated tomato generally exhibit a glycophytic response to salinity, while salt tolerant accessions within wild tomato species, such as *L. pennellii*, *L. cheesmanii* and *L. peruvianum*, usually exhibit a halophytic response to salinity (Foolad, 2007). In this study, the irrigation of both entries plants with 300 mM NaCl stimulated the dry matter production in relation to untreated plants with more pronounced effect in Ciettaicale (Table 3.1 and Fig. 3.1); interestingly, this is a characteristic response of halophyte species to salt stress and water deficit conditions (Flowers et al., 1977). Many greenhouse experiments where tomato has been subjected to moderate salt magnitude range (50-150 mM NaCl) for several weeks showed an increase in plant root/shoot ratio resulting from limitations of epigeal growth and changes in plant hormone concentrations (Albacete et al., 2008; Lovelli et al., 2012; Maggio et al., 2007). Concordantly, Ciettaicale root/shoot ratio (Fig. 3.1E and F) also increased under salt treatments suggesting a major investment of photosynthates towards belowground organs to explore better the ground in order to escape from the osmotic pressure (Lynch, 1995). On the contrary, a reduced root/shoot ratio, as we found in San Marzano, may improve salinity tolerance by restricting the flux of toxic ions to the shoot or it could be the consequence of limitation of root morphogenesis during salt adaptation (Maggio et al., 2001; 2007).

Our study showed that Ciettaicale was able to keep lower Na^+/K^+ ratio than San Marzano especially when exposed to highest salt treatment (Fig. 3.2E and F). This strategy has been considered a salt tolerance indicator (Dasgan et al., 2002) which could be related to the exclusion of Na^+ from the shoots *via* an efficient compartmentalization and/or exclusion ionic system (Pompeiano et al., 2016b). In some cases, the effect of the cations in saline-treated roots was translated into an increase in biomass greater than that of the control (Carvajal et al., 2000).

Changes in tomato growth rate could be also associated with a limitation of plant photosynthesis performance (Lovelli et al., 2012). Our results showed that g_s and E were considerably decreased by salinity in both cultivars as we expected considering the magnitude of salt level tested (Fig. 3.3B and C). In fact, the reduction of g_s and E can be considered a biophysical strategy to reduce water losses from stomata and reduce salt uptake according to the transpiration stream (Chaves et al., 2009).

Syvertsen et al. (2010) reported a negative relationship between leaf salt content and leaf WUE in Citrus, supporting the idea that increased leaf WUE under salinity stress can be an indicator of salt tolerance in Ciettaicale (Fig. 3.3F). However, under salt stress the hydraulic conductivity of roots can decrease significantly resulting in a reduction of WUE values, as reported in a comparative study of tomato cultivars and in our study using San Marzano (Al-Karaki, 2000). In our study, the salt-induced reduction of g_s resulted in a limitation on A and a decrease of C_i (Fig. 3.3A, B and D). Thus, the observed reduction of A and C_i and the strong increase of A/g_s and WUE at 300-450 mM NaCl (Fig. 3.3E and F), indicated that stomatal limitations dominate irrespective of any metabolic impairment. However, at the highest salt concentration, despite similar values of g_s recorded in the two entries, the trend of A and C_i highlighted a different behaviour. In San Marzano, C_i increased significantly, concomitantly with the decrease of WUE and F_v/F_m (Fig. 3.3F and Fig. 4A), indicating the occurrence of non-stomatal limitations on the decline of A and photoinhibition (Flexas and Medrano, 2002). Conversely, Ciettaicale was able to maintain higher values of F_v/F_m and WUE than San Marzano at the highest saline level, underlying a predominance of stomatal effects on the reduced photosynthetic activity. A decline in Φ_{PSII} reflected a lower electron transport and could be accompanied in parallel by an enhancement of NPQ (Fig. 3.4B and C). This defence mechanism also includes changes in photosynthetic pigments to sustain thermal dissipation of excess radiant energy preserving PSII activity (Fusaro et al., 2014). At 450 mM NaCl, San Marzano showed a significant increase of NPQ compared to control as well as high values of the $Car/Chl_{(a+b)}$ ratio (Fig. 3.5B). Changes in $Car/Chl_{(a+b)}$ and Chl *a/b* ratios (Fig. 3.5) reflect rearrangements in the stoichiometry of PSII core and its associated light harvesting complexes (LHCII) promoting excessive energy dissipation (Pompeiano et al., 2016a). However, the increase in NPQ may be insufficient to dissipate the excess of energy, and thus this excess may be also diverted to form ROS inducing the plant antioxidant system as it was observed in San Marzano leaves under high salt stress (Fig. 3.6A). On the other hand, Ciettaicale dissipates the excess of energy *via* mainly NPQ at foliar level limiting the investment on TAC (Fig. 3.4C and Fig. 3.6A). Interestingly, completely different patterns were found at root level; while Ciettaicale seems to rationally use antioxidants in order to mitigate the oxidative damage caused by salt stress, San

Marzano may not show an efficient TAC production caused by a high root damage related to salt stress (Fig. 3.6B). It should be mentioned that additional strategies to mitigate salt-dependent oxidative stress could be occurred as previously reported by Mittova et al. (2002, 2004).

Previous investigations suggested that the accumulation of sugars in tomato may play another potential ROS scavenger as well as an important role in the osmotic adjustment (Haque et al., 2015; Hunsche et al., 2010). In our study, Ciettaicale and San Marzano showed a different non-structural sugars partitioning between leaf and root tissues in response to the salt gradient (Fig. 3.7). The increasing accumulation of TSS in San Marzano source organs according to salt gradient suggests a sink-limitation reducing sucrose export and in a feedback regulation of photosynthesis as previously reported (Li et al., 2015; Pompeiano et al., 2016a). Different response strategy was observed in Ciettaicale which maintains stable the ability to regulate the sugar balance between source and sink organs under intermediate salt stress conditions.

In conclusion, our results showed that a comprehensive assessment of salinity tolerance in different accessions could be also obtained evaluating plant response to high salinity levels in the short-term at vegetative stage. A clear differentiation of Ciettaicale compared to San Marzano in response to seawater dilutions emerged above 300 mM NaCl (Fig. 3.8). After a week of salt treatment, salinity effects culminated in photoinhibition and/or photodamage events at 600 mM NaCl in San Marzano plants resulting in a source-sink imbalance. At the same extreme conditions, Ciettaicale plants showed an efficient physiological and metabolic plasticity provided by improved photosynthesis efficiency and osmotic adjustment resulting in a greater energy availability to be allocated in root exploration. Thus, Ciettaicale plants strategy allowed themselves to survive or at least to slow down the appearance of actual damage at higher NaCl concentrations. Since salt tolerance is a complex trait influenced greatly by genetic, physiological and environmental factors, our future perspective will be to investigate more deeply the tolerance strategy adopted by Ciettaicale plants, at different phenological stages, in an experimental set up closest as possible to the field reality to enhance the possibility of introducing this ecotype in tomato genetic improvement programs.

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Chapter 4

Drought induced changes of leaf-to-root relationships in two tomato genotypes

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Abstract

Water deficit triggers a dynamic and integrated cross-talk between leaves and roots. Tolerant plants have developed several physiological and molecular mechanisms to establish new cell metabolism homeostasis, avoiding and/or escaping from permanent impairments triggered by drought. Two tomato genotypes (a Southern Italy landrace called Ciettaicale and the well-known commercial cultivar Moneymaker) were investigated at vegetative stage to assess leaf and root metabolic strategies under 20 days of water deficit. Physiological and metabolic changes, in terms of abscic acid (ABA), indole-3-acetic acid (IAA), proline, soluble sugars and phenols contents, occurred in both tomato genotypes under water stress. Overall, our results pointed out the higher plasticity of Ciettaicale to manage plant water status under drought in order to preserve the source-sink relationships. This aim was achieved by maintaining a more efficient leaf photosystem II (PSII) photochemistry, as suggested by chlorophyll fluorescence parameters, associated with a major investment towards root growth and activity to improve water uptake. On the contrary, the higher accumulation of carbon compounds, resulting from reduced PSII photochemistry and enhanced starch reserve mobilization, in leaves and roots of Moneymaker under drought could play a key role in the osmotic adjustment, although causing a feedback disruption of the source-sink relations. This hypothesis was also supported by the different drought-induced redox unbalance, as suggested by hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents. This could affect both PSII photochemistry and root activity, leading to a major involvement of non-photochemical fluorescence quenching (NPQ) and antioxidant system in response to drought in Moneymaker than Ciettaicale.

Keywords

Water deficit; tomato; source-sink relations; chlorophyll *a* fluorescence; leaf water potential; carbon partitioning; redox status

4.1 Introduction

Water deficit events of unpredictable duration and severity are progressively increasing worldwide, also due to global warming, exacerbating the current harsh situation in warm and arid ecosystems (Trenberth et al., 2014).

The main impact by drought stress is usually observed in crop growth rate and yield production injury, which are related to alterations of sink-source relations (Albacete et al., 2014). This induces a complex signalling network, in which hormones, reactive oxygen species (ROS) and sugars are mainly involved. The source-sink cross-talks modulate carbon and nutrient utilization within the whole plant resulting essentially crucial for crop productivity (Jogaiah et al., 2013; Albacete et al., 2014).

Typically, plants can be classified in two distinct categories on the basis of their drought response strategies: the "drought escape", which expedites their life cycle in order to anticipate the potential harmful drought effects, and the "drought avoidance", which are able to conduct their life cycle under water deficit conditions. However, it is not simple to differentiate uniquely these two strategies and a trade-off between them could occur (Franks, 2011).

Once the hypogeal apparatus has sensed soil water breakdown, root signals are loaded into xylem sap to reach shoot tissues with the primary aim to limit the transpiration flux and then water losses. In the leaves, the alarm state is converted in biophysical responses related to cell turgor decrease and stomatal conductance regulation. The resulting stomatal-dependent CO_2 uptake reduction can affect net photosynthesis, causing an alteration of carbohydrates metabolism (Chaves et al., 2009). The upset carbon status is converted in adaptive accumulation of carbon-rich compounds whose main representatives are soluble sugars, like the disaccharide sucrose and the hexoses glucose and fructose, which in turn can also derived from an increased starch hydrolytic activity (Bartels and Sunkar, 2005). In addition to their roles as energetic and signalling molecules (Rolland et al., 2006), non-structural carbohydrates and other compatible solutes, like proline and glycine betaine, regulate osmotic and redox adjustments, thus preserving membrane and macromolecules structure and functionality (Bartels and Sunkar, 2005).

Under drought cell metabolism is invested by an energy surplus that triggers free radicals production, such as hydrogen peroxide (H_2O_2), superoxide (O_2^-) and nitric

oxide (NO). To counteract stress-related oxidative burst, plant strengthens its scavenging system that consists of several specific components with or without enzymatic activity (Miller et al., 2010). If ROS overproduction is uncontrolled, cell damages, like lipid peroxidation, and biochemical limitations on respiratory and photosynthetic pathways occur (Chaves et al., 2009). On the contrary, if ROS formation/scavenging processes ratio reached a new redox equilibrium, ROS themselves can act as effective secondary messengers interplaying with the carbohydrates status and hormonal regulatory networks, leading to adaptative responses (Miller et al., 2010).

Drought-induced changes in biomass production are regulated by hormone signalling (Sharp and LeNoble, 2002). Abscisic acid (ABA) and indole-3-acetic acid (IAA) play a critical role, often synergistically, in plant drought-induced acclimation processes like stomata closure, shoot branching inhibition, synthesis of storage molecules, root hydraulic conductivity and lateral root formation improvement (Dodd, 2003; Pasternak et al., 2005; Woodward and Bartel, 2005; Tognetti et al., 2012).

Tolerant plants have developed several physiological and molecular mechanisms to establish new cell metabolism homeostasis, avoiding and/or reducing permanent impairments triggered by drought. Landraces represent an important part of this biodiversity, often well-adapted under harsh environmental conditions, such as drought, due to phenotype plasticity and/or evolutionary selection (Franks, 2011). Investigating local accessions germplasm for these beneficial stress-related traits could be a sustainable strategy to provide food and valorise marginal lands with a view to counteract the future climate changes (Halford and Foyer, 2015). Interestingly, drought-tolerance traits have been found among Mediterranean tomato accessions, which are typically cultivated in limited water supply scenario (Galmés et al., 2013; Patanè et al., 2016, Guida et al., 2017).

With this aim, we compared the biometric and metabolic responses to water deficit between the well-known cultivar Moneymaker, used as standard, and a Southern Italy landrace (Ciettaicale), recently identified as a well-adapted accession to marginal soil, characterized by intense drought and salt contaminated water (Moles et al., 2016).

4.2 Materials and methods

4.2.1 Plant material and growth condition

Ciettaicale seeds (De Angelis S.r.l., Tolve, Italy) and Moneymaker seeds (Thompson and Morgan, Ipswich, UK) were germinated directly in plastic pots (1.5 L) containing a mixture of soil and perlite (3:1, v/v) in which a controlled release fertilizer (Osmocote® Bloom NPK ratio 2:1:3, Everris) was added (2 g/L). Pots were placed in growth chamber (temperature 25°C/20°C, 16 h photoperiod, irradiation intensity 300 μmol photons m^{-2} s^{-1}) for four weeks. Then, plants were divided into two groups: control group was daily watered, while stressed group received water equal to 50% of pot-capacity every 48h for 20 days. We chose these condition of stress according to preliminary experiments.

4.2.2 Biometric analysis

After 20 days of drought treatment (20 DAT), plants were separated into shoot organs and root tissues and then weighed (fresh weight, FW). Roots were previously washed with water to remove soil and perlite and then carefully dried with paper. The dry weights (DW) of plant organs were recorded after drying at 60°C for a week. Leaf and root samples were collected from an additional set of plants not used for biometric evaluations. Collected samples were ground in liquid nitrogen and then stored at -80°C for further biochemical analyses.

4.2.3 Stomatal conductance and leaf water potential measurement

Stomatal conductance (g_s) was measured using an AP4 Delta-T dynamic porometer (Delta-T Devices, Cambridge, UK). The fully expanded and exposed apical leaf of the third branch was chosen for measurements. The g_s value derived from the average of four measurements in different positions on the leaf abaxial side of the selected leaf. Leaf water potential ($L\Psi_w$) was measured using a Scholander pressure chamber according the procedure reported in Ferdous et al. (2017). A fully expanded, mature leaf was cut and placed immediately through the chamber lid with the cut end of the leaf outside and the remaining part of the leaf inside the chamber. Pressure was increased slowly. A magnifying glass was used to observe the cut end of the leaf. As soon as a drop of sap appeared from the cut end of the leaf sample, the pressure shown on the chamber gauge was recorded as a measure of the $L\Psi_w$.

4.2.4 Chlorophyll a fluorescence measurements

After 20 DAT, chlorophyll fluorescence was measured using a miniaturized pulse-amplitude-modulated fluorometer (Mini-PAM; Heinz Walz GmbH, Effeltrich, Germany) on fully-expanded and exposed apical leaf of the third branch at growth chamber light condition. The potential efficiency of PSII photochemistry (F_v/F_m), the actual photochemical efficiency of photosystem II in the light (Φ_{PSII}) and the non-photochemical fluorescence quenching (NPQ) parameters were calculated according to the equations and nomenclature reported in Chapter 3.

4.2.5 ABA and IAA quantification

After 20 DAT, the same apical leaves used for the *in vivo* measurements and roots were ground in a mortar with 80% methanol (1:5 w/v) and $^{13}C_6$ IAA (Cambridge Isotopes Laboratories Inc., Andover,MA, USA) and [$^2H_6$]-ABA (OlChemlm Ltd, Olomouc, Czech Republic) were added as internal standards, acidified (pH = 2.8-3) and thrice partitioned with ethyl-acetate (1:1 v/v). The organic phase was purified by high performance liquid chromatography (HPLC) apparatus (Kontron, Munich, Germany) equipped with a C18 column (150 mm long, 4.6 i.d., particle size 5 μm, Hypersil ODS, Thermo Fisher Scientific, Waltham, MA, USA), as previously reported in Mariotti et al. (2011). The column was eluted with a linear gradient of methanol and water (0.01% of acetic acid) from 10% to 100% of methanol at the flow rate of 1 mL min^{-1} and fractions corresponding ABA and IAA standards retention time were collected, respectively All fractions were dried and trimethylsilylated with N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% of trimethylchlorosilane (Pierce, Rockford, IL, USA) at 70 ◦C for 1 h. Finally, IAA were identified and quantified through chromatography–tandem mass spectrometry (GC–MS/MS). Analysis were carried out with a Saturn 2200 quadrupole ion trap mass spectrometer coupled to a CP-3800 gas chromatograph (Varian Analytical Instruments, Walnut Creek, CA, USA) using a 1MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness, Mega, Milan, Italy). The concentrations of ABA and IAA in the original extracts were determined from the peak area ratio of labelled and non-labelled ions of internal standards and endogenous hormones, respectively.

4.2.6 H_2O_2 content and lipid peroxidation estimation

H_2O_2 was measured in leaf and root tissues according to the potassium iodide method described in Chapter 2. Leaf and root level of lipid peroxides was estimated as malondialdehyde (MDA) content by thiobarbituric acid method as reported in Cotrozzi et al. (2016). Samples were sonicated in 0.1% trichloroacetic acid (TCA) then centrifugated at 16000×g for 20 min at room temperature. An aliquot of the supernatant was mixed with 20% TCA containing 0.5% thiobarbituricacid (TBA) and incubated for 30 min at 90°C. After incubation, the extract were cooled on ice and centrifuged at 8000×g for 10 min at 4°C. The new supernatant was used to determine MDA content at 532 nm (ε = 155 mM^{-1} cm^{-1}), corrected fo non-specific turbidity by subtracting the absorbance at 600 nm.

4.2.7 Starch and total soluble sugars quantification

Frozen leaves and roots samples were extracted and assayed for glucose, fructose and sucrose contents through coupled enzymatic assay methods reported in Chapter 2. Starch in the insoluble pellet was washed twice in 70% (v/v) ethanol and resuspended in water (Paparelli et al., 2013). Starch was solubilized by boiling for 15 min at 95°C and subsequently digested to glucose using α-amylase and amyloglucosidase (dissolved in 15mM Na-acetate, pH 4.8) for 2 h at 37°C. The glucose equivalents were determined through an enzymatic reaction (25 mM HEPES, 1mM $MgCl_2$, 1mM ATP, 1mM NAD, 2 units hexokinase and 2 units glucose-6-phosphate dehydrogenase) which converts NAD to NADH in an equimolar ratio. The increase in the absorption spectrum at 340 nm specific for NADH was assayed in a spectrophotometer.

4.2.8 Root activity determination

Root activity, an indicator of root catabolic metabolism, was determined by 2,3,5-triphenyltetrazolium chloride (TTC) reduction method reported in Comas et al. (2000). Control roots were boiled for 10 min in dH_2O and then carefully dried with paper. Roots were homogenized in a solution containing 50mM sodium phosphate, pH 7.4, 0.05% Triton X-100 and 0.6% (w/v) TTC and vacuum-infiltrated for 5 min. Samples were incubated for 24 h at 30°C; then, washed twice with dH_2O and

extracted four times in 95% (v/v) ethanol for 5 min at 85°C in a waterbath. Finally, an aliquot of the total ethanolic solution extracted was measured at 490 nm.

4.2.9 Free phenols, proline and total antioxidant capacity determinations

Total free phenolic content was assayed in leaf and root samples according to Caser et al. (2016). Frozen samples powder was homogenized in 70% (v/v) methanol and then incubated for 30 min at 4°C in darkness. After centrifugation, an aliquot of the supernatant was mixed by inversion with the Folin-Ciocalteau's reagent and 7.5% (w/v) of saturated Na_2CO_3 solution. The samples were incubated at room temperature for 2 h in the dark. The absorbance was measured at 765 nm.

Leaf and root proline content was quantified using the acid-ninhydrin procedure reported in Cotrozzi et al. (2016). Frozen samples powder was homogenized in 3% sulfosalicylic acid and then centrifuged for 20 min at 16000×g at room temperature. The supernatant was first filtered and then mixed with equal volumes of glacial acetic acid and of ninhydrin reagent (dissolved in glacial acetic acid and 6M H_3PO_4), respectively. The samples were incubated for 1 h at 100°C in the dark. The reaction was stopped by cooling the tubes on ice. Finally, the samples were vigorously mixed with toluene. After 15-20 s, the light absorption of the toluene phase was estimated at 520 nm, using pure toluene as a blank.

Leaf and root total antioxidant capacity (TAC) was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay as previously reported in Chapter 2.

4.2.10 Statistical analysis

The experimental design was completely randomized with five replicates. Data were subjected to one-way and two-way analysis of variance (ANOVA) and the mean values were compared using Tukey's test ($P<0.05$). Statistical analysis was performed with Statgraphics Centurion XVII (Statpoint Technologies, Inc. Warrenton, Virginia, U.S.A.) software.

4.3 Results

3.1 Biometric analysis

Water deficit affected negatively root biomass FW and DW in both genotypes, but the magnitude of the observed reduction was significantly higher in Moneymaker (Table 4.1).

Table 4.1. Effect of drought stress on biometric traits after 20 days of treatment.

Genotype-Treatment*	Root FW (g plant^{-1})	Root DW (g plant^{-1})	Root/Shoot FW	Root/Shoot DW	Leaf H_2O (%)	Root H_2O (%)
CE-C	8.07±0.39 a	0.76±0.04 a	0.099±0.006 b	0.094±0.009 a	87.90±0.25 a	90.54±0.61 b
CE-DRO	6.05±0.11 b	0.43±0.01 b	0.125±0.005 a	0.073±0.007 a	84.50±0.41 b	92.87±0.43 a
MM-C	5.22±0.14 b	0.51±0.01 b	0.079±0.008 b	0.078±0.007 a	87.66±0.46 a	90.23±0.32 bc
MM-DRO	1.39±0.13 c	0.16±0.02 c	0.034±0.004 c	0.038±0.006 b	86.76±0.18 a	88.35±0.32 c

Means±SD within a column followed by the same letter are not significantly different based on Tukey's test (P<0.05). * Genotypes: Ciettaicale (CE) and Moneymaker (MM); Treatments: well-watered (C) and water deficit (DRO).

In fact, root production DW in Moneymaker stressed plants was 68.6% less than control ones, while a reduction of about 40% was recorded in Ciettaicale plants. ANOVA indicated that drought treatment and tomato genotypes affected shoot FW as well as shoot DW with no interactions drought *versus* genotypes (Table 4.2).

Table 4.2. Mean effects of tomato genotype and drought treatment on biological traits.

Factor		Shoot FW (g plant^{-1})	Shoot DW (g plant^{-1})	g_s (mol m^{-2} s^{-1})	Root phenols (mg g^{-1})
Genotype	Ciettaicale	65.13 a	7.00 a	NS	3.40 a
	Moneymaker	53.70 b	5.37 b		2.39 b
Treatment	Control	73.93 a	7.34 a	0.363 a	1.81 b
	Drought	44.90 b	5.03 b	0.026 b	3.97 a

Means within a column followed by the same letter are not significantly different based on Tukey's test (P<0.05); NS, not significant.

Overall, root/shoot FW showed that drought induced opposite trends in Ciettaicale (increasing) and Moneymaker (decreasing) (Table 4.1). On the other hand, ANOVA indicated that root/shoot DW was not affected by drought in Ciettaicale, while it was reduced in Moneymaker. At the end of experiment, stressed Ciettaicale leaf and root tissues had less and more water content comparing to the well-watered conditions, respectively. On the contrary, no significant changes in water content were recorded in Moneymaker tissues.

3.2 Physiological and metabolic changes

Drought treatment induced a significant drop in g_s in both genotypes (Table 4.2). Well-watered plants showed $L\Psi_w$ values in the range of -0.4 and -0.5 MPa (Fig. 4.1B). Water deficit resulted in a significant lower osmotic potential in both genotypes, but more pronounced in Moneymaker (-1.5 MPa) than in Ciettaicale (-0.9 MPa).

Drought induced a decline in Φ_{PSII} of 52.4% and 22.6% in Moneymaker and Ciettaicale, respectively (Fig. 4.2A).

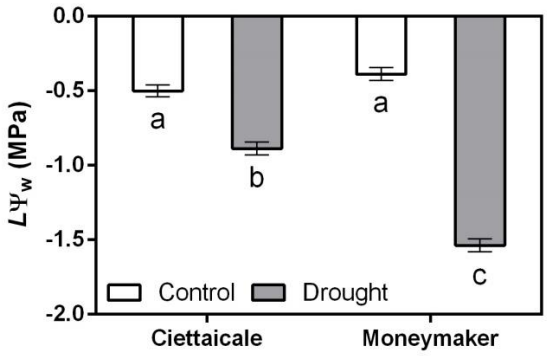

Fig. 4.1. Leaf water potential ($L\Psi_w$) in Ciettaicale and Moneymaker tomato after 20 days of water deficit. Error bars represent the standard error of the mean (n = 5). Bars with same similar letters are not statistically different from one another according to Tukey's test (P<0.05).

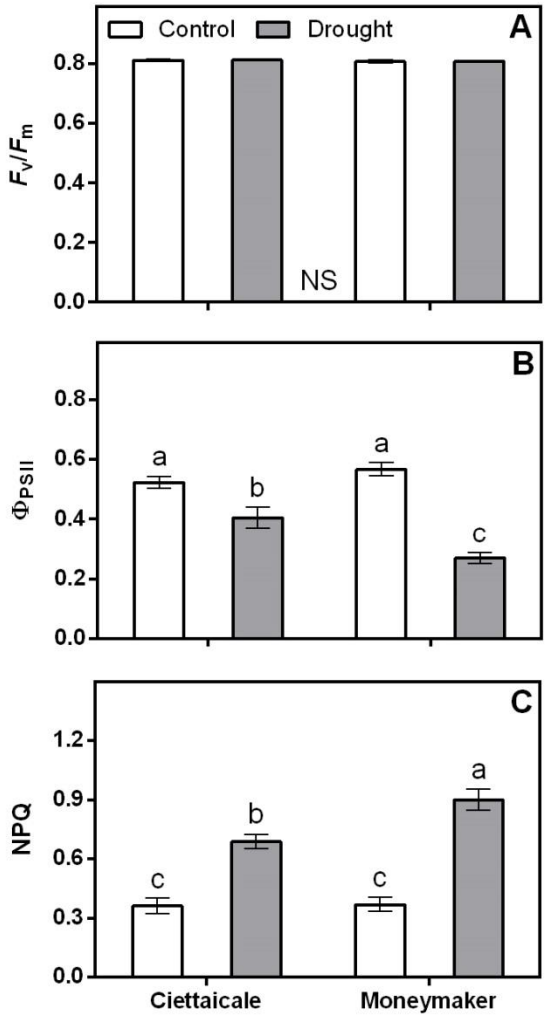

Fig. 4.2. Leaf fluorescence parameters of Ciettaicale and Moneymaker tomato after 20 days of water deficit at growing light conditions. [A] maximum photochemical efficiency of PSII (F_v/F_m), [B] actual photochemical efficiency of PSII in the light (ΦPSII), and [C] non-photochemical fluorescence quenching coefficient (NPQ). Error bars represent the standard error of the mean (n = 5). Bars with same similar letters are not statistically different from one another according to Tukey's test ($P<0.05$); NS, not significant.

A decrease in Φ_{PSII} was accompanied in parallel by an enhancement of NPQ (Fig. 4.2B). This trend is well evident in Moneymaker under stress in which NPQ values increased 2.4 fold than control. F_v/F_m of both genotypes was not significantly affected by drought, remaining at values above 0.8 (Fig. 4.2A).

The endogenous hormonal levels were analysed by GC-MS in tomato leaf and root tissues after 20 DAT. A common increasing trend of ABA and IAA contents in source and sink organs in both genotypes under stress was observed (Fig. 4.3).

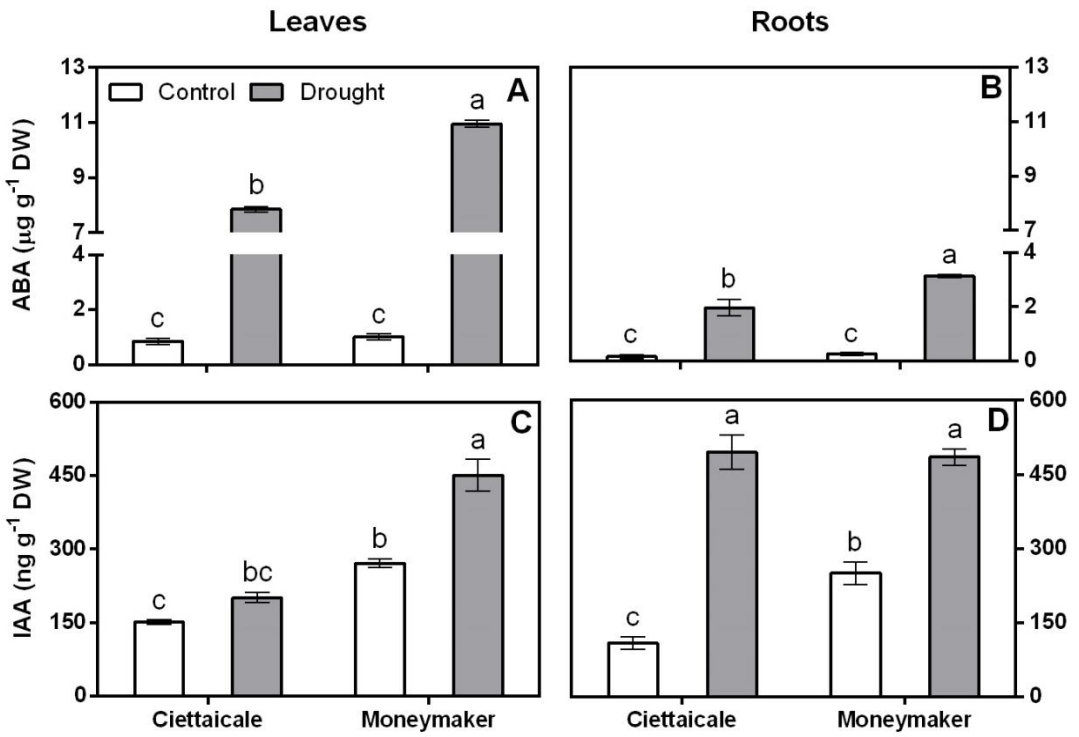

Fig. 4.3. Hormone profiling in Ciettaicale and Moneymaker tomato after 20 days of water deficit. ABA in [A] leaves and [B] roots. IAA in [C] leaves and [D] roots. Error bars represent the standard error of the mean (n = 5). Bars with same similar letters are not statistically different from one another according to Tukey's test ($P<0.05$).

Ciettaicale showed lower leaf ABA amount comparing to Moneymaker (7.86 µg g^{-1} DW and 10.95 µg g^{-1} DW, respectively) (Fig. 4.3A), as well as in Ciettaicale root tissues (1.97 µg g^{-1} DW) respect to Moneymaker (3.14 µg g^{-1} DW) (Fig. 4.3B). Ciettaicale and Moneymaker differed in leaf and root IAA contents already in normal water condition (Fig. 4.3C and D). The highest values of leaf IAA (450.87 ng g^{-1} DW) were recorded in Moneymaker drought stressed plants (Fig. 4.3C).

Indeed, under drought root IAA content was approximately the same in both genotypes, but interestingly these values resulted from 4.6 fold more and 1.9 fold more increase in Ciettaicale and in Moneymaker, respectively (Fig. 4.3D).

Exposure to water deficit resulted in changes of H_2O_2 production rate in leaf and root tissues of both genotypes which were directly correlated to the degree of lipid peroxidation estimated as MDA content (Fig. 4.4). However, Ciettaicale source and sink tissues under drought suffered a minor oxidative pressure than Moneymaker as indicated by lower levels of H_2O_2 and lipid peroxidation.

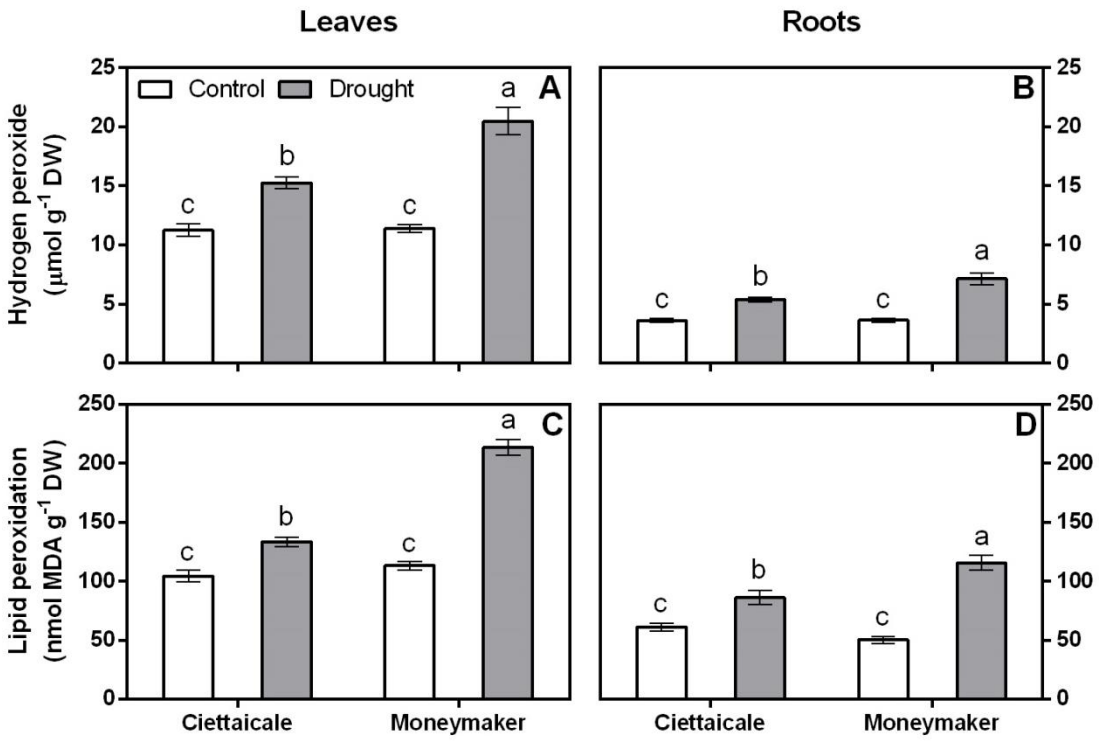

Fig. 4.4. H_2O_2 content and lipid peroxidation estimated, as malondialdehyde (MDA) content, in Ciettaicale and Moneymaker tomato after 20 days of water deficit. H_2O_2 in [A] leaves and [B] roots. MDA in [C] leaves and [D] roots. Error bars represent the standard error of the mean (n = 5). Bars with same similar letters are not statistically different from one another according to Tukey's test ($P<0.05$).

Ciettaicale and Moneymaker shared a common drought-induced decreasing trend of leaf starch (Fig. 4.5A). Particularly, under stress leaf starch level was reduced by about 36% in Ciettaicale and about 51% in Moneymaker comparing to respective controls. On the other hand, no changes in starch level occurred in Ciettaicale roots under drought, while a reduction of about 38% was observed in Moneymaker root starch level (Fig. 4.5B).

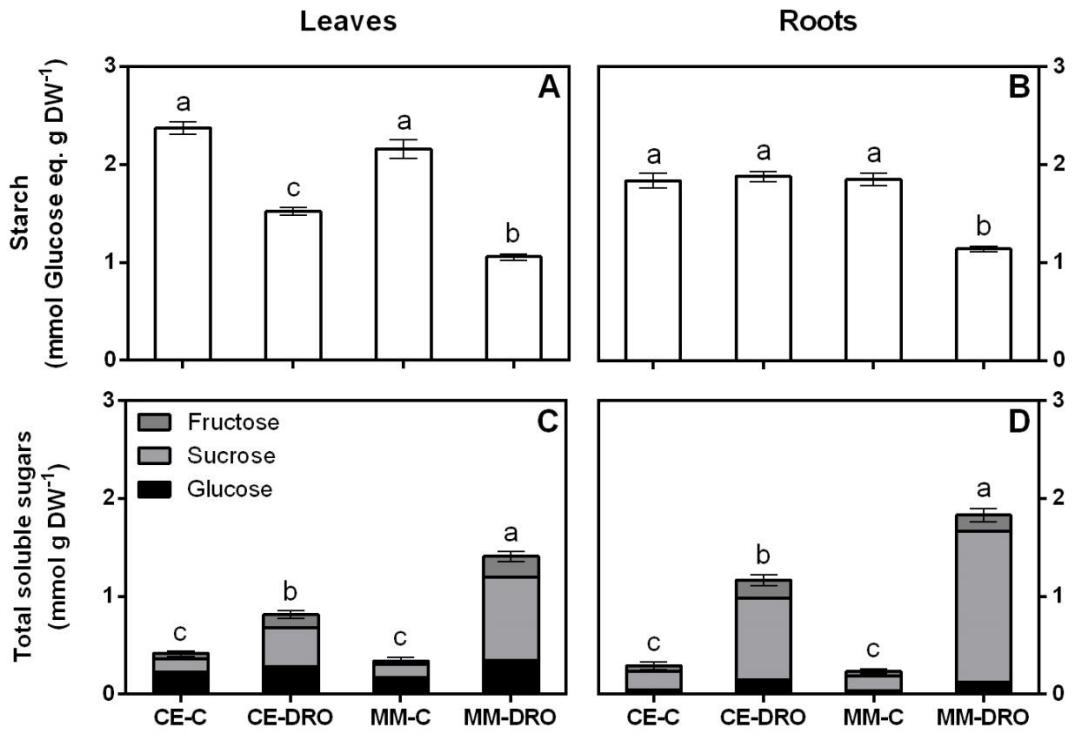

Fig. 4.5. Starch and total soluble sugars (TSS) contents in Ciettaicale (CE) and Moneymaker (MM) tomato in well-watered condition (C) and after 20 days of water deficit (DRO). Starch in [A] leaves and [B] roots. TSS in [C] leaves and [D] roots. Error bars represent the standard error of the mean (n = 5). Bars with same similar letters are not statistically different from one another according to Tukey's test ($P<0.05$).

After 20 DAT, higher concentrations of total soluble sugars (TSS) were found in leaf than in root tissues in both genotypes (Fig. 4.5C and D). However, more significantly increasing rate was recorded in the sink organs. Compared to the control, Moneymaker had 4-fold more and 8-fold more TSS content in leaves and roots, respectively. Conversely, TSS increase was about 2-fold and 4-fold more in Ciettaicale source and sink organs, respectively. Sucrose was the main contributor to these differences in TSS profiling. In fact, leaf sucrose was 6-fold and 3-fold more in Moneymaker and in Ciettaicale comparing to their respective controls (Fig. 4.5C). Moreover, sucrose increased about 10-fold more in Moneymaker roots, representing around 84% of TSS, while in Ciettaicale roots sucrose content increased about 4.5–fold more (Fig. 4.5D).

In Ciettaicale, a 50%-increased root metabolic activity was recorded under drought condition compare with control (Fig. 4.6). On the contrary, a reduction of root activity (around 24% less than controls) was observed in Moneymaker.

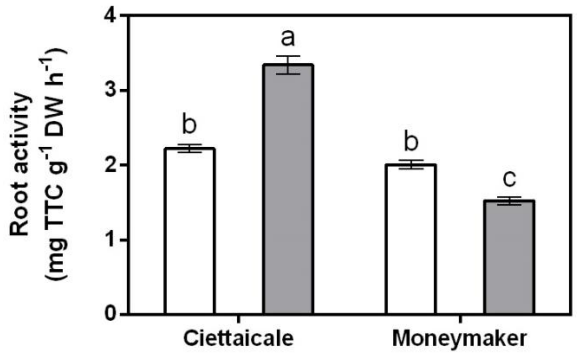

Fig. 4.6. Root activity in Ciettaicale and Moneymaker tomato after 20 days of water deficit. Error bars represent the standard error of the mean (n = 5). Bars with same similar letters are not statistically different from one another according to Tukey's test (P<0.05).

We observed significant differences in leaf phenolic content between both tomato genotypes in well-watered condition, but not after 20 DAT (Fig. 4.7). In fact, under drought Ciettaicale and Moneymaker showed statistically comparable levels of leaf free phenols, but these levels resulted from decreasing and increasing trends, respectively. Otherwise, Ciettaicale and Moneymaker differed for root total phenols level when subjected to drought (Table 4.2).

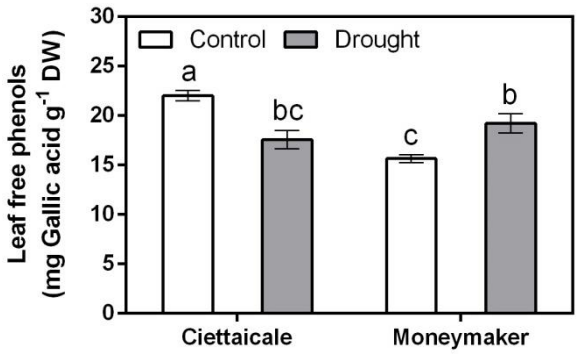

Fig. 4.7. Leaf free phenolic content in Ciettaicale and Moneymaker tomato after 20 days of water deficit. Error bars represent the standard error of the mean (n = 5). Bars with same similar letters are not statistically different from one another according to Tukey's test (P<0.05).

Under stress proline content increased both in leaf and root tissues in Ciettaicale and Moneymaker (Fig. 4.8A and B). However, the increasing rate of proline amount in leaves was stronger in Ciettaicale (10.4 fold more) than in Moneymaker (6.9 fold more), respectively (Fig. 4.8A). On the contrary, Moneymaker had on average more root proline (6.1 fold more) than in Ciettaicale (3.3 fold more) (Fig. 4.8B).

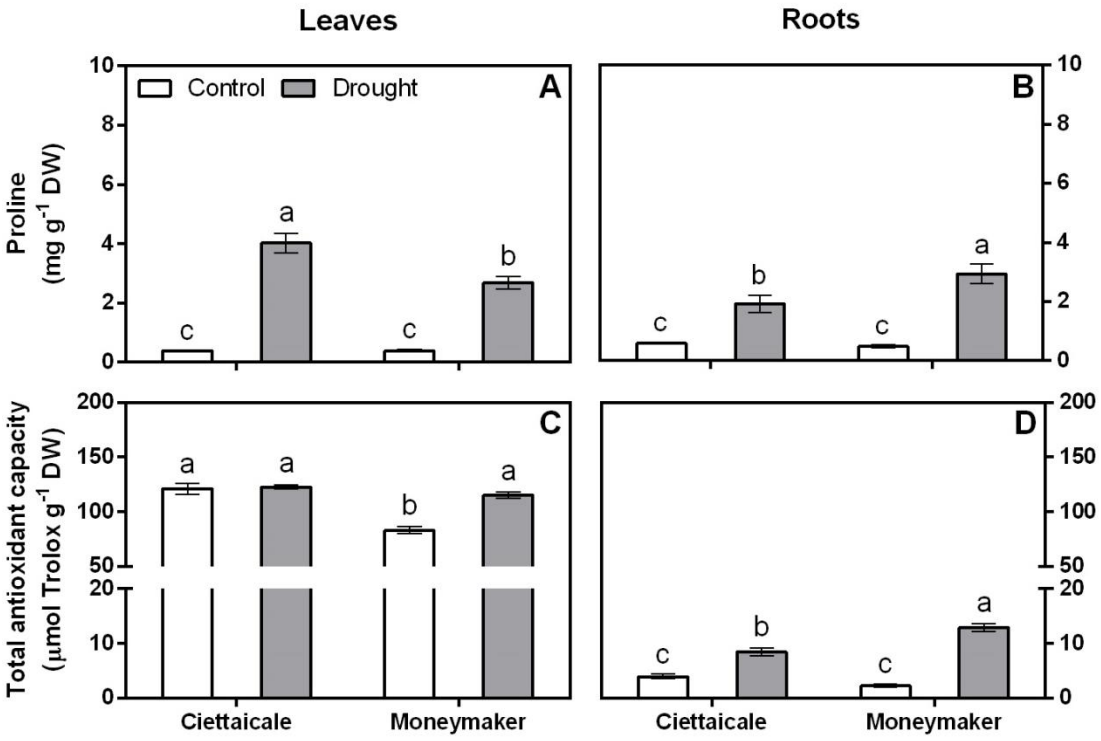

Fig. 4.8. Proline content and total antioxidant capacity (TAC) in Ciettaicale and Moneymaker tomato after 20 days of water deficit. Proline in [A] leaves and [B] roots. TAC in [C] leaves and [D] roots. Error bars represent the standard error of the mean (n = 5). Bars with same similar letters are not statistically different from one another according to Tukey's test (P<0.05).

Water deficit induced no significant changes in leaf TAC in Ciettaicale (Fig. 4.8C). Conversely, Moneymaker had lower TAC in well-watered condition and then increased leaf TAC around 40% when subjected to drought. However, both Ciettaicale and Moneymaker increased TAC in root tissues, despite the increasing magnitude was about 2.1 and 5.6 fold more in Ciettaicale and Moneymaker, respectively (Fig. 4.8D).

4.4 Discussion

In response to drought, tomato activates alternative physiological, biochemical and molecular processes (Iovieno et al., 2016). Most of the commercial tomato varieties, cultivated normally under optimal water condition, are more drought-sensitive than tomato accessions which are originated from arid or warm regions (Galmés et al., 2013). Traditional tomato landraces from the semi-arid Mediterranean Basin can be included in the drought-tolerant category, showing interesting profile that should be more investigated (Galmés et al., 2013; Landi et al., 2017). With this aim, we

explored physiological and biochemical adjustments trigged by 20 days of drought in the Southern Italy tomato landrace Ciettaicale and in the commercial standard variety Moneymaker at vegetative stage.

Drought induced biomass changes in Ciettaicale and Moneymaker plants. Plant growth reprogram could be a flexible and coordinated mechanism to save water and energy, leading to a different partitioning of carbon assimilates among plant tissues (Rogers et al., 1996; Dubois et al., 2017). Root/shoot ratio can be considered an indicator of source-sink carbon allocation changes as well as water status rearrangements within the plant due to external stimuli (Rogers et al., 1996). More water content, as well as an enhanced root activity, could sustain higher investment in root biomass of Ciettaicale comparing to Moneymaker under drought, resulting in a higher root/shoot ratio. Increased root/shoot is a frequent trait observed in some tolerant tomato genotypes in response to different drought conditions (Mingo et al., 2004; Manoj and Uday, 2007). Notably, Ciettaicale also showed high root/shoot under short-term severe salt stress (Moles et al., 2016). However, a reduced root/shoot ratio, as well as a low root activity, observed in Moneymaker, have been previously considered as parts of alternative strategies activated by crops to cope with drought in which losses in biomass compensate for the maintenance of the water status in the tissues (Liu and Li, 2005).

A discrepancy of about 0.4–0.5 MPa in $L\Psi_w$ due to water deficit has been considered as start point for tomato physiological and biochemical changes (Haupt-Herting and Fock, 2002; Mishra et al., 2012). Indeed, since drought trigged a sudden decline in g_s in both genotypes, the changes in $L\Psi_w$ recorded in Ciettaicale and Moneymaker could be associated to a different profiling of soluble sugars, proline and phenols contents, which take part in the water management within plant tissues adjusting the osmotic potential. It is well established that sucrose accumulation in vacuole might play a role as osmoprotectant becoming not available to fuel growth (Hummel et al., 2010; Zhou et al., 2017), as well as higher free phenolic and proline levels have been commonly associated with drought tolerance traits in tomato (Alian et al., 2000; Tahi et al., 2008; Sanchez-Rodriguez et al., 2010). Moreover, free phenols could be invested to form covalent cross-bridges with carbohydrate chains of the cell wall (cell wall-bound phenols) preventing water loss from the apoplast and protecting photosynthetic apparatus (Hura et al., 2012).

Overall, Moneymaker drought-stressed leaves had similar water content comparing to the well-watered ones, associated with a more negative $L\Psi_w$ value. This lower $L\Psi_w$ could affect Moneymaker electron transport rate, causing a stronger drought-induced reduction of Φ_{PSII} (Chaves et al., 2009). Meanwhile, the enduring excess energy is channelled both in alternative electron transport processes like photorespiration and the Mehler-peroxidase pathway (Haupt-Herting and Fock, 2002), or dissipated as NPQ (Mishra et al., 2012). On the contrary, Ciettaicale kept less water in leaves under drought, a condition that required minor adjustments in $L\Psi_w$, but still sufficient to guarantee a more efficient primary photochemistry. In addition, the absence of significant changes in F_v/F_m suggested that the photoprotective mechanisms were able to avoid photoinhibition events, thus preserving the efficiency of primary photochemical processes at PSII in both genotypes, according with Zhou et al. (2017).

ABA accumulation is one of the early response to drought, participating actively in root-to-shoot and shoot-to-root signalling (Christmann et al., 2007; Manzi et al., 2015). The coordinated variation between $L\Psi_w$ and leaf ABA content, especially in Moneymaker, supported ABA involvement in leaf hydraulics impairments (Comstock, 2002). In addition, Manzi et al. (2015) pointed out the substantial contribute of ABA basipetal transport into tomato root under long-term water stress. The effects of ABA on root growth have intensely investigated, revealing a complex and intense cross-talking with auxin and ethylene, although ABA role at root level is still under debate (Sharp and LeNoble, 2002; Mahdieh and Mostajeran, 2009; Thole et al., 2014). The coordinated variation between IAA and ABA found in Moneymaker leaves and roots under drought suggested an interfering mechanism as previously reported in other works (Suzuki et al., 2001; He et al., 2012). The different balance between IAA and ABA found in drought-stressed Ciettaicale in comparison with Moneymaker could positively promote root biomass, conferring improved tolerance to drought stress (Shi et al., 2014).

During dehydration, ROS overproduction primarily targets membrane lipids leading to oxidative damage. H_2O_2 is considered the main compound involved in the membrane lipid peroxidation (Miller et al., 2010). In addition, nowadays it is well established the connection between H_2O_2 and hormones in several physiological processes, including ABA-dependent stomatal closure (Bright et al., 2006) and

auxin-induced lateral root formation (Lavenus et al., 2013). As previously observed by Sanchez-Rodriguez et al. (2010), we found a positive relationship between H_2O_2 level and lipid peroxidation in both genotypes, although more marked in Moneymaker.

In response to a redox unbalance and potential damages, plants react activating own enzymatic and non-enzymatic antioxidant defences (Miller et al., 2010). Total non-enzymatic antioxidant activity significantly increased in Moneymaker leaves under drought, while was unchanged in Ciettaicale, suggesting a different rearrangement of antioxidant machinery.

Leaf sucrose accumulation during stress events limiting photosynthetic uptake, such as drought, can derive from reduction of both *in situ* utilization and export towards sinks. Alterations in soluble sugars level induced by stress events have been associated in tomato source and sink organs with a complex modulation of the carbon metabolism enzymes (Osorio et al., 2014). It is well investigated that the degradation of starch sustains the production of soluble sugars helping osmotic adjustment and providing carbon precursors (Thalmann and Santelia, 2017). Interestingly, the mobilization of root starch reserves observed in Moneymaker could reinforce the hypothesis of the disrupted translocation of photosynthates from leaves. Hence, radical cells could be forced to counteract a prolonged soil drying condition using own storage reserve to produce osmolytes. On the contrary, starch availability in root tissues, as we found in Ciettaicale, could contribute to plant survival under drought (Sala et al., 2010).

In conclusion, physiological and metabolic changes, in terms of ABA, IAA, proline, soluble sugars and phenols contents, occurred in Ciettaicale and Moneymaker under water stress. Overall, our results highlighted the ability of Ciettaicale to manage plant water status under drought in order to preserve both leaf and root activities. This aim was achieved thanks to the preservation of the source-sink relations, though a more efficient PSII photochemistry at leaf level associated with a major investment in root growth and activity in order to improve water uptake. On the contrary, drought-stressed Moneymaker plants reduced Φ_{PSII} and enhanced starch reserve mobilization in both leaves and roots, possibly suggesting a major role of the osmotic adjustment to counteract tissue dehydration, but meantime a feedback potential disruption of source-sink relations. This hypothesis was also supported by

the more pronounced redox disequilibrium, as suggested by higher H_2O_2 and MDA contents, that affected both PSII photochemistry and root activity and markedly trigged in turn the NPQ and antioxidant responses by Moneymaker plants compared to Ciettaicale.

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Chapter 5

Salinity in autumn-winter season and fruit quality of tomato landraces

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Abstract

Tomato landraces, originated by adaptive responses to local habitats, are considered a valuable resource for many traits of agronomic interest, including fruit quality. Primary and secondary metabolites are essential determinants of fruit quality, and some of them, such as carotenoids and phenolics, have been associated with beneficial proprieties for human health. Seasonal variations of environmental parameters, such as temperature and light intensity, as well as the occurrence of salt and drought stress conditions, could significantly modulate organoleptic fruit properties. In an autumn-winter greenhouse hydroponic experiment, we evaluated the response of three Southern-Italy tomato landraces (Ciettaicale, Linosa and Corleone) and one commercial cultivar (UC-82B) to different concentrations of sodium chloride (0 mM, 60 mM or 120 mM NaCl). At harvest, no losses in marketable yield were noticed in all genotypes. Instead, fresh and dry fruit yield, as well as cation concentrations, were markedly affected under salt stress in the commercial cultivar as compared to landraces. Different trends of lycopene content and soluble sugars amount were found in the fruits among all investigated accessions. Data obtained by UPLC-MS revealed differential accumulation of glycoalkaloids, phenolic acids, flavonoids and their derivatives in the landrace fruits under stress in all genotypes. Our results point to the feasible use of tomato landraces as a target to select interesting genetic traits to improve fruit quality under stress conditions.

Keywords

Tomato, landraces, off-season, salinity, fruit quality, metabolites

5.1 Introduction

Tomato is the most consumed berry fruit worldwide as well as one of the most important constituents of the Mediterranean diet representing a key source of minerals, vitamins and antioxidants. Tomato fruit yield, in term of quantity and quality, is the result of what the plant has experienced during its life. Environmental conditions, such as seasonal changes, the occurrence of biotic/abiotic stress and agronomic practices (water availability and fertilizer supply), and genetic factors can affect fruit quality (Poiroux-Gonord et al., 2010), despite the mechanisms behind are not completely clear. To enhance health-related compounds, different agronomic strategies have been applied, namely grafting (Sánchez-Rodríguez et al., 2012; Casals et al., 2018) or controlled water management techniques (Barbagallo et al., 2008).

Salinity induces changes in physiology and metabolism that affect the final crop yield. Tomato is generally considered a salt moderately-tolerant crop, often cultivated in areas polluted by salinization of aquifers and consequent use of saline water for irrigation (Santa-Cruz et al, 2002). In accordance, salinity can positively modulate tomato fruit metabolism that in turn improves the sensorial/nutritional value of the production (D'Amico et al., 2003). Salinity can increase the total soluble content (°Brix) and the titratable acidity, which are two parameters influencing the sweetness of tomato fruits. Moreover, a high salt concentration in irrigation water generally stimulates the defence system of the plant, thereby leading to accumulation of the secondary metabolites in different tissues. One common feature of plant secondary compounds classes, such as carotenoids, polyphenols and terpenoids, is ROS scavenging activity (Ndhlala et al., 2010). Due to their strong antioxidant activity, these bioactive metabolites have been recognized as beneficial players against human cardiovascular and chronic degenerative diseases (Borguini and Torres, 2009) and tumours (Barone et al., 2018). High salinity can accelerate lycopene biosynthesis in hydroponically-grown tomato plants (Wu and Kubota, 2008). Several water management techniques applying controlled and moderate drought/salt stress in the pre-harvesting period of tomato fruits have been implemented to maintain a sufficient yield and also to produce fruits with improved nutritional level (Kubota et al., 2012).

Yield and quality of tomato fruits from off-season greenhouse cultivation are often reduced compared to open-field production (Hu et al., 2006). This effect depends on the association of the different climatic conditions with the covering materials used in the protected environment – that can deplete the intensity and the quality of the light spectrum inside the greenhouse (Toor et al., 2006; Gent, 2007; Mariz-Ponte et al., 2019). In fact, light quality and intensity are major constraints influencing quality parameters in tomato fruit (Slimestad and Verheul, 2005). Generally, °Brix and titratable acidity are positively correlated with increasing solar radiation (Claussen et al., 2006) and temperature (Adams et al., 2001). High light intensity and the modulation of UV-B in the light spectra enhance the flavonoids accumulation in tomato fruit tissues. Low night temperature, which often occurs in non-heated greenhouse during winter, drastically affects plant growth and crop yield (Jing et al., 2016). The diurnally and seasonally changes regarding light intensity, vapour pressure and temperature can also explain the differences observed between seasonal experiments (spring-summer *vs* autumn-winter) and cultivation systems (open-field *vs* greenhouse) (Incerti et al., 2009; Asensio et al., 2019).

The genetic background is also another critical factor that could significantly influence fruit quality (Steward et al., 2000; Vallverdú-Queralt et al., 2011). Among tomato genetic biodiversity, landraces (or traditional varieties) that are originated by adaptive responses to local habitats, are considered a valuable resource for many traits of agronomical interest (Baldina et al., 2016; Patanè et al., 2017; Siracusa et al., 2018). Comparing to the wild relatives, landraces may represent a promising target to improve stress tolerance and nutritional value of modern commercial cultivars (Gascuel et al., 2017).

This work aimed to evaluate fruit quality attributes of different tomato genotypes (three Southern Italy landraces and one commercial variety) in response to combined non-optimal environmental conditions, such as salt stress (moderate and high concentration of sodium chloride in the hydroponically irrigation system) during an autumn-winter (off-season) experiment.

5.2 Materials and methods

5.2.1 Plant material and growth condition

Three Southern Italy tomato landraces (Ciettaicale, Corleone and Linosa) and a tomato standard variety (UC-82B, kindly supplied by the Tomato Genetics Resource Center, Department of Plant Sciences, University of California-Davis, CA, USA) were grown in Grodan Rockwool cube in an open nutrient solution system at a plant density of approximately 3 m^{-2} in a glasshouse at the University of Pisa (Italy) from September 2016 to February 2017. The plants were grown vertically with only three trusses left on the plants. Climatic parameters were continuously monitored by means of a weather station located inside the glasshouse. The mean temperature was 12.9°C (T_{min} = 8.5°C and T_{max} = 17.2°C). Mean values of daily global radiation was 7.4 MJm^{-2} (GR_{min} = 1.9 MJm^{-2} and GR_{max} = 12.9 MJm^{-2}). Two salinity levels of nutrient solution were used with electrical conductivities (EC) of 8.3 and 14.6 mS cm^{-1}, which corresponded roughly to 60 mM and 120 mM NaCl, respectively. The concentration of nutrient solution was as reported by Incerti et al. (2007). Salt stress was initiated 3 weeks after planting; the process was stepped up in roughly 2.1 mS cm^{-1} (20 mM NaCl) daily increments to avoid osmotic shock. Irrigation was controlled by a timer that opened the irrigation lines for 1 min up to 12 times per day, depending on growing stage and environmental conditions.

5.2.2 Biometrical and quality parameters

Fruit yield was determined on the basis of fully ripe fruits picked two times per week from the different trusses. The fruits from the second truss were picked at the red-ripe stage, as usually occurs for marketing and immediately transferred to the laboratory where whole-fruits were ground at 4°C. Aliquots of the obtained material were stored at 80°C for one week to record the dry matter and for cation quantifications; additional aliquots were used to determine °Brix and titratable acidity (Beckles, 2012); finally, other samples were collected in tubes and stored at -80°C for further metabolic analyses.

5.2.3 Total soluble sugars measurements

Tomato fruit homogenate aliquots were extracted in 0.7 M perchloric acid and then neutralized in MES-KOH as previously described in Hostettler et al. (2011). Glucose, fructose, and sucrose were quantified enzymatically according to Thalmann et al. (2016). Briefly, an aliquot of the sample was added to into 50 mM HEPES buffer, pH 7.5, containing 1 mM ATP, 1 mM NAD and 1 mM $MgCl_2$. To measure glucose, hexokinase and glucose 6-phosphate dehydrogenase were used to convert glucose to 6-phosphogluconate with concomitant reduction of NAD to NADH, which was monitored spectrophotometrically at 340 nm. Subsequently, phosphoglucoisomerase was added to determine the amount of fructose. Finally, invertase was added to cleave sucrose into fructose and glucose. The further increase in OD_{340} represented sucrose.

5.2.4 Cation determination

Fruit dried samples were powdered and mineralized (60 min at 220 ºC) using a solution of HNO_3:$HClO_4$ (2:1, v/v). Sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}) were determined using an atomic absorption spectrometer (Varian AA 24FS, Australia).

5.2.5 Lycopene determination

Lycopene content was assayed according to Giovannetti et al. (2012). Briefly, an aliquot of tomato fruit homogenate was extracted in a solution of acetone:ethanol:hexane (1:2:1, v/v) and put in agitation on an orbital shaker for 15 min. Then a volume of distillate water was added, followed by 5 min agitation. After centrifugation, the hexane phase was measured at 503 nm, blanked with pure hexane.

5.2.6 Total flavonoids, total phenols and total antioxidant activity contents

Tomato fruit homogenates were mixed in 70% (w/v) methanol and incubated overnight in agitation at 4°C in the dark. After incubation, the extracts were centrifuged at 12000 x g for 15 min at 4°C and the supernatants were utilized for the analyses indicated below. Total soluble phenols content (TPHE) and total flavonoids content (TFL) were assayed using the respective protocols reported in Caser et al. (2016). Total antioxidant capacity (TAC) was determined by the 2,2-diphenyl-1-

picrylhydrazyl (DPPH) assay as previously reported in Moles et al. (2016), with some modifications. Briefly, an aliquot of the methanolic leaf extract was added to a 0.1 mM methanolic DPPH solution. After 30 min of incubation at room temperature in the dark, absorbance was measured at 515 nm, and the results were expressed as μmol of Trolox per gram of plant material on dry basis.

5.2.7 Metabolite profiling

Tomato fruit homogenate (100 mg) was extracted with 100% methanol. The samples were ground using a mixer mill with 3 glass beads for 1.5 min at 30 Hz, centrifuged at 15,000 x g at 4°C and the supernatants collected. All samples were de-salinized over a silica-based classic cartridge (WAT051910, Waters) accordingly to the manufacturer instructions. The eluted samples were concentrated using a Savant SpeedVac concentrator (Thermo Fisher Scientific) at 42°C. Prior to LC-MS/MS analysis the samples were re-suspended in 80% methanol, 0.1% formic acid. After sonication for 5 min, the samples were centrifuged at 15,000 x g, at 4 °C for 5 min, and transferred to liquid chromatography vials. Samples were analysed on a UHPLC (Dionex UltiMate 3000, Thermo Fisher Scientific) coupled to a Bruker compact electrospray ionisation-quadrupole-time-of-flight tandem-mass spectrometer (Bruker Daltonics). The UHPLC separation was performed at 28 °C with a C18 reverse-phase column (ACQUITY UPLC TM BEH C18, 1.7 μm, 2.1 × 150 mm, Waters) using the following gradient of solvent B (acetonitrile with 0.1% (v/v) formic acid) and solvent A (water with 0.1% (v/v) formic acid): 0–0.5 min, 5% B; 0.5–12 min, 5–100% B; 12–14 min, 100% B; 14–16 min, 100–5% B. The flow rate was set-up to 0.3 mL min^{-1} and 5 μL of each sample was injected. The ESI source was operated in positive mode and parameters were set as follows: gas temperature, 220 °C; drying gas, 9 l min^{-1}; nebuliser, 2.2 bar; capillary voltage, 4500 V; end plate offset, 500 V. The instrument was set to acquire an m/z range of 50–1300. Conditions for MS/MS were set as described by Christ et al. (2016). All data were recalibrated internally using pre-run injection of 10 mM sodium hydroxide in 0.2% formic acid, 49.8% water, 50% isopropanol (v/v/v). Data Analysis v.4.2 and TargetAnalysis v.1.3 softwares (Bruker Daltonics) were used to analyse the data. Metabolites were identified and annotated by comparison MS and MS/MS data spectrum either

generated by authentic reference standards or deposited in the literature and databases, such as PubChem and MoTo (Moço et al. 2006).

5.2.8 Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) and the mean values were compared using Duncan's test ($P<0.05$) to check the significant differences. Statistical analysis was performed with Statgraphics Centurion XVII (Statpoint Technologies, Inc. Warrenton, Virginia, U.S.A.) software.

5.3 Results

5.3.1 Yield, quality parameters and soluble sugars

Salinity treatments affected differently fruit size (Fig. 5.1) as well as the yield FW and DW in the tomato genotypes (Fig. 5.2).

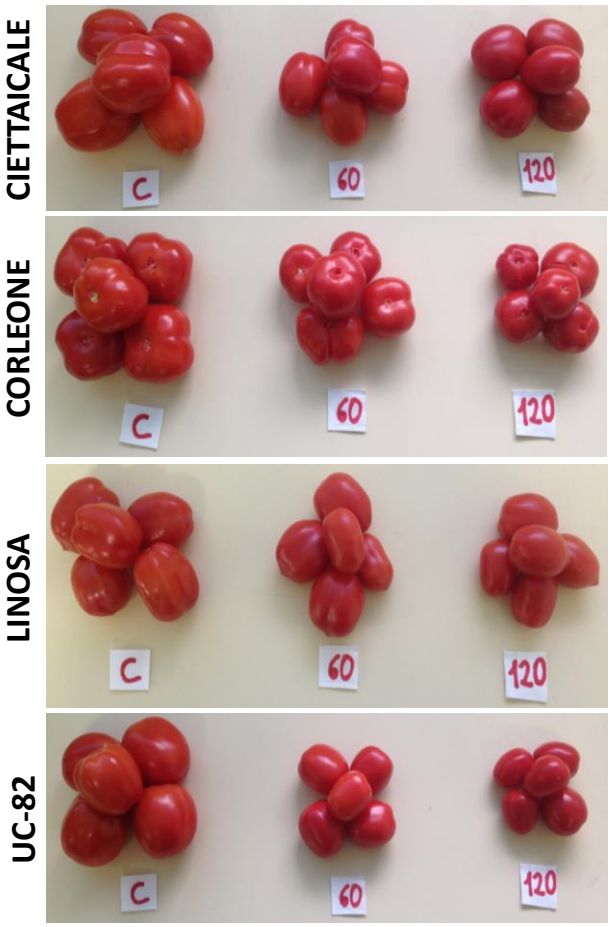

Fig. 5.1. Effect of different concentrations of sodium chloride (60 mM or 120 mM NaCl) on fruit size of three tomato landraces (Ciettaicale, Corleone and Linosa) and tomato commercial variety (UC-82B) compared to respective control condition (C).

Ciettaicale and UC-82B showed a progressive reduction in yield FW according to the salt gradient, but with different magnitude: in fact, under 60 mM and 120 mM NaCl Ciettaicale lost around 19% and 43%, respectively, while in the same conditions UC-82B showed yield FW reduced approximately by 43% and 55.5%, respectively (Fig. 5.2A).By contrast, Corleone and Linosa decreased yield FW only under 120 mM NaCl, reducing fresh fruit production of around 47% and 20%, respectively. We did not recorded a significant effect of salinity on the dry matter (yield DW) in Ciettaicale, Linosa and UC-82, despite the commercial variety showed lower values of this parameter comparing to the landraces already from control condition (Fig. 5.2B). Interestingly, Corleone increased yield DW under 60 mM NaCl (around 14%) and then decreased under 120 mM around 27% comparing to the control condition.

In all genotypes 120 mM NaCl treatment caused significant increase in fruit °Brix and titratable acidity (Table 5.1). We did not detected sucrose in fruits samples, but we observed different trends in glucose and fructose contents among tomato genotypes (Fig. 5.3). Under 120 mM NaCl Ciettaicale and Linosa increased glucose and fructose contents, while Corleone accumulated just more glucose. However, no significant changes in glucose and fructose levels were observed in the commercial variety according to the salt gradient.

Table 5.1. Effect of different concentrations of sodium chloride (60 mM or 120 mM NaCl) on fruit total soluble solids (°Brix) and titratable acidity (TA) of three tomato landraces (Ciettaicale, Corleone and Linosa) and tomato commercial variety (UC-82B). Data are means ± SE of six replicates. Means within a column followed by the same letter are not significantly different based on Duncan's test ($P < 0.05$).

Genotype	[NaCl]	°Brix	TA
Ciettaicale	Control	3,84±0.25 e	0,26±0.01 d
	60 mM	4,94±0.15 cd	0,29±0.01 c
	120 mM	6,28±0.13 b	0,33±0.01 b
Corleone	Control	4,10±0.10 e	0,29±0.01 c
	60 mM	4,60±0.18 d	0,30±0.01 c
	120 mM	6,44±0.13 b	0,39±0.01 a
Linosa	Control	5,04±0.11 c	0,27±0.01 cd
	60 mM	6,02±0.12 b	0,33±0.01 b
	120 mM	7,14±0.12 a	0,29±0.01 c
UC-82B	Control	2,50±0.16 f	0,17±0.01 e
	60 mM	4,14±0.12 e	0,25±0.01 d
	120 mM	4,70±0.07 cd	0,36±0.02 a

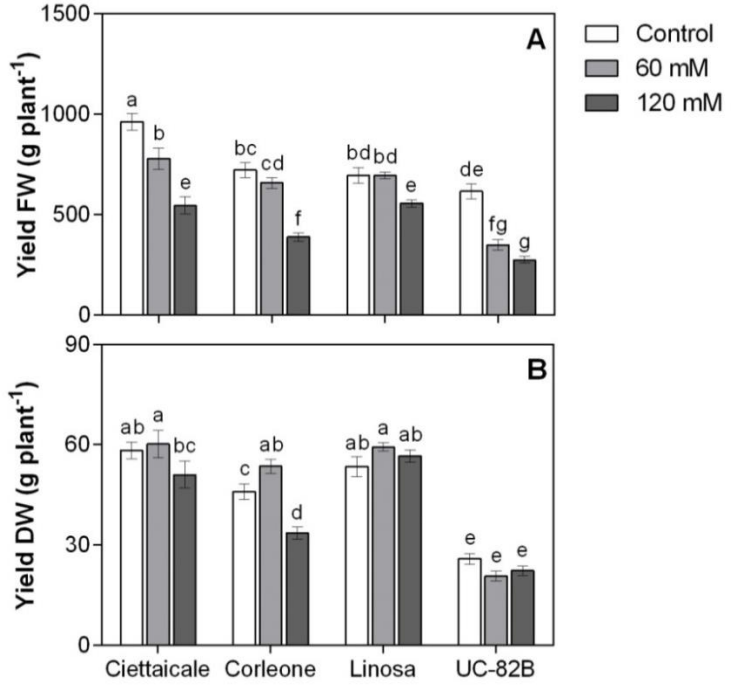

Fig. 5.2. Effect of different concentrations of sodium chloride (60 mM or 120 mM NaCl) on fruit yield of three tomato landraces (Ciettaicale, Corleone and Linosa) and tomato commercial variety (UC-82B). [A] Yield fresh weight (FW) and [B] yield dry weight (DW). Error bars represent the standard error of the mean (n=16). Bars with same letters are not statistically different from one another according to Duncan's test ($P < 0.05$).

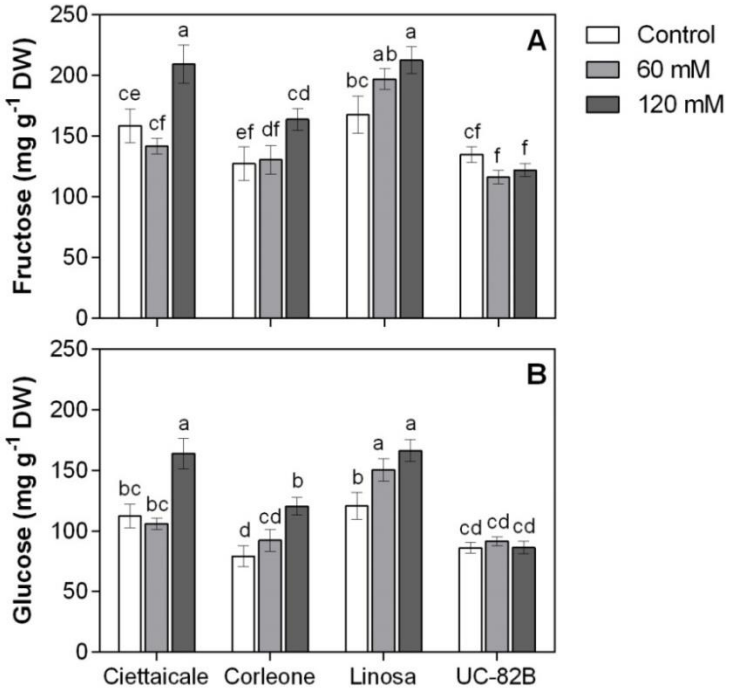

Fig. 5.3. Effect of different concentrations of sodium chloride (60 mM or 120 mM NaCl) on fruit hexoses contents of three tomato landraces (Ciettaicale, Corleone and Linosa) and tomato commercial variety (UC-82B). [A] Fructose content and [B] glucose content. Error bars represent the standard error of the mean (n=6). Bars with same letters are not statistically different from one another according to Duncan's test ($P < 0.05$).

5.3.2 Cations

As increasing salinity in the nutrient solution, the contents of Na^+, as well as K^+ and Ca^{2+}, were affected in tomato fruits (Table 5.2). In particular, Ciettaicale showed the highest fold increase among all the genotypes, reaching 3.4 fold more under 60 mM and 5.3 fold more under 120 mM comparing to its control. Under 120 mM NaCl the lowest Na^+ concentration was found in Linosa fruits (2.5 fold more than its control), while the highest Na^+ level was recorded in UC-82B (4 fold more than its control). Notably, Ciettaicale accumulated more K^+ under both salinity treatments than in control, as well more K^+ was found in Linosa under 60 mM NaCl. However 120 mM salt induced a decrease of K^+ concentration in Corleone and UC-82B. Commercial variety fruits showed reduced Ca^{2+} content already from 60 mM NaCl, as well Linosa had less Ca^{2+} under 120 mM comparing to its controls. Overall, Linosa maintained higher K^+/Na^+ and Ca^{2+}/Na^+ ratios at 60 mM and 120 mM NaCl comparing to the other genotypes, while Ciettaicale and UC-82B more markedly decreased Ca^{2+}/Na^+ ratio.

Table 5.2. Effect of different concentrations of sodium chloride (60 mM or 120 mM NaCl) on fruit cation contents of three tomato landraces (Ciettaicale, Corleone and Linosa) and tomato commercial variety (UC-82B). Data are means ± SE of six replicates. Means within a column followed by the same letter are not significantly different based on Duncan's test ($P < 0.05$).

Genotype	[NaCl]	Na^+	K^+	Ca^{2+}	K^+/Na^+	Ca^{2+}/Na
Ciettaicale	Control	0.62±0.03 f	30.03±0.94 f	1.70±0.09 cd	48.96±2.92 a	2.76±0.20 a
	60 mM	2.12±0.22 cd	36.47±2.26 bd	1.61±0.13 cd	17.38±1.03 d	0.77±0.06 e
	120 mM	3.31±0.31 b	36.29±1.22 bd	1.71±0.09 bd	11.18±1.27 ef	0.52±0.04 eg
Corleone	Control	0.95±0.09 f	39.21±0.81 ab	1.57±0.14 cd	41.94±3.35 b	1.65±0.04 e
	60 mM	1.99±0.02 de	38.22±0.89 ac	1.56±0.12 cd	19.20±0.30 d	0.79±0.06 c
	120 mM	3.48±0.11 b	34.38±0.65 de	1.45±0.04 d	9.90±0.43 ef	0.42±0.01 fg
Linosa	Control	0.89±0.07 f	36.93±1.39 bd	1.78±0.13 ac	42.10±3.68 b	2.02±0.20 b
	60 mM	1.67±0.06 e	41.29±1.04 a	2.02±0.03 a	24.79±1.23 c	1.21±0.05 d
	120 mM	2.26±0.61 cd	35.38±0.47 ce	1.49±0.03 cd	15.73±0.53 de	0.66±0.02 ef
UC-82B	Control	1.00±0.02 f	37.46±0.70 bd	1.99±0.10 b	37.50±1.21 b	2.00±0.12 b
	60 mM	2.50±0.20 c	35.83±1.06 bd	1.16±0.06 e	14.61±1.69 de	0.47±0.03 fg
	120 mM	3.98±0.14 a	32.00±0.94 ef	1.10±0.06 e	8.05±0.04 f	0.28±0.01 g

5.3.3 Lycopene

Landraces treated by 60 mM NaCl did not show changes in lycopene content (Fig. 5.4). However, Corleone fruits under 120 mM NaCl contained around 40% less of lycopene level comparing to controls. Notably, UC-82B in control conditions showed the highest values of fruit lycopene among the tomato genotypes. Conversely, the commercial variety showed a progressive decrease in lycopene content already from 60 mM NaCl (-38%) and then more marked under 120 mM (-55%) comparing to its control.

5.3.4 Total flavonoids, total phenols and total antioxidant activity

Salt conditions did not affect Ciettaicale and Linosa TFL (Fig. 5.5A). Moreover, an increase (+ 21%) in TFL was recorded in Corleone fruits under 120 mM NaCl comparing to controls. Conversely, salinity negatively affected TFL in UC-82B. We did not determine differences in TPHE in the landraces under 60 mM NaCl (Fig. 5.5B). In the same stress condition, UC-82B reduced around 29% fruit TPHE comparing to its control. The highest salt concentration induced a decrease in fruit TPHE with a similar magnitude in Corleone, Linosa and UC-82B comparing to respective controls (-26%, -28% and -33%, respectively). At harvesting point, fruits of Ciettaicale and Linosa maintained roughly control TAC values under 60 mM NaCl (Fig. 5.5C). Corleone and UC-82B decreased TAC under 60 mM (-17% and -29% comparing to their respective controls). However, a common reduction in TAC was observed in all genotypes under 120 mM comparing to controls in a scale between -17% found in Linosa to -48% recorded in UC-82B.

5.3.5 Metabolite profiling

An untargeted UPLC-MS/MS analysis profiled the same sample sets as described above. We were able to identify 34 metabolites (Table 5.3) based on accurate mass measurements and MS/MS spectra from biological standards or publicly available data, namely literature and/or databases such as PubChem and Moto. ANOVA results were reported in Tables 5.S1-S4 and graphically represented in Fig. 5.6. Most metabolites detected were phenylpropanoids (11 hydroxycinnamic acids and 7 flavonoids) and glycoalkaloids (9). The rest of the metabolites were assigned as phenylamides (4), amino acids (2) and vitamins (1).

From the hydroxycinnamic acids (Fig. 5.6A and Table 5.S1), coumaric acid (detected as two isomers) was the compound that presented the most intense mass signal, especially in Linosa. We could observe only a significant decrease of coumaric acid under 120 mM NaCl in both Linosa and Ciettaicale if compared to UC-82B. Interestingly, the same trend was observed in coumaric acid-hexose accumulation. Two ferulic acid isomers were detected: ferulic acid I significantly increased in all genotypes under salt stress compared to control conditions, whereas ferulic acid II did not significantly change in the case of Ciettaicale and Corleone upon salt stress. Linosa and UC-82B displayed a similar trend of reduction of the intensity of ferulic acid II mass signal upon 120 mM NaCl. All three 1,3-O-Dicaffeoylquinic acid isomers showed a remarkably low mass signal intensity in UC-82B fruits when compared to the landraces. At 120 mM NaCl Corleone fruits showed a 3 fold increase of 1,3-O-Dicaffeoylquinic acid I mass signal intensity. Also, 3,4,5-Tricaffeoylquinic acid showed a significant increase in mass signal intensity in Corleone fruits upon 120 mM NaCl.

Apart from hydroxycinnamic acids, the phenylamides conjugated to caffeic acid (Caffeoylputrescine I and II isomers) and ferulic acid (Feruloylputrescine I and II isomers) were also identified (Fig. 5.6C and Table 5.S1). Caffeoylputrescine I was significantly reduced in all landraces upon 120mM NaCl, whereas the same salt concentration induced an increased in UC-82B fruits. Feruloylputrescine I was significantly reduced in all salt treatment conditions for the three landraces, whereas the commercial variety showed a significant increase upon 120 mM NaCl. Remarkably, Feruloylputrescine II increased in Corleone and UC-82B fruits upon salt stress, but in Ciettaicale and Linosa fruits the opposite trend was observed.

Regarding the flavonoids identified, rutin was the compound that showed the most intense mass signal (Fig. 5.6B and Table 5.S2). Remarkably, UC-82B fruits showed the highest signal for all the flavonoids detected. Nevertheless, rutin and rutin-O-pentoside did not significantly change upon salt stress in all landraces, whereas UC-82B fruits showed a significant decrease of rutin upon 60 and 120 mM NaCl.

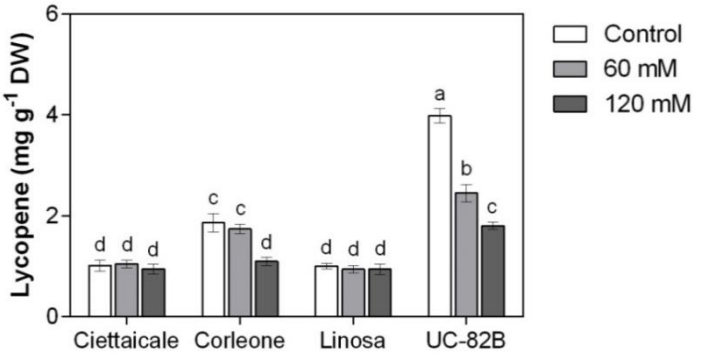

Fig. 5.4. Effect of different concentrations of sodium chloride (60 mM or 120 mM NaCl) on fruit lycopene content of three tomato landraces (Ciettaicale, Corleone and Linosa) and tomato commercial variety (UC-82B). Error bars represent the standard error of the mean (n=6). Bars with same letters are not statistically different from one another according to Duncan's test ($P < 0.05$).

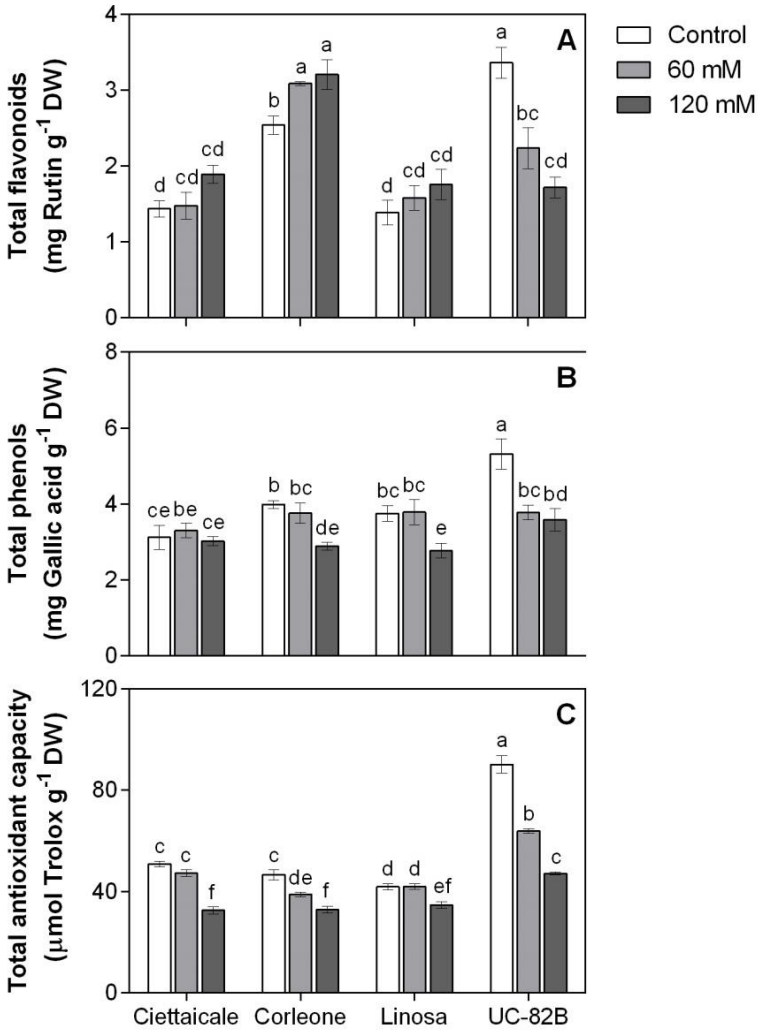

Fig. 5.5. Effect of different concentrations of sodium chloride (60 mM or 120 mM NaCl) on fruit phenolic content and antioxidant capacity of three tomato landraces (Ciettaicale, Corleone and Linosa) and tomato commercial variety (UC-82B). [A] Total flavonoids, [B] total phenols and [C] total antioxidant capacity. Error bars represent the standard error of the mean (n=6). Bars with same letters are not statistically different from one another according to Duncan's test ($P < 0.05$).

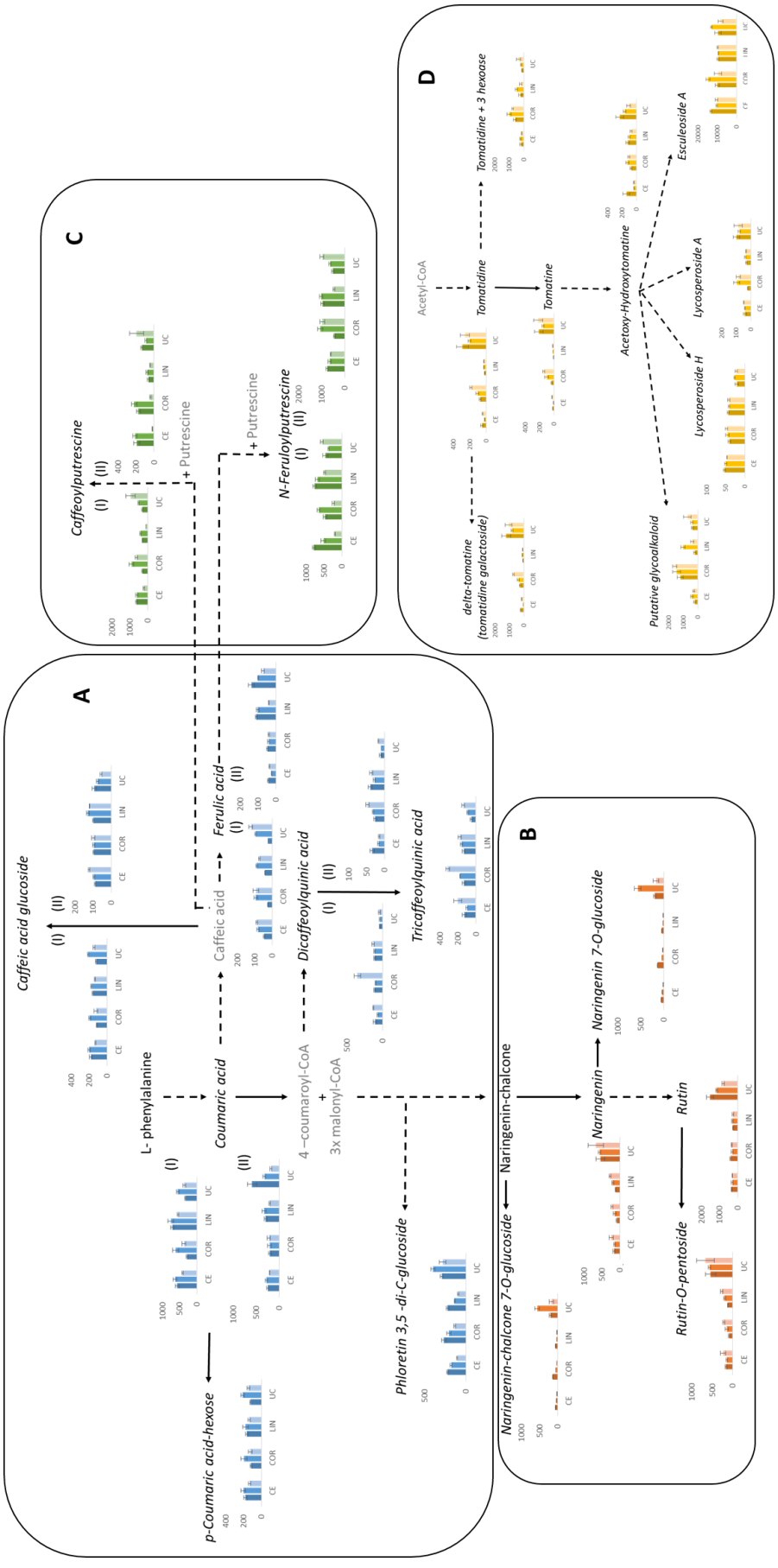

Fig. 5.6. Overview of the plant specialized metabolites responsive to salinity stress in Italian tomato landraces. [A] Hydroxycinnamic acids, [B] Flavonoids, [C] Phenylamides and [D] Glycoalkaloids. Metabolites that were identified in the present study are represented in black, whereas non identified metabolites are in grey. Metabolites that showed statistically significant changes (landraces x salinity treatment) are represented in italic and graphical representations are presented. The graphs represent the mean of six biological replicates ± SE. Dark, middle and light colours (blue, orange, green and yellow) represent 0, 60 and 120 mM NaCl, respectively; CE, Ciettaicale, LIN, Linosa, COR, Corleone, UC, UC-82B.

Table 5.3. Peak list and diagnostics of the plant specialized metabolites responsive to salinity stress in Italian tomato landraces. For the identification level: A, database; B, database, S, standard.

Peak #	Ret time (min)	Metabolite name	Molecular formula	$[M+H]_{ob}$	$[M+H]_{theo}$	MS/MS fragments	Metabolite class	Identificaton level	Reference
1	3,33	Phenylalanine	C9H11NO2	166,0869	166,0863	120, 103	Amino acid	B	PubChem
2	4,52	Tryptophan	C11H12N2O2	205,0976	205,0971	188, 170, 159, 146, 118	Amino acid	B	PubChem
3	7,9	Naringenin chalcone	C15H12O5	273,0763	273,0757	153, 147, 119	Flavonoids	S, A, B	Moço et al., 2006; MoTo
4	8,05	Naringenin	C15H12O5	273,0764	273,0757	153, 147, 119	Flavonoids	S, A, B	Moço et al., 2006; MoTo
5	6,43	Naringenin 7-O- glucoside	C21H22O10	435,1304	435,1285	273, 153	Flavonoids	A, B	Moço et al., 2006; MoTo
6	6,8	Naringenin chalcone 7-O- glucoside	C21H22O10	435,1298	435,1285	273, 153	Flavonoids	A, B	Moço et al., 2006; MoTo
7	5,81	Phloretin 3',5'-Di-C-glucoside	C27H34O15	599,1970	599,1990	497, 479, 461, 449, 431, 419, 413, 407, 395, 383, 377, 365, 353, 341, 329, 301, 259, 247, 235, 107	Flavonoids	A	Slimestad et al., 2008; Beelders et al., 2014
8	5,69	Rutin	C27H30O16	611,1616	611,160661	303	Flavonoids	S	
9	5,39	Rutin-O-pentoside	C32H38O20	743,2041	743,20292	465, 303	Flavonoids	A, B	Moço et al., 2006; MoTo
10	6,52	Tomatidine	C27H45NO2	416,3524	416,3523	398, 273, 255, 161	Glycoalkaloids	A, B	Caprioli et al., 2015; MoTo
11	6,5	Delta-tomatine	C33H55NO7	578,4057	578,40513	417, 273, 255, 161	Glycoalkaloids	A, B	Cataldi et al., 2005; PubChem
12	5,95	Tomatidine+3hexoase	C45H75NO18	918,5081	918,505691	432, 245, 162	Glycoalkaloids	A	Iijima et al., 2008
13	6,5	Tomatine	C50H83NO21	1034,5538	1034,5530		Glycoalkaloids	A, B	Moço et al., 2006; MoTo
14	5,72	Lycoperoside H	C50H83NO22	1050,5475	1050,5480	1032, 594, 432, 325, 273, 255, 163, 145, 127	Glycoalkaloids	A	Adato et al., 2009
15	6,45	Lycoperoside A	C52H85NO23	1092,5577	1092,5585		Glycoalkaloids	A	Moço et al., 2006
16	5,93	Acetoxy-Hydroxytomatine	C52H85NO24	1108,5517	1108,5534		Glycoalkaloids	A	Iijima et al., 2008
17	5,55	Esculeoside A	C58H95NO29	1270,6011	1270,6063	1210, 1090, 1048, 1030, 652, 592	Glycoalkaloids	A	Iijima et al., 2008
18	6,47	Putative glycoalkaloid	C53H76N8O15	1065,5503	1065,5504	903, 741, 579, 417, 325, 273, 255, 163, 145, 127	Glycoalkaloids		
19	4,33	Coumaric acid I	C9H8O3	165,0550	165,0546	147, 119, 91	Hydroxycinnamic acids	B	Moço et al., 2006; MoTo
20	4,99	Coumaric acid II	C9H8O3	165,0552	165,0546	147, 119, 91	Hydroxycinnamic acids	B	Moço et al., 2006; MoTo
21	4,59	Ferulic acid I	C10H10O4	195,0659	195,0651	177, 145, 117	Hydroxycinnamic acids	B	Moço et al., 2006; MoTo
22	6,25	Ferulic acid II	C10H10O4	195,0663	195,0651	177, 145, 117	Hydroxycinnamic acids	B	Moço et al., 2006; MoTo
23	4,33	Coumaric acid-hexose	C15H18O8	327,1082	327,1074	165, 147, 119	Hydroxycinnamic acids	A, B	Moço et al., 2006; MoTo
24	4,28	Caffeic acid glucoside I	C15H18O9	343,1035	343,1023	181, 163	Hydroxycinnamic acids	A, B	Moço et al., 2006; MoTo
25	4,8	Caffeic acid glucoside II	C15H18O9	343,1033	343,1023		Hydroxycinnamic acids	A, B	Moço et al., 2006; MoTo
26	6,4	1,3-O-Dicaffeoylquinic acid I	C25H24O12	517,1351	517,134053	163	Hydroxycinnamic acids	B	PubChem
27	6,77	1,3-O-Dicaffeoylquinic acid II	C25H24O12	517,1354	517,134053	323, 295, 273, 163	Hydroxycinnamic acids	B	PubChem
28	7,15	1,3-O-Dicaffeoylquinic acid III	C25H24O12	517,1367	517,134053	163	Hydroxycinnamic acids	B	PubChem
29	7,15	3,4,5-Tricaffeoylquinic acid	C34H30O15	679,1679	679,165747	499, 163	Hydroxycinnamic acids		
30	3,24	Caffeoylputrescine I	C13H18N2O3	251,1399	251,1390	234, 163, 145, 135,117	Phenylamides	A	Gaquerel et al., 2010
31	3,96	Caffeoylputrescine II	C13H18N2O3	251,1398	251,1390	234, 163, 145, 135,117	Phenylamides	A	Gaquerel et al., 2010
32	4,36	Feruloylputrescine I	C14H20N2O3	265,1539	265,1546	177, 145, 117	Phenylamides	B	PubChem
33	4,6	Feruloylputrescine II	C14H20N2O3	265,1532	265,1546	177, 145, 117	Phenylamides	B	PubChem
34	3,92	Pantothenic acid -hexose	C15H27NO10	382,1722	382,1707	252, 220, 202, 184, 116, 90	Vitamins	A	Mintz-Oron et al., 2008

The list of identified compounds also includes 9 glycoalkaloids that presented a variable accumulation pattern in the studied conditions (Fig. 5.6D and Table 5.S4). Esculeoside A was the glycoalkaloid that showed highest mass signal intensity. Under control Ciettaicale accumulated significant higher amounts of Esculeoside A than all the other genotypes, while 60 mM NaCl promoted its accumulation in Corleone and UC-82B fruits. Under 120 mM NaCl all genotypes accumulated similar levels of Esculeoside A. Tomatidine was significantly induced in the commercial variety and Corleone when compared to Ciettaicale and Linosa. Tomatidine+3hexoase showed the highest mass intensity signal in Corleone fruits, in particular upon 60 mM NaCl treatment. Tomatine, delta-Tomatine and Lycosperoside A were significantly induced in the commercial variety in all conditions and Corleone upon 120 mM NaCl. By the other hand, Lycoperoside H was significantly higher in all landraces compared to UC-82B variety, and its content was not modulated by salt treatment.

We also identified the amino acids phenylalanine and tryptophan, and the vitamin derivative, pantothenic acid–hexose (Table 5.S3). Phenylalanine showed a marked increase in mass signal intensity upon 60 mM NaCl treatment in Ciettaicale fruits, whereas for the other genotypes the levels remained unchanged except for Corleone fruits at 120 mM NaCl, where a significant reduction of mass signal was observed. The lowest mass signal intensity of tryptophan was observed in Ciettaicale in particular at 120 mM NaCl treatment. Under 120 mM NaCl, all the other genotypes showed the same mass signal intensity for this compound. Finally, the levels of pantothenic acid –hexose were promoted upon 60 mM NaCl in all genotypes except for Linosa, which remained with unaltered levels of this metabolite in all treatments.

5.4 Discussion

Tomato landraces are a valuable resource for many traits of agronomic interest, namely due to their resilience against abiotic stresses, which contributes to yield stability and adaptation to low input and/or adverse growth conditions. Landraces are also associated with distinctive organoleptic and nutritional quality traits. According to the literature, landraces can exhibit particular and often contrasting metabolic profiles. High contents in functional compounds are frequent traits found in Mediterranean traditional tomato varieties (Pinela et al., 2012; Berni et al., 2018), but

often no differences in sensory profile have been identified between commercial varieties and landraces (Ruiz et al., 2005; Sinesio et al., 2007; Casals et al., 2011). Moreover, the promoting effect on fruit quality metabolites is frequently ascribed to the concentration effect and not to the active accumulation (Zushi and Matsuzoe, 2015). However, all the studies unanimously concluded that the interaction between genotype and environment is the key component that modulates the expression of specific metabolic patterns (Berni et al. 2019). For example, Massaretto et al. (2018) reported that 100 mM NaCl affected differently the metabolism of two Spanish tomato landraces, but both genotypes increased fruit quality: one accumulating more carotenoids and the other redirecting the metabolic pathway toward other metabolites still unknown.

In the current work, we assessed the effects of high salt concentrations in terms of yield and quality traits in three Southern Italy tomato landraces: Ciettaicale, Corleone and Linosa. Most commercial tomato cultivars are classified as moderately sensitive to salinity (Cuartero et al., 2006). For this reason, we conducted this study in autumn-winter off-season climatic conditions, which allowed us to use salt concentrations that normally are considered high for a moderately salt tolerant crop such a tomato and that is known to deeply affect fresh marketable production when tomato plants are cultivated in a cropping season. In fact, 60 mM and 120 mM NaCl concentrations can be considered as 10% and 20% seawater dilutions, respectively. Many studies showed that high salt concentrations can improve tomato functional fruit quality with minimal impact on the commercial yield (D'Amico et al., 2003; Incerti et al., 2007; Sgherri et al., 2007).

In our study, salinity promoted the anticipation of fruit ripening in all genotypes, but differentially caused fresh fruit yield losses. Notably, under 60 mM NaCl all landraces showed better performance in terms of yield FW compared to the commercial variety. The most interesting results were the absence of yield loss in Corleone and Linosa under 60 mM NaCl and the observation that Linosa reduced only around 20% fresh fruit production under 120 mM NaCl. The capacity to maintain an adequate yield has been also found in a Kenyan tomato landrace grown in site soil polluted by high salty water irrigation (Agong et al., 1997).

Yield and functional quality can be influenced by salinity mainly due to sodium competition for other cations, such as K^+ and Ca^{2+} (Adams, 1991; Petersen et al.,

1998). Among the genotypes, Linosa maintained higher K^+/Na^+ according to the salt gradient, as well as a higher Ca^{2+}/Na^+ ratio. Calcium participates both in the alleviation of sodium toxicity and in the fruit size development (Manaa et al., 2013; Plieth, 2005). This observation could support the ability of Linosa to maintain an adequate yield under salt stress. Additionally, fresh tomato fruits with high Ca^{2+} content represent a functional natural mineral supply indispensable in human dietary (Soetan et al., 2010). On the contrary, in the commercial variety the Ca^{2+}/Na^+ ratio was more affected by the salinity gradient, leading to a more marked reduction of fruit size and weight. Several studies reported that calcium deficiency affects tomato fruit development (Park et al., 2005), often resulting with the appearance of the blossom-end rot (Taylor and Locascio, 2004). However, in our study, as well as in the winter greenhouse experiment conducted by Zushi and Matsuzoe (2009), the fruits of all genotypes were not affected by this marketable injury.

The increase in soluble solids and titratable acidity are common responses of tomato fruits under salt stress (Balibrea et al., 2003; Zushi and Matsuzoe, 2015). High values of these parameters have been found in Spanish tomato landraces under salt stress (Massaretto et al., 2018). Soluble solids content (°Brix) estimated mainly the sugars amount in tomato fruit pulp, but also organic acids, amino acids, soluble pectins, phenolic compounds and mineral (Beckles et al. 2012). Nevertheless, the sugar/acid ratio generally increases during summer and decreases during winter. Primary metabolism is more affected when light and temperature changes occurred during early fruit development than when environmental conditions mutate during the ripening phase (Gautier et al., 2008). Under 120 mM NaCl all genotypes showed higher °Brix content compared to the respective controls. High °Brix improves the taste of tomato fruits and is a desirable characteristic for the processing of tomato products (De Pascale et al., 2001). Also 120 mM NaCl promoted the accumulation of fructose and glucose in the landraces, but not in the commercial variety. Zushi and Matsuzoe (2015) reported that the cherry tomato cv. Mini Carol fruits accumulated more glucose and fructose under 50 mM NaCl, while the normal-fruited tomato cv. House Momotaro increased total soluble sugars only under 100 mM NaCl. These authors concluded that the salt effect on sugars levels depends essentially on the genotype. Interestingly, we did not detect sucrose in the fruits for any of the studied tomato accessions. This result could be explained by the fact that salt stress promotes

invertase activity and consequently the release of hexoses during tomato ripening (Balibrea et al., 2003). Also, the fact that low solar radiation conditions, such as the ones experienced in our study, affect sugar concentration in sink tissues due to a limitation in carbon fixation/transport in/from source leaves (Hu et al., 2006) can contribute to such results.

The content of lycopene, the main carotenoid that confers the red pigmentation to the tomato fruit, is a genotype-dependent trait. Lycopene metabolism can be modulated by water deficit (Atkinson et al., 2011; Coyago-Cruz et al., 2017), low light radiation as well low temperature (Dumas et al., 2003; Jarquín-Enríquez et al., 2013). Even though several tomato landraces have been identified with constitutive high carotenoid content (Pinela et al., 2012; Figàs et al., 2015; Zushi and Matsuzoe, 2015), our landraces showed lower fruit lycopene content under control conditions compared to the commercial variety. Also upon 60 mM NaCl treatment, the landraces maintained roughly control values of lycopene, while the commercial variety significantly reduced its content. Ciettaicale and Linosa displayed under 120 mM NaCl no changes in lycopene amount. Interestingly, the decrease in K^+ content, as we observed in Corleone and UC-82B under 120 mM NaCl, could affect lycopene production. Indeed, K^+ has a role as cofactor of several enzymes involved in the biosynthesis of isopentenyl diphosphate, the first precursor of carotenoids in the mevalonate pathway (Trudel and Ozbun, 1971). Li and Yuan (2013) and Coyago-Cruz et al. (2019) suggested that the accumulation of fruit lycopene during ripening can be also delayed due to limited quantity of sucrose. Overall, our results were in agreement with those found by Peterson et al. (1998), who reported that salinity did not promote lycopene levels per dry weight of tomato fruits. Nevertheless, moderate salinity stress has been used to improve lycopene content in tomato (De Pascale et al., 2001; Kubota et al., 2012).

Phenolic acids and flavonoids represent a complex class of chemical structures with particular biological activity. Their profile in tomato fruits have been widely investigated and often used as taxonomical markers to discriminate tomato varieties (Minoggio et al., 2003; Vallverdú-Queralt et al., 2011). Flavonoids content has been positively correlated with environmental radiation, resulting in seasonal changes (Incerti et al., 2009). For example, one light-dependent effect is the up-regulation of the gene expression of *chalcone synthase*, the first committed enzyme in the

flavonoid biosynthesis (Feinbaum and Ausubel, 1988). Flavonoids have been found highly concentrated in epidermal and placental tissues of tomato fruits, acting as chemical defences against pathogens and UV radiation (Slimestad et al., 2008). Besides, flavonoids, also known as vitamin P, have been recently targeted as an important functional compound with benefits for human health (Perez-Vizcaino and Fraga, 2018). New emerging studies showed that flavonoids may interfere with the signalling of several kinases that in turn modulate cellular functions by altering the phosphorylation state of target molecules and by modulating gene expression (Williams et al., 2004; Van Der Rest, 2006).

Most of the flavonoids found in tomato are mainly present as O-glycosides, but also as non-conjugated forms (aglycones). The main flavonoid reported in tomato fruits is rutin (quercetin 3-rutinoside) (Le Gall et al., 2003). However, chalconaringenin and its more stable cyclized form naringenin, as well as naringenin-7-O-glucoside, have been also detected as additional frequent flavonoids in fresh tomato skins (Moço et al., 2006) and have been investigated *in vitro* as potential anti-allergic compounds (Yamamoto et al., 2004). In addition, the presence of the aglycones kaempferol, quercetin and naringenin is also noticed, but with a discrepancy in the amount among experimental studies (Stewart et al., 2000). Overall, the content of flavonoids in terms of quantity and quality greatly varies depending on genotype, growth conditions, stage of ripeness and tissue, as well as on the detection method (Slimestad and Verheul, 2005; Slimestad et al., 2008). In our study, we identified most of the abovementioned flavonoids, except the aglycones, what may be explained by the fact that we did not perform acid hydrolysis of the analysed sample extracts. Furthermore, the commercial fruit variety clearly differed from the landraces for naringerin and rutin contents both under control and also under salinity. Overall, the TFL content in the commercial variety revealed a decreasing trend induced by salt treatments, similar to that observed for rutin content. In Ciettaicale and Linosa TFL content was not affected by salinity, while 120 mM NaCl induced an accumulation of TFL in Corleone fruits.

Tomato fruits also contain hydroxycinnamic acids and their ester derivatives with quinic acid. During ripening, an increase of these esters generally occurs, especially in the pulp. Caffeic acid and its ester chlorogenic acid, as well as ferulic acid and coumaric acid, occur in quite high level in tomato fruits (Whitaker and Stommel,

2003). In particular, chlorogenic acid and its derivatives reduce the incidence of fungal disease in tomato (Ruelas et al., 2006; Wojciechowska et al. 2014). Moreover, hydroxycinnamic acids can modulate auxin and ethylene metabolism, which are both involved in fruit size development and fruit ripening (Fleuriet and Macheix, 1981). Drought stress can decrease hydroxycinnamic acids and derivatives contents in tomato fruits (Barbagallo et al., 2008; Sanchez-Rodriguez, 2012). We observed that in the landraces salt stress conditions promoted Di- and Tri-caffeoylquinic acids accumulation compared to the commercial variety. Coumaric acid showed a similar profile of accumulation in the landraces, while a decreasing trend was observed in the commercial variety according to the salt gradient. Cinnamate 3-hydroxylase (CH3), a key limiting enzyme for hydroxycinnamic acids and its derivatives biosynthesis (Ferrer et al., 2008), was shown to be up-regulated at transcript and enzymatic activity levels by salinity (Martinez et al., 2016). Our data suggests that CH3 is differentially regulated in the landraces compared to the commercial variety, but further investigations need to be conducted to validate this hypothesis.

Furthermore, we observed that high salt induced a decrease in TPHE. This may be the result of the reallocation of phenolic compounds in lignin polymers as a protective mechanism (Humphreys and Chapple, 2002). However, decreased values of phenolic contents often observed when fruiting tomato grown under low temperature (Rivero et al, 2001; Gautier et al., 2008).

Tomato, a fully-fledged member of the Solanaceae family, produces steroidal glycoalkaloids which belong to the terpenoids family. Tomatine represents the main glycoalkaloid in tomato fruit. From a pharmaceutical point of view, dietary tomatine leads to a reduction of plasma cholesterol content thanks to the capacity of this terpenoid to form with cholesterol insoluble complexes which are poorly absorbed from the intestinal tract (Friedman, 2013). During fruit ripening, tomatine is normally converted in esculeoside (Katsumata et al., 2011) and this trend is often associated with an increase of lycopene content. However, as for many other metabolites, this relationship is deeply depending on agronomic practices, genotype and environmental conditions (Kohn et al., 2013). The pattern of accumulation of glycoalkaloids was variable in the landraces and in the commercial variety upon salt stress. The levels and accumulation trends of Esculeoside A were similar in all genotypes. Although salinity was shown to induce accumulation of these compounds

in tomato leaves (Han et al., 2016), we could only observe in one of the landraces, Corleone, such a general trend.

Polyamines class, which includes putrescine, spermidine and spermine, is required for tomato fruit development (Cohen et al., 1982). These metabolites can donate amino groups to different other plant compounds, such as hydroxycinnamic acids. Hydroxycinnamic amides seem to play a key role in the reproductive tissues since their catabolism provides nitrogenous and phenolic carbon skeletons for the reproductive development (Balint et al., 1987). Caffeoylputrescine and Feruloylputrescine isomers contents showed different trends in the genotype according to the salt gradient. Since polyamines competes with ethylene for the biosynthetic precursor S-adenosylmethionine, high content of hydroxycinnamic amides may delay the fruit softening inhibiting ethylene production (Liu et al., 2006).

Overall, despite the salinity-induced rearrangement in the stoichiometry of the antioxidant metabolites identified by UPLC-MS, UC-82B and Corleone progressively decreased fruit TAC according to the salt gradient, while in Ciettaicale and Linosa TAC only declined under 120 mM NaCl.

5.5 Conclusion

As expected, the combination of moderate/high salt concentrations with low light irradiance in our greenhouse experiment differently affected the metabolism of all studied tomato genotypes, resulting in changes in yield and functional metabolites content. Despite these non-optimal environmental conditions, our results showed that the Italian landraces were differentiated from the commercial variety UC-82B under moderate salinity stress, showing a tolerable compromise between yield and quality attributes. Conversely, salt stress markedly reduced yield and functional metabolites contents in the commercial variety. Among the landraces investigated, Linosa showed better performance in terms of yield/quality parameters under 60 mM NaCl. However, off-season high salinity stress (120 mM NaCl) significantly reduced the antioxidant activity both in UC-82B and in the landraces. In conclusion, we point to the use of tomato landraces germplasm remains both a suitable strategy to counteract detrimental environmental factors, such as salinity and off-season cropping, and also a valuable/rich genetic resource to improve commercial varieties.

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Table 5.S1. Hydroxycinnamic acids identified in the current study. Data are means ± SE of six replicates. Means within a column followed by the same letter are not significantly different based on Duncan's test ($P < 0.05$).

`	Coumaric acid I			Coumaric acid II			Ferulic acid I			Ferulic acid II			Coumaric acid-hexose I			Caffeic acid glucoside I			Caffeic acid glucoside II		
CVxSALT	Mean	SE	Signif.	Mean	SE	Signif.	Mean	SE	Signif.	Mean	SE	Signif.	Mean	SE	Signif.	Mean	SE	Signif.	Mean	SE	Signif.
CE-0	574.709	50.8874	bc	256.528	18.0752	be	47.2568	4.28793	c	43.8749	4.05888	de	238.209	19.2859	ac	181.36	15.5718	ac	88.955	4.60321	bc
CE-60	635.058	59.8263	ab	282.807	22.7778	be	80.9363	6.74085	b	24.8992	1.81659	e	268.489	25.8408	ab	202.13	14.0568	a	96.5867	5.11082	b
CE-120	419.568	22.3877	cd	210.147	3.02565	de	88.7068	6.15293	b	37.1028	2.0631	de	173.189	11.5737	ce	132.382	6.89968	de	122.412	4.05608	a
COR-0	289.89	23.1782	d	206.605	33.8312	de	25.2841	2.05803	c	49.3625	4.91376	d	114.806	9.34735	e	115.882	7.04587	e	96.5817	3.84654	b
COR-60	617.749	86.4568	ab	215.048	38.2822	ce	94.5983	10.6655	b	42.2651	4.67193	de	241.299	33.1991	ab	195.026	11.2591	ab	97.132	6.03547	b
COR-120	382.327	56.0163	d	234.428	37.4829	be	92.9381	16.3681	b	41.222	4.15232	de	150.459	21.6911	de	125.457	19.5863	de	99.4617	8.31624	b
LIN-0	713.678	44.5852	ab	300.845	29.497	bd	41.4615	1.70612	c	110.607	4.19675	b	279.41	15.5796	a	162.392	6.42574	bd	100.558	3.9624	b
LIN-60	763.532	80.2815	a	331.047	45.3635	b	89.0667	4.67772	b	103.839	7.74215	b	301.53	32.5352	a	177.605	3.38303	ac	129.812	7.31445	a
LIN-120	551.676	21.2188	bc	209.455	13.171	de	73.6211	4.67125	b	41.0001	2.03934	de	203.726	15.073	bd	138.327	5.87376	de	121.27	1.37476	a
UC-0	339.207	15.0903	d	595.768	101.032	a	23.6981	0.45601	c	138.578	16.5567	a	134.831	6.92424	e	124.838	7.84331	de	94.31	12.9027	b
UC-60	561.042	37.0305	bc	326.703	33.3999	bc	94.451	3.88478	b	100.749	2.36291	b	206.22	19.431	bd	211.291	9.45128	a	75.2317	6.19296	c
UC-120	373.709	42.4268	d	182.792	18.7488	e	123.726	11.4634	a	73.4563	8.59709	c	147.21	16.1834	de	146.454	10.5531	ce	56.112	5.78161	d

`	1,3-O-Dicaffeoylquinic acid I			1,3-O-Dicaffeoylquinic acidII			3,4,5-Tricaffeoylquinic acid			Caffeoylputrescine I			Caffeoylputrescine II			Feruloylputrescine I			FeruloylputrescineII		
CVxSALT	Mean	SE	Signif.	Mean	SE	Signif.	Mean	SE	Signif.	Mean	SE	Signif.	Mean	SE	Signif.	Mean	SE	Signif.	Mean	SE	Signif.
CE-0	108.421	22.6418	b	35.2311	5.95423	ab	131.974	22.6611	ce	644.726	59.8992	b	193.938	35.5752	ab	788.905	37.9015	a	769.43	50.3677	b
CE-60	69.7706	9.35622	bd	17.1799	2.24864	b	99.9529	11.0147	df	631.584	65.0435	b	215.557	24.9346	ab	524.179	67.7802	cd	659.044	69.5777	bc
CE-120	132.757	7.27435	b	15.2566	1.64662	cd	204.134	41.8932	b	236.643	32.7209	de	19.4383	1.54986	d	200.47	13.3778	g	618.768	26.2434	bc
COR-0	115.397	8.66485	b	26.7215	3.78927	bc	134.346	18.2139	be	313.216	29.9344	cd	175.943	20.8402	ab	473.403	57.1818	de	455.782	40.2572	c
COR-60	100.584	5.26473	bc	32.1161	1.5933	cd	177.747	3.83417	bd	922.13	128.769	a	223.53	30.3001	a	651.688	39.5235	ac	1072.3	108.834	a
COR-120	356.109	45.0797	a	48.48	4.52537	a	317.394	19.1899	a	655.264	65.1272	b	42.5533	6.23284	d	247.357	31.9705	fg	983.155	101.109	a
LIN-0	113.352	13.5724	b	40.4724	5.41795	ab	137.021	15.739	be	310.444	16.3465	cd	65.2783	8.33091	cd	766.255	43.2242	a	969.088	63.6522	a
LIN-60	115.001	9.76245	b	29.0046	3.17663	bc	163.374	13.4088	bd	415.784	38.965	bd	80.4467	13.3066	cd	684.079	58.0073	ab	1036.26	74.4728	a
LIN-120	133.526	14.9411	b	37.729	4.74981	ab	183.582	16.5684	bc	116.557	7.21616	e	44.0383	4.66644	d	486.744	20.4314	de	473.753	34.326	c
UC-0	42.5395	6.43025	cd	11.9129	3.35018	d	53.0031	12.7497	f	312.793	33.6008	cd	136.382	10.44	bc	463.77	71.9371	de	526.797	54.1259	c
UC-60	38.5934	8.47113	d	9.46837	0.55953	c	91.7924	10.5955	ef	517.375	32.001	bc	91.315	12.1451	cd	372.702	22.8745	ef	652.793	49.2839	bc
UC-120	46.6101	14.3729	cd	16.9419	1.40873	d	143.832	16.2682	be	979.612	252.199	a	197.14	77.828	ab	561.369	37.7092	bd	998.197	83.3063	a

Table 5.S2. Flavonoids identified in the current study. Data are means ± SE of six replicates. Means within a column followed by the same letter are not significantly different based on Duncan's test ($P < 0.05$).

	Naringenin chalcone			Naringenin			Naringenin 7-O- glucoside			Naringenin chalcone 7-O- glucoside			Phloretin 3 5 -di-C-glucoside			Rutin			Rutin-O-pentoside		
CVxSALT	Mean	SE	Signif.	Mean	SE	Signif.	Mean	SE	Signif.	Mean	SE	Signif.	Mean	SE	Signif.	Mean	SE	Signif.	Mean	SE	Signif.
CE-0	1388.22	298	a	965.3	366.87	ab	31.6037	2.37362	d	55.5759	7.45743	c	220.769	12.5878	cd	338.081	30.8652	d	164.512	27.1122	b
CE-60	453.784	216.478	a	457.439	165.923	b	39.4281	7.92131	d	34.3999	7.49796	c	178.217	10.7213	de	300.153	45.3106	d	145.321	19.3278	b
CE-120	591.983	287.198	a	327.087	100.295	b	29.9063	3.07684	d	16.9003	3.76437	c	104.127	5.45898	e	293.817	21.1229	d	238.621	59.4657	b
COR-0	419.275	180.853	a	535.197	108.165	b	138.989	14.0603	c	139.235	7.09486	b	266.213	28.1947	bc	390.394	34.3177	d	88.5385	13.3327	b
COR-60	544.478	93.5376	a	273.78	41.1457	b	37.7371	6.75622	d	45.782	0.72583	c	204.199	23.9137	d	301.635	54.9241	d	144.743	37.7759	b
COR-120	714.857	171.508	a	459.001	139.589	b	28.5393	3.76911	d	25.2356	2.94312	c	135.127	12.2499	ef	341.485	27.9101	d	223.435	23.793	b
LIN-0	654.454	338.462	a	442.541	79.0787	b	24.2351	1.2909	d	51.4262	5.34671	c	221.06	15.7323	cd	262.912	24.3142	d	118.706	11.6533	b
LIN-60	982.731	251.613	a	707.456	128.61	b	13.2592	1.38598	d	20.6122	2.35656	c	136.867	5.37629	ef	286.108	35.5881	d	198.921	25.9991	b
LIN-120	752.957	313.466	a	601.38	211.579	b	17.7763	3.55512	d	15.5813	3.78944	c	92.4357	6.32926	e	273.553	35.948	d	276.04	29.6974	b
UC-0	569.247	265.911	a	1919.73	1006.68	a	426.676	99.4302	b	200.846	28.0403	b	291.1	17.1743	b	1543.06	199.732	a	546.307	123.925	a
UC-60	946.212	334.071	a	696.205	155.353	b	629.174	58.2255	a	569.398	68.7171	a	398.771	25.4873	a	1170.75	60.2837	b	568.015	27.9264	a
UC-120	1133.15	497.209	a	1187.72	603.073	ab	93.9497	12.6856	cd	171.027	62.1305	b	281.579	38.4877	b	810.166	70.492	c	671.368	217.933	a

Table 5.S3. Amino acids and vitamin derivatives identified in the current study. Data are means ± SE of six replicates. Means within a column followed by the same letter are not significantly different based on Duncan's test ($P < 0.05$).

	Phenylalanine			Tryptophan			Pantothenic acid-hexose		
CVxSALT	Mean	SE	Signif.	Mean	SE	Signif.	Mean	SE	Signif.
CE-0	1101.46	142.92	b	760.096	44.3955	bc	120.277	9.65506	ab
CE-60	4855.47	819.35	a	938.884	152.538	ab	142.011	19.7469	a
CE-120	997.75	188.24	b	628.779	73.4603	cd	69.5019	11.9444	cd
COR-0	480.44	53.49	bc	399.947	50.814	ef	73.886	9.91246	cd
COR-60	493.50	49.37	bc	360.427	8.29038	ef	130.205	14.4292	ab
COR-120	196.76	43.20	c	251.858	43.8432	f	64.7964	12.1606	cd
LIN-0	880.93	38.85	bc	462.102	22.4131	df	75.8617	5.00391	cd
LIN-60	793.08	166.50	bc	437.213	34.7199	df	64.4423	4.35411	cd
LIN-120	1173.31	67.04	b	576.21	20.1943	ce	59.2167	2.96528	d
UC-0	1093.01	50.60	b	1014	117.842	a	96.6553	3.27366	bc
UC-60	949.37	153.99	bc	574.288	68.2154	ce	126.816	11.957	ab
UC-120	849.37	156.77	bc	630.199	46.7113	cd	66.8142	7.52462	cd

Table 5.S4. Glycoalkaloids identified in the current study. Data are means ± SE of six replicates. Means within a column followed by the same letter are not significantly different based on Duncan's test ($P < 0.05$).

`	Tomatidine			delta-Tomatine			Tomatidine+3hexoase			Tomatine			Lycoperoside H			Lycoperoside A			Acetoxy-Hydroxytomatine			Esculeoside A			Putative glycoalkaloid		
CVxSALT	Mean	SE	Signif.	Mean	SE	Signif.	Mean	SE	Signif.	Mean	SE	Signif.	Mean	SE	Signif.	Mean	SE	Signif.	Mean	SE	Signif.	Mean	SE	Signif.	Mean	SE	Signif.
CE-0	55.9256	14.0613	cd	230.251	54.9625	de	191.744	83.1499	d	19.5494	3.00145	c	53.5917	2.75951	a	41.397	10.4549	c	140.772	45.8233	bc	13360.4	450.224	ab	211.97	70.9876	e
CE-60	22.6478	1.61615	d	57.4801	6.7051	e	231.496	53.5904	d	7.89751	0.7166	c	50.4918	2.97983	ab	40.1965	2.26466	c	34.5412	2.66854	d	10354.1	321.788	cd	411.23	74.2081	de
CE-120	46.737	3.68622	cd	170.377	17.6009	de	145.207	30.6888	d	29.6114	2.42886	c	46.9207	0.51396	ac	48.1256	2.51338	bc	35.1245	4.31232	d	10493.7	718.835	bd	281.631	56.3573	de
COR-0	76.9731	13.3758	cd	277.451	50.2541	de	607.493	105.956	bc	35.2753	6.53098	c	40.85	3.21517	c	20.8729	1.33548	c	76.6921	13.0895	cd	10061.8	1111.86	d	1218.04	207.081	ab
COR-60	111.294	20.4205	c	403.725	82.8948	d	1028.86	146.228	a	102.914	27.6403	b	45.9916	4.37292	ac	97.9094	19.9437	a	126.817	20.3269	bc	14887.7	1030.86	a	1469.46	269.283	a
COR-120	187.594	14.1901	b	743.953	58.8369	c	768.677	61.4962	b	144.881	13.1104	b	48.1884	2.91969	ac	85.8211	14.3472	a	106.089	13.1248	bd	9668.25	1835.03	d	1598.01	149.289	a
LIN-0	21.5983	3.25922	d	69.2444	9.88727	e	238.092	108.902	d	9.59674	2.23118	c	43.6968	2.52256	bc	29.8786	4.98446	c	124.38	22.4651	bc	9636.89	546.493	d	200.877	82.6455	e
LIN-60	32.5421	1.44868	d	122.212	27.4784	de	506.676	86.1835	c	15.0906	1.26277	c	41.2519	1.92363	bc	37.517	4.98698	c	101.673	12.6285	bd	9477.54	127.901	d	1024.11	155.621	bc
LIN-120	29.7008	2.12133	d	96.0839	7.2785	de	215.66	72.8544	d	17.1555	1.81731	c	42.4132	2.63266	bc	32.8025	1.98631	c	75.6729	11.5505	cd	9825.75	295.072	d	388.038	102.95	de
UC-0	289.514	71.4275	a	1243.78	316.034	a	120.596	18.9362	d	212.89	60.1867	a	20.8704	4.59148	d	98.898	22.8384	a	233.335	54.392	a	9705.64	1638.29	d	376.689	71.1728	de
UC-60	205.171	15.9925	b	828.163	69.6093	bc	172.892	37.7494	d	154.39	12.8365	ab	26.9981	1.49137	d	79.7854	6.68339	ab	165.395	16.6875	ab	13067.4	420.478	ac	405.544	78.8202	de
UC-120	261.202	55.8656	ab	1081.37	231.471	ab	375.065	145.262	cd	221.816	62.5518	a	22.6612	3.10489	d	87.5821	29.7675	a	109.553	26.323	bd	8278.33	1313.26	d	719.522	266.985	cd

Chapter 6

General conclusions

Our study reported an evaluation of the effect of salinity and drought stress on the physiology and the metabolism of the Southern Italy tomato landrace Ciettaicale compared to well-known commercial cultivars (San Marzano, Moneymaker and UC-82B) at different plant phenological stages.

In **Chapter 2**, a moderate concentration of sodium chloride (25 mM NaCl) revealed clear differentiation in the biochemical profile and then in the germination rate of Ciettaicale and San Marzano germinating seeds during 5 days-experimental time-course. Salt induced a promotion of endo-β-mannanase and β-mannosidase activities in Ciettaicale, as well as starch mobilization, contributing to the increment of total soluble sugars and providing more energy to maintain organogenesis and scavenge oxidative stress, as indicated by increased total antioxidant activity and catalase activity. Conversely, in San Marzano, we found some salinity-induced physiological changes only at the end of our observations, suggesting that seeds were not dormant, but impaired by the stress to conclude properly the germination process.

In **Chapter 3** we evaluated Ciettaicale and San Marzano responses to short-time high salinity stress (0, 300, 450 and 600 mM NaCl). A clear differentiation of Ciettaicale compared to San Marzano emerged above 300 mM NaCl. After a week of salt treatment, salinity effects culminated in photoinhibition and/or photodamage events at 600 mM NaCl in San Marzano plants resulting in a source-sink imbalance. At the same extreme conditions, Ciettaicale plants showed an efficient physiological and metabolic plasticity provided by improved photosynthesis efficiency and osmotic adjustment resulting in a greater energy availability to be allocated in root exploration. Thus, Ciettaicale plants strategy allowed themselves to survive or at least to slow down the appearance of actual damage at higher NaCl concentrations.

We also combined chlorophyll *a* fluorescence measurements and metabolic determinations to investigate the profile of Ciettaicale and Moneymaker plants

subjected to a drought stress (**Chapter 4**). Physiological and metabolic changes, in terms of abscisic acid (ABA), indol-3-acetic acid (IAA), proline, soluble sugars and phenols contents occurred in Ciettaicale and Moneymaker under water stress. However, our results highlighted the ability of Ciettaicale to manage plant water status under drought in order to preserve both leaf and root activities. This aim was achieved thanks to the preservation of the source-sink relations, though a more efficient PSII photochemistry at leaf level associated with a major investment in root growth and activity in order to improve water uptake. On the contrary, drought-stressed Moneymaker plants reduced Φ_{PSII} and enhanced starch reserve mobilization in both leaves and roots, possibly suggesting a major role of the osmotic adjustment to counteract tissue dehydration, but meantime a feedback potential disruption of source-sink relations. This hypothesis was also supported by the more pronounced redox disequilibrium, as suggested by higher hydrogen peroxide and malondialdehyde contents, that affected both PSII photochemistry and root activity and markedly trigged in turn the non-photochemical fluorescence quenching (NPQ) and antioxidant responses by Moneymaker plants compared to Ciettaicale.

In **Chapter 5**, we evaluated the yield and the fruit quality of Ciettaicale under salt stress in an off-season (autumn-winter) greenhouse trial. In the experiment we added other two Southern Italy tomato landraces, Linosa and Corleone, and one commercial cultivar, UC-82B. Salt treatments (60 mM and 120 mM NaCl) promoted the anticipation of fruit ripening in landraces and UC82-B compared to non-salt conditions. At harvest, no losses in marketable yield were noticed in all genotypes. Instead, fresh and dry fruit yields, as well as cation concentrations, were more affected under stress in the commercial cultivar as compared to landraces. Different trends of lycopene content and soluble sugars amount were found in the fruits among all investigated accessions. Data obtained by UPLC-MS revealed differential accumulation of glycoalkaloids, phenolic acids, flavonoids and their derivatives in the landrace fruits under stress in all genotypes. Overall, despite the non-optimal environmental conditions, our results showed a differentiation between the Italian landraces and the commercial variety UC-82B under 60 mM NaCl, showing a tolerable compromise between yield and quality attributes. Conversely, off-season high salinity stress (120 mM NaCl) significantly reduced the antioxidant activity both in UC-82B and in the landraces.

Our data suggested that Ciettaicale could carry interesting traits at vegetative stage such as improved root/shoot ratio, water use efficiency, osmotic regulation and maintenance of shoot-to-root relationships under high salinity and water deficit, as well as good performance in term of germination rate and fruit yield and quality under salt stress. We point to the feasible use of the tomato landrace Ciettaicale as a target to select interesting genetic traits to improve stress response and fruit functional values. Thus, deep investigations are required in order to enhance the possibility of introducing this landrace in tomato genetic improvement programs.

Author's CV and publications

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09/2010 – 01/2014	Bachelor degree in Agro-Industrial Biotechnology (class L-2), awarded by University of Pisa - Department of Agriculture, Food and Environment, score 110/110 *cum laude* Title of the thesis: "Increasing ascorbic acid in Lactuca sativa by the overexpression of *L-galactono-1,4-lactone dehydrogenas*e (*L-GaLDH*) gene" – Supervisors: Prof. Claudio Pugliesi and Prof. Lucia Guidi
09/2006 – 07/2010	High school Diploma (specializing in classical studies), awarded by Liceo Classico "Aldo Moro" – Praia a Mare (CS), score 100/100

PUBLICATIONS

Moles, T.M., Pompeiano, A., Huarranca Reyes, T., Scartazza, A., Guglielminetti, L. (2016). The efficient physiological strategy of a tomato landrace in response to short-term salinity stress. Plant Physiol. Biochem. 109, 262-272.

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Pompeiano, A., Huarancca Reyes, T., Moles, T.M., Guglielminetti, L., Scartazza, A. Photosynthetic and growth responses of *Arundo donax* L. under different oxygen deficiency stresses and re-oxygenation. Front. Plant Sci., *submitted*.

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CONFERENCES AND MEETINGS

Attendance at "Geo&Geo" (season 2015-2016), Rai TV show aired on 25/01/16: topic of the program section "Storie" was an overview on Ciettaicale tomato landrace including a reference to my PhD project (http://www.rai.it/dl/RaiTV/programmi/media/ContentItem-c98d7280-3f12-4843-9090-57a5af2e1066.html#p=).

Moles TM, Pompeiano A, Huarranca Reyes T, Scartazza A, and Guglielminetti L. "Effetti della salinità sul pomodoro Ciettaicale di Tolve". ORAL PRESENTATION. XI National Conference on Biodiversity: "Biodiversity and Sustainable Intensification". 9-10 June 2016 – Matera, Italy.

Huarranca Reyes T, Moles TM, Pompeiano A, Scartazza A, and Guglielminetti L. "The efficient physiological strategy of an Italian tomato landrace in response to high salinity stress". POSTER. 34th Annual Meeting of the Japanese Society of Plant Cell and Molecular Biology. 1-3 September 2016 – Nagano, Japan.

Moles TM, Mariotti L, De Pedro LF, Di Baccio D, Scartazza A, Guglielminetti L, and Picciarelli P. "Source-sink physiological changes and metabolic adjustments trigged by water deficit in two *Solanum lycopersicum* genotypes". POSTER. Joint Congress SIBV-SIGA. 19-22 September 2017 – Pisa, Italy.

Moles TM, Mariotti L, De Pedro LF, Di Baccio D, Scartazza A, Guglielminetti L, Santelia D and Picciarelli P. "Source-sink physiological changes and metabolic adjustments trigged by water deficit in two *Solanum lycopersicum* genotypes". POSTER. PSC Symposium "Dynamics in Plant Development and Evolution". 30 November-1 December 2017 – Zürich, Switzerland.

Yokota A, Moles TM, Thalmann M, and Santelia D. "Elucidating β-amylase 1 (BAM1) regulation for ABA-dependent starch degradation upon osmotic stress". POSTER. 5th International Conference "Plant Abiotic Stress Tolerance". 5-6 July 2018 – Vienna, Austria.

Yokota A, Moles TM, Thalmann M, and Santelia D. "Elucidating β-amylase 1 (BAM1) regulation for ABA-dependent starch degradation upon osmotic stress". POSTER. PSC Symposium "Breakthroughs in Plant Sciences". 5 December 2018 – Zürich, Switzerland.