DIPARTIMENTO SCIENZE DELLA VITA

DOTTORATO DI RICERCA IN SCIENZE DELLA VITA

CICLO XXXIV

COORDINATORE Prof. Massimo Valoti

Locally-adapted Italian cultivars of tomato (*Solanum lycopersicum* L.) under drought stress: morpho-physiological, biochemical and nutraceutical aspects underlying stress tolerance

SETTORE SCIENTIFICO-DISCIPLINARE: ad es. BIO/01

TUTOR: Prof. Giampiero Cai

DOTTORANDO: Dr.ssa Veronica Conti

CO-RELATORI: Dr. Claudio Cantini
(Institute for Bioeconomy (CNR-IBE), 58022 Follonica, Italy)

Dr. Marco Romi
(Department of Life Sciences, University of Siena, 53100 Siena, Italy)

A.A. 2021-2022

Table of Contents

Abstract

Background: Water deficit is one of the 21st century's major challenges, agriculture being both the cause and the victim since 70% of global available water is used for its practices. Irrigation is fundamental but, as climate change becomes more persistent, there is a need to conserve water and use it more efficiently. Exposure of plants to drought stress can cause morphological, anatomical, physiological, and biochemical changes in many tissues and organs. Drought can affect the growth and development of plant organs causing drastic reduction in productivity and commercial performance. It is therefore crucial to identify cultivars that can tolerate drought. When dealing with economically relevant crops like tomatoes, this purpose is even more incisive and local agrobiodiversity is a large genetic reservoir of promising cultivars.

Aims and Methods: Nine local Italian and four commercial tomato cultivars were considered. All experienced approximately 20 days of drought during the vegetative and reproductive phases. Plants were studied for three aspects. Morpho-physiology: several physio-morphological parameters were monitored, such as stomatal conductance, photosynthesis, water use efficiency, growth, and soil water content. The different responses and behaviors allowed the cultivars to be divided into three groups: tolerant, susceptible, and intermediate. The classification was also confirmed by the principal component analysis. Biochemistry: the expression of proteins related to drought stress tolerance in four local tomato cultivars was evaluated. Cultivars were selected after the results of previous analyses and corresponded to different tolerance levels. Only the vegetative stage was considered in this section. The approach consisted of extraction, separation, and immunological analysis of proteins such as dehydrins, osmotin, HSP70s, sucrose synthase, and cyclophilin. We also analyzed the pattern of phosphorylated proteins and the isoforms of RuBisCO. Nutraceutics: Genetic factors, ripeness and the impact of environmental conditions lead to differences in the bio-metabolic and nutraceutical composition of tomato fruit. We tested the hypothesis that local tomato cultivars subjected to drought stress showed an increased capacity for biosynthesis of compounds with antioxidant activity. The antioxidant power and the total content of polyphenols and flavonoids in both pulp and peel were evaluated by colorimetric assays. In addition, flavonoids (such as rutin, naringenin, and caffeic acid), vitamin C, and lycopene were identified and quantified using HPLC methods.

Results and Conclusions: At last, the data obtained at the morphological, physiological, biochemical, and metabolic levels indicate that specific locally adapted tomato cultivars respond much more efficiently to drought stress, even more than widespread commercial cultivars. In addition, this study lays the foundations for an application aspect, namely the use of moderate and controlled drought stress conditions to increase the nutritional quality of tomato fruits. Given that many bioactive compounds are found in the peel of tomatoes, this supports the reuse of waste components and therefore their sustainable recovery.

Chapter 1. General introduction

Global warming, primarily due to the intense emission of carbon dioxide caused by human activities, is constantly increasing the average temperature and is responsible for the reduction of rainfalls in highly vulnerable areas such as the Mediterranean, which is considered a "hot spot" in the 21st century [1]. It is very unlikely that the world will experience an inversion in climate changes, especially in the coming years, with the effects being even worse. It therefore becomes pressing to determine the effects that water scarcity can cause at the level of natural and anthropogenic ecosystems. Considering that agriculture is both the cause and the victim of drought stress, as 70% of globally available water is used for its practices [2], the impact that water deficit can have on the productivity of cultivated plants and the relative costs must not be underestimated. The severity of drought conditions can be quantified globally with the Palmer Drought Severity Index (PDSI) represented in **Figure 1.1**, which is widely used to make assessments on drought and agricultural productivity. It is within this highly uncertain context that we see population growth; therefore, water for agriculture is expected to compete with water used in emerging industries, which are steadily increasing. At the same time, population growth will require an intensification of agricultural productivity to meet food needs, with an estimated 45% increase in the amount of water required for irrigation by 2080 [3]. Given these future predictions, the study of crop responses to drought stress takes on increased importance, through the development of mitigation and adaptation strategies.

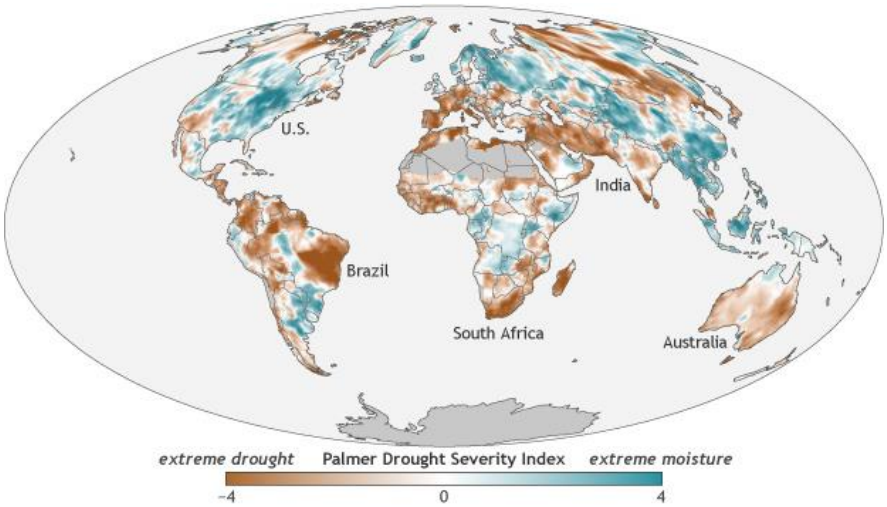


Figure 1.1. Worldwide distribution of the PDSI in 2017 (https://www.climate.gov/news-features/featured-images/2017-state-climate-global-drought). The PDSI, considering rainfall, evapotranspiration, and soil moisture, returns a value between -4 (extremely dry) and 4 (extremely humid).

Plants have developed physiological, morphological, cytologic, and biochemical responses to avoid and/or limit the consequences of drought stress. Plants sense the lack of water primarily through root cells, which respond by causing an immediate increase in the

synthesis of abscisic acid (ABA) that, once accumulated in the leaves, leads to stomatal closure [4]. Plants commonly respond to water stress with the accumulation in the cell cytoplasm of molecules such as osmolytes. Among the latter are proline, sucrose and other sugars that function as osmoprotectants, interacting with proteins and preventing their denaturation [5]. Due to stomatal closure, there is a decrease in CO_2 influx and an increase in O_2 concentration in the leaf, which in turn causes an increment in the production of reactive oxygen species (ROS). ROS, being unstable compounds, are likely to react with several molecules (proteins, lipids, DNA) causing functional alterations of the cells [6]. In response to drought stress there are also changes in the regulation of the photosynthetic process since photosynthesis is limited due to the low availability of reagents such as H_2O and CO_2 [7]. Plants respond to oxidative stress by synthesizing specific enzymes able to scavenge ROS [8] and/or by producing antioxidant molecules such as polyphenols [9].

Locally adapted cultivars are the result of a process of domestication of wild species that have undergone selective pressures due to both contingent environmental conditions and human needs [10]. In addition, local cultivars are adapted to the various climatic changes that a given environment may experience and, therefore, exhibit resilient traits to changing climatic conditions [3]. In this context, maintaining and protecting local agrobiodiversity becomes a resource for food availability [11]. Many studies identify local cultivars as a patrimony of genetic traits that can make plants more tolerant to abiotic stresses, such as drought [3,12–17]. For example, in countries such as Peru, Brazil, and India, recent repatriations of genbank accessions raise questions about whether and how crop biodiversity can be included in production systems in climate change-prone areas [18]. Furthermore, agrobiodiversity is one of the global keys in agriculture to ensure stable harvests and livelihoods under changing environmental conditions [19]. The greater the supply of genetic diversity, the greater the opportunities for farmers to adapt crops to local environmental conditions. In this context, access to a wide range of locally adapted cultivars is and will be critical for sustainable agriculture under extreme climate change [20]. Nor should it be forgotten or underestimated that the search for plants more tolerant to environmental conditions such as drought is part of sustainable development goals such as SDG12 (responsible consumption and production), SDG13 (climate action) and SDG15 (life on land) (https://sdgs.un.org/goals).

Tomato (*Solanum lycopersicum* L.) is one of the most important plant crops in the world, ranking second only to the potato [21]. Globally, almost five million hectares of cultivated land are used for its farming, for a total of more than 180 million tons of fruits harvested [22]. To date, Italy is among the top 10 tomato producers in the world with 5.2

million tons per year [22]. Tomatoes are particularly susceptible to water shortage because prolonged water deficit limits growth and yield of the crop. Both the vegetative and reproductive stages of existing tomato cultivars can be severely affected by drought, which inhibits seed development and reduces the growth of stems and fruits [23,24].

Nowadays, a more sustainable agriculture, which therefore requires less water resources, must consider genetic biodiversity as a key factor to improve crop yield and quality, as well as tolerance to biotic and abiotic stresses. Previously (during my master's thesis), I evaluated the drought resilience of seven tomato cultivars grown locally in Tuscany, Italy [25]. In that study, plants were cultivated in a growth chamber and analyzed for some key features related to water deficit stress. In this Ph.D. thesis, I extended the study to all nine tomato cultivars currently cataloged in the Regional Germplasm Bank of Tuscany as at risk of genetic erosion.

The purpose of my Ph.D. project was originally targeted at characterizing the differences that locally adapted cultivars might exhibit in response to drought stress. Several objectives were planned to be achieved. The first target was to carry out a screening of local cultivars in both vegetative and reproductive stages. Thus, by studying physiological and morphological aspects, it was possible to classify cultivars based on their tolerance to drought stress. Among the physiological and morphological aspects, photosynthetic efficiency (Fv/Fm and PI), stomatal conductance, stomatal density, Soil Water Content (SWC), plant growth (i.e., growth index and stem diameter), leaf area and length and Leaf Relative Water Content (RWC) were analyzed. A more detailed study on metabolomic aspects such as pigment, sugar, abscisic acid, and jasmonic acid content was therefore required. Because of drought stress, oxidative stress was also consequently measured by monitoring the leaf content of H_2O_2, O_2 and determining the ROS scavenging potential through the content of antioxidants and polyphenols. After ranking cultivars based on drought stress tolerance, a more detailed study would then be performed on the most promising and significant cultivars. A study on RuBisCO and proteins involved in drought response (such as HSP70, dehydrins, osmotins, and aquaporins) was planned on this limited number of selected cultivars. During the reproductive stage, the aim was also to qualitatively characterize the fruits and assess the productive capacity of plants. By monitoring flower and fruit development, fruit set, and seed germination, it became possible to assess cultivar productivity. Finally, the nutraceutical evaluation of fruits in terms of content of antioxidants, polyphenols and flavonoids was used to estimate the capacity of fruits to retain quality even after drought stress. Once the most promising cultivars have been identified, it would be worthwhile and interesting to investigate

the genetic aspects in future works, thus identifying the genes that are activated during drought stress and that confer more tolerance to specific cultivars.

1.1. Plant Material

Seeds of nine Tuscan tomato cultivars, namely ‘Costoluto Fiorentino’, ‘Canestrino di Lucca’, ‘Fragola’, ‘Rosso di Pitigliano’, ‘Giallo di Pitigliano’, ‘Pisanello’, ‘Quarantino ecotipo Valdarno’, Tondino Liscio da Serbo Toscano’ and ‘Perina a Punta della Valtiberina’, were obtained from the Regional Germplasm Bank of Tuscany (Tuscany, Italy). No permissions were necessary to collect seeds. The Regional Germplasm Bank of Tuscany undertook the formal identification of samples. Four commercial cultivars, namely 'Cuore di Bue', 'Datterino', 'Pantano' and 'Pearson', were chosen among many other commercial cultivars because of their wide commercialization throughout Italy; the corresponding seeds were provided by local retailers.

1.2. Growth Conditions and Stress Treatment

The parameters used for plant growth and drought treatment are described in this section and will therefore not be repeated in subsequent chapters. Seeds were germinated in Petri dishes on filter paper soaked with distilled water at a constant temperature of 25 °C in the dark. Afterwards, seedlings were transferred to a greenhouse (Botanical Garden, University of Siena) and planted in a tray with wells (4 × 5 × 6 cm) at 25 °C. For each cultivar, 10 plants were studied during the vegetative phase and 8 plants during the reproductive growth phase (**Figure 1.1**). For studies at the vegetative phase, plants were transferred into square PE pots (15 cm side, 20 cm height), while for studies at the reproductive stage PE pots had an upper diameter of 28 cm, a base diameter of 22 cm, and a height of 24 cm. The substrate used for repotting operations was the VIGOR PLANT® RADICOM BIO. For each cultivar and growth phase, half of plants were used as control (CTRL) and the other half were subjected to drought stress (DS). Until the beginning of water deficit treatment, all plants were well-watered. For studies at the vegetative phase, the drought treatment began when plants were 30/40 cm high, corresponding to 45 d after germination; the stress condition was maintained for 16 d and consisted in complete watering withdrawal; the CTRL group was kept in a fully irrigated regime for the whole period. For studies at the reproductive phase, the drought treatment began when plants were flowering, and the first fruits started to grow. Plants were around 120 cm high at the beginning of stress, the drought treatment lasted for 20 d and consisted in complete watering withdrawal; the CTRL group was kept in a fully irrigated regime for the whole period.

The timing of the drought was chosen following Landi [26], Sànchez-Rodrìguez [27], Nuruddin [24] and our previous work in a growth chamber [25]. The experimental period was divided into 3 time points for each phase: time point 0 (t_0) corresponds to the beginning of stress; time point 1 (t_1) is the intermediate stage of stress; time point 2 (t_2) is the end of stress. Plants in the reproductive phase were also subjected to a recovery step, consisting of full irrigation of drought-stressed plants after t_2 for two weeks (recovery time point, RW). At each time point, required parameters were taken, and samples were harvested, immediately put in liquid nitrogen, and stored at −80 °C until use.

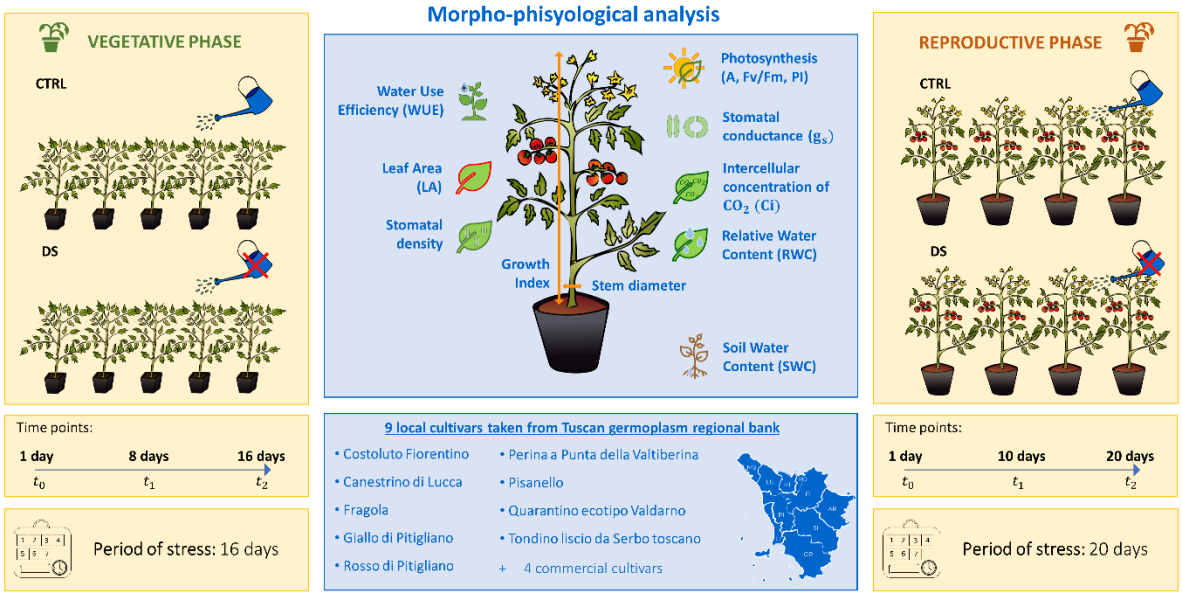
Figure 1.1. Pictures of the greenhouse taken during the period of plant cultivation and stress; on the left the plants in vegetative phase, on the right the plants in reproductive phase

1.3. Temperature and Relative Humidity

Each phase was performed during July in a greenhouse with a complete randomized scheme. The greenhouse facility prevented accidental wetting of plants but allowed solar illumination, temperature, and humidity parameters to be comparable to those outside. However, temperature and humidity values were collected hourly by an EBI 20-TH1 (ebro®) datalogger, daily mean and standard deviation computed separately for day and night hours. During the vegetative phase, the mean temperature and humidity in daytime hours were 34.7 ± 2.6 °C and 46.8 ± 6.2 %, respectively; during nighttime hours, the mean temperature was 25.3 ± 1.7 °C while the mean humidity was 60.9 ± 6.3 %. During the reproductive phase, an average temperature of 32.7 ± 3.8 °C and humidity of 50.7 ± 8.4 % was recorded during daytime hours, while temperature and humidity were 23.9 ± 2.1 °C and 64.7 ± 3.2 %, respectively, during nighttime hours. The values were very close to those usually recorded in Siena in July.

<u>Chapter 2.</u> Morpho-physiological traits of vegetative and reproductive growth as a tool to classify Italian tomato cultivars according to drought stress tolerance

<u>Collaborators:</u> *Marco Romi, Sara Parri, Iris Aloisi, Giovanni Marino, Giampiero Cai and Claudio Cantini*



Graphical abstract: Nine local Italian cultivars of tomatoes plus four widely used commercial cultivars were considered. These experienced about 20 d of drought, either at vegetative or reproductive phase. Various physio-morphological parameters were monitored, such as stomatal conductance (g_s), photosynthesis (A), water use efficiency (WUE), growth (GI) and soil water content (SWC). The presence of different responses and behaviors allowed to divide the cultivars into three groups: tolerant, susceptible, and intermediate. The classification was also confirmed by a principal component analysis (PCA).

2.1. Introduction

Since water is fundamental for the life of plants in all the physiological processes [28], drought triggers a multitude of different responses affecting morphological and molecular traits in each phenological phase of plant growth [29]. Plants have evolved various adaptation mechanisms to counteract water scarcity, one of the most important is the opening/closing of stomata, driven by changes in guard cell shape. When roots perceive water shortage, plants respond by increasing the synthesis of abscisic acid (ABA) [4], which leads to stomata closure [30]. Although stomatal conductance is then partially affected, a slight decrease in conductance has a protective effect against stress allowing plants to safeguard water reservoirs and improve water use efficiency [4]. In addition, morphological adaptations, such as stomatal density and leaf area, are involved in maintaining the water balance because a decrease of stomata number [31,32] as well as of the transpiring leaf surface [33] contribute significantly to reducing water loss. Defenses are not without side effects; when stomata close excessively, plants were compelled to activate scavenging systems, such as the water-water

cycle, that counteract excess Reactive Oxygen Species (ROS) [6]. In addition, the synthesis of carotenoids allows to capture excess energy from chlorophylls and dissipate it as heat [6] although under extended stress conditions this process is not sufficient [34]. Drought also affects mitosis and consequently plant development reducing both cell number and expansion [35]. These events lead to a reduction in plant growth and yield lowering the revenues of the crop. For all these reasons, the selection of plants tolerant to water deficit has become a high priority. Plants were analyzed for physiological (stomatal conductance, photosynthetic efficiency, water use efficiency, leaf relative water content) and morphological parameters (growth index, stem diameter, leaf area, stomatal density) as well as for soil water content. Plants were grown in a greenhouse and analyzed either at vegetative or reproductive phases. The goal was to highlight the differences in drought tolerance that each cultivar might exhibit specifically in relation to either developmental stage [24,36]. Therefore, this study aimed to identify the most drought tolerant cultivars for future breeding to reduce irrigation demands in sustainable agriculture.

2.2. Materials and Methods

2.2.1. Soil Water Content

The Soil Water Content (θ_g) was evaluated for each pot. Soil samples were weighted (m_{wet}), put overnight in an oven at 105 °C and then weighted again (m_{dry}). Soil water content was calculated as:

$$\theta g = \frac{m_{water}}{m_{soil}} = \frac{m_{wet} - m_{dry}}{m_{dry}},$$

where

- θ_g = Gravimetric Water Content,
- m_{water} = mass of water contained in the samples,
- m_{soil} = sample soil mass,
- m_{wet} = wet soil sample mass,
- m_{dry} = dry soil sample mass.

The mean and standard deviation for each cultivar and phase was calculated at t_0, t_1 and t_2.

2.2.2. Relative Water Content

The leaf relative water content (RWC) was determined as follows [37,38]. Completely expanded and mature leaves at t_2 were cut, leaving a petiole of about 1 cm, immediately inserted into plastic bags with the petiole down, closed and stored in the dark. Each leaf was

weighed with their own plastic bag (TFW-Total Fresh Weight) using a Gibertini-EUROPE_500 balance. Then, 2–3 mL of $CaCl_2$ were added. Samples were incubated for 8 h, allowing them to absorb the $CaCl_2$ solution. Subsequently, leaves were removed from the plastic bag and placed between two paper towels to absorb the excess water. To determine the turgid weight (TW-Turgid Weight), each leaf was weighed. Then, leaves were placed into a paper bag and heated in an oven at 60 °C for 3–4 d. Finally, samples were weighed to determine the dry weight (DW-Dry Weight). The RWC of leaves was calculated as:

$$RWC = \frac{(TFW - BW) - DW}{TW - DW} \; x \; 100 \, ,$$

where

• RWC = Relative Water Content,

• TFW = Total Fresh Weight,

• BW = Bag Weight,

• DW = Dry Weight,

• TW = Turgid Weight.

The mean and standard deviation for each cultivar were calculated.

2.2.3. Growth Index

The growth index (GI) was calculated as:

$$GI_{f,i} = \frac{h_f - h_i}{2} \, ,$$

where

• h_f = final height,

• h_i = initial height.

Heights were measured at t_0, t_1 and t_2 for both vegetative and reproductive phases. The height of each plant was measured with a meter stick parallel to the stem, from the base up to the highest internode. Three GIs were calculated for each plant: $GI_{1.0}$ indicates the growth between t_0 and t_1, $GI_{2.1}$ between t_1 and t_2, while the total growth is expressed by $GI_{2.0}$. The mean and standard deviation of GI for each time-point, cultivar and growth phase were computed.

For pictures on the difference in height of all cultivars in both the vegetative and reproductive stages, click here [1].

2.2.4. Stem Diameter

The stem diameter was measured with a digital caliber (POWERFIX®, Neckarsulm, Germany) at t_0, t_1 and t_2. The diameter was measured about 7 cm from the base of stems, which was marked during the first measurement. The mean and standard deviation for each plant and growth phase were computed.

For more information on the trend of stem diameter in all cultivars, both in vegetative and reproductive phases, click here [2].

2.2.5. Efficiency of Photosynthesis

Photosynthetic efficiency was evaluated by using a fluorometer Handy PEA 2000 (Hansatech Instruments King's Lynn, Norfolk, UK) analyzing Fv/Fm and the performance index (PI). The parameter Fv/Fm indicates the maximum quantum efficiency of Photosystem II, where Fv is the difference between the maximum fluorescence signal (Fm) and the basic fluorescence. The parameter PI shows variations of the entire photosynthetic apparatus, including photosystem I (PSI) and II (PSII). For each growth phase and cultivar, Fv/Fm and PI were collected at t_0, t_1 and t_2. Finally, the mean and standard deviation were calculated.

For more information on the trend of Fv/Fm and PI in all cultivars, both in vegetative and reproductive phases, click here [3].

2.2.6. Leaf Gas Exchange: Stomatal Conductance and Photosynthesis

The LI-6400XT Portable Photosynthesis System (LI-COR Inc., Lincoln, NE, USA) equipped with 6400-40 Leaf Chamber Fluorometer were used to analyze CO_2 and H_2O gas exchange, and intercellular concentration of CO_2 (Ci), stomatal conductance (g_s), and net photosynthesis (A) were calculated. Inside the chamber, the relative humidity (30/70) and the temperature (set to 30 °C) were measured. The light in the chamber, the CO_2 concentration was maintained at 400 µmol mol^{-1}, the relative humidity at 40 to 50%, temperature at 30 °C and the PAR was set to 1500 µmol s^{-1} (values close to the average growth conditions in the greenhouse). The first fully expanded leaves from the apex of plants were used for

[1] https://drive.google.com/file/d/1NqpwqcXx843hwJkbHunHJpfM8GBVOtOP/view
[2] https://drive.google.com/file/d/1tk80fq-CFxd_xh0A3K6SfV-UlBnmwxag/view
[3] https://drive.google.com/file/d/1N6LyZbNLT9KrEPDv-rz6bIpjtQOwBPV8/view

measurements. The measurements of each plant and phase were carried out four times: at t_0, t_{0-1} (between t_0 and t_1) t_1, and at t_2 for the vegetative phase; at t_0, t_1, t_2 and t_R for the reproductive phase. Finally, the mean and standard deviation were computed. The A/g_s ratio, which expresses the water use efficiency (WUE), was calculated for all cultivars.

2.2.7. Morphometric Evaluation of Leaf

In the vegetative phase, at each time-point (t_0, t_1 e t_2) and for selected cultivars, pictures of three leaves per plant at the same developmental stage were taken. Pictures were examined with the software ImageJ (National Institute of Health, Bethesda, MD, USA) to determine:

- Leaf Area (LA),
- Lamina Length (LaL),
- Lamina Width (LaW) (for this parameter 3 measures were taken for each leaf).

Finally, the mean and standard deviation were computed.

2.2.8. Stomatal Density

The stomatal density was calculated at t_2 during the vegetative phase of selected cultivars. Three leaves from each plant were sampled at the same developmental stage. On the lower surface of leaves, a thin layer of transparent nail polish was uniformly applied according to Xu and Zhou [39]. Once dried, the nail polish was pulled away and the molds obtained were put onto a microscope slide. Samples were examined with the optical microscope Zeiss Axiophot (Oberkochen, Germany). For each mold, 10 pictures were taken, and stomata were counted using ImageJ. Stomata number per leaf area (mm^2) expresses stomata density. Finally, the mean and standard deviation were calculated.

2.2.9. Statistical Analysis

Principal Component Analysis (PCA), correlograms and the dendrogram were performed with RStudio IDE (RStudio PBC, Boston, MA, USA). In particular, the corrplot package was used for the analysis of correlation coefficients and their visualization. Raw data were normalized then KMO adequacy and Bartlett's test were performed before factor analysis while orthogonal varimax rotation method was chosen for PCA. Clustering was performed by UPGMA hierarchical cluster analysis on the base of Mahalanobis distance metric.

2.3. Results

2.3.1. Vegetative Phase

2.3.1.1. Drought Stress Highlights Differences among Tomato Cultivars

At first, the multiple physiological traits were measured to perform a principal component analysis (PCA). The complete set of measured parameters at t_1 was integrated to depict the correlation between the various traits. The time-point t_1 (middle of stress treatment) was considered instead of t_2 (end of stress treatment) since the latter was not determined by a varietal difference. Indeed, at t_2 all the cultivars indiscriminately showed a too high deficit in most of the parameters examined. The first factor (PC1), to which the parameters A, g_s, SWC and Fv/Fm contribute most, explains 47.65 % of the total variance, while the second factor (PC2), to which WUE and Ci contribute most, about 19.8 %. In total, both PCs explain 67.45 % of the total variance of all analyzed variables. **Figure 2.1** indicates that photosynthesis (A), conductance (g_s), soil water content (SWC) and photosynthetic efficiency (Fv/Fm and PI) share a positive correlation. The height of plants (h) and the diameter of stem (sd) has a correlation between photosynthetic efficiency and Ci. The water use efficiency (WUE) is inversely correlated to the intercellular concentration of CO_2 (Ci).

Figure 2.1. Principal component analysis (PCA) for physiological and morphological traits in the stress treatment at the vegetative stage: Water Use Efficiency (WUE), intercellular concentration of CO_2 (Ci), photosynthesis (A), stomatal conductance (g_s), Soil Water Content (SWC), photosynthetic efficiency (Fv/Fm), Performance Index (PI), height (h), stem diameter (sd).

In **Figure 2.2.** it is possible to notice that all control plants (blue) are distributed in a restricted area without much difference between the cultivars. On the contrary, all the stressed plants (orange) are distributed in a much larger space that extends mostly along the PC2 axis.

This indicates that drought stress differentiates the behavior of plants in a genotype-dependent manner. It is important to observe that WUE and, correspondingly, Ci are the parameters that drive the differentiation between the genotypes. Secondly, to evaluate the behavior of each cultivar a PCA was performed with each parameter of the stressed plants in relation to their own control (**Figure 2.3**). Tomato cultivars can be divided into two main groups mainly by differences in PC1 values, which accounts for 47.3 % of variation with high loadings of Ci, WUE, A, and g_s. One group consists of Costoluto Fiorentino, Rosso di Pitigliano, Pisanello, Pantano, Datterino, Pearson, Giallo di Pitigliano and Canestrino di Lucca; the other group contains Perina, Cuore di bue, Fragola, Tondino. The genotypes of Quarantino and Pearson are at an intermediate position.

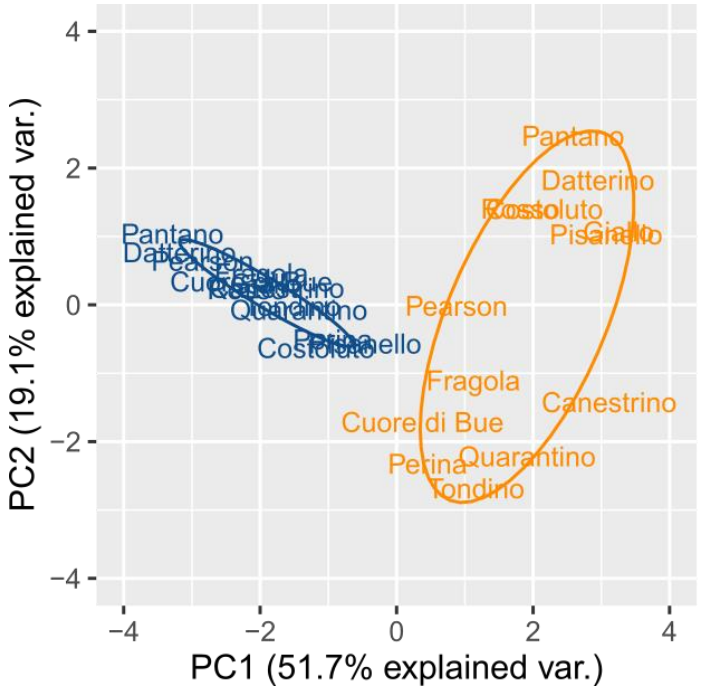


Figure 2.2. Principal component analysis (PCA) for genotypes based on control (blue) and stress (orange) indices calculated for physiological traits at t_1 in the stress treatment at the vegetative stage.

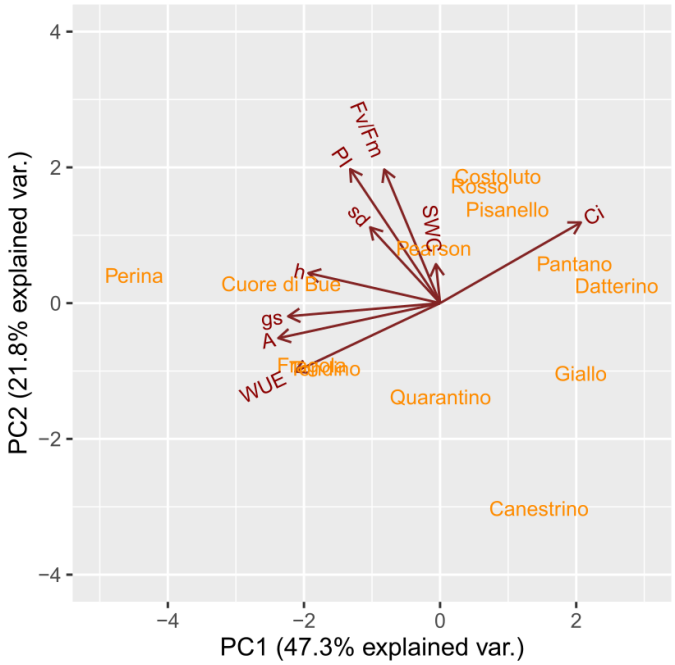


Figure 2.3. Principal component analysis (PCA) for genotypes based on stress indices in relation to control indices calculated for physiological traits at t_1 in the stress treatment at the vegetative stage.

2.3.1.2. Clusterization

Nine traits have been correlated for each cultivar according to their time course. Firstly, each parameter relative to stressed plants was normalized to its own control (in percentage). Then, a correlogram for each cultivar was constructed (for a representative example see **Figure 2.4**).

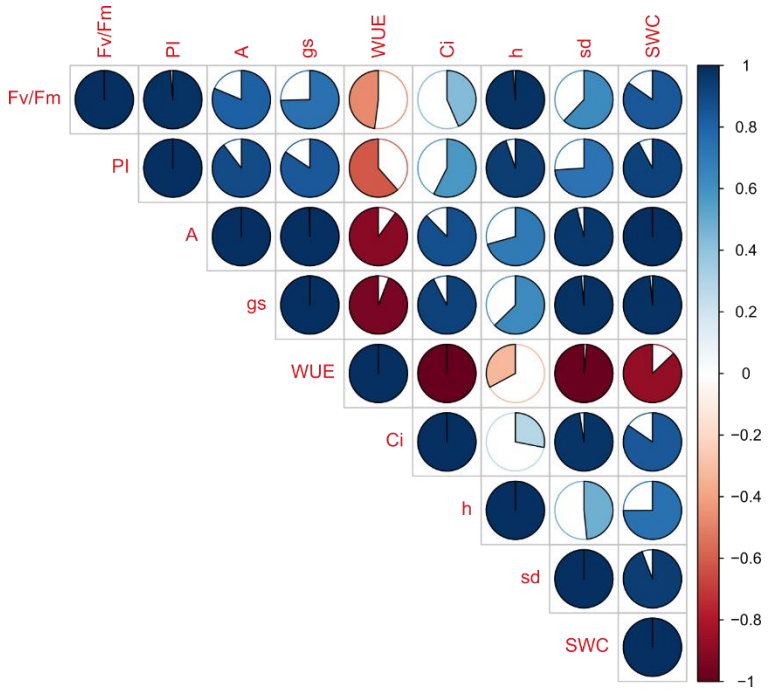


Figure 2.4. Correlogram of 9 physiological and morphological traits evaluated in Perina cultivar in the stress treatment at the vegetative stage. Each trait of DS plants is normalized to that of CTRL and then correlated according to the time course (t_0, t_1, t_2). The filling of the cake corresponds to the value of the correlation

coefficient (full cake means unit correlation, in absolute value) while the color indicates the sign (blue/red means positive/negative correlation coefficient).

From the PCA previously described, WUE and Ci turned out to be the parameters that most influenced the differentiation between cultivars. Hence, the correlations of all the traits with respect to WUE were used to construct the dendrogram in **Figure 2.5** showing the cultivars distributed within two main clusters. One of them is clearly distinguishable and is formed by Cuore di bue, Quarantino, Fragola, Tondino and Perina. The other is composed by Costoluto, Rosso, Pantano, Canestrino, Datterino, Pisanello, Giallo, Pearson. A dendrogram corresponding to correlations with respect to Ci was also obtained, but it was not reported in this thesis as it revealed the same two distinct groups.

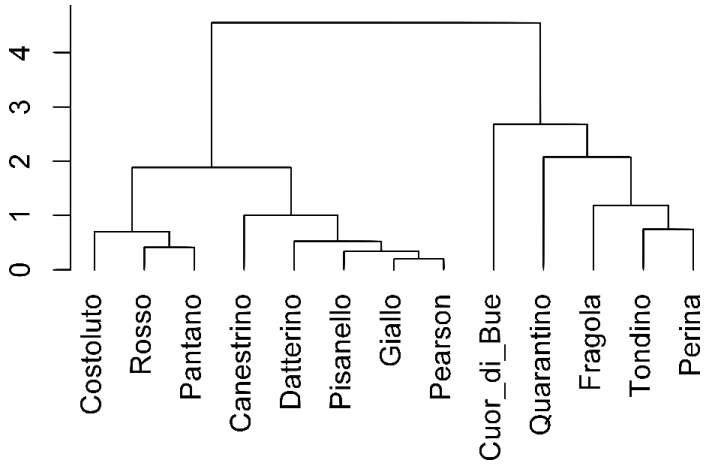


Figure 2.5. Dendrogram assembled by multivariate cluster analysis using correlation coefficients of all parameters with respect to WUE in the stress treatment at the vegetative stage.

2.3.1.3. Susceptible and Tolerant Cultivars

Analysis of clusterization and PCA revealed two very similar groups. Differentiation in these two groups can be encompassed by individual parameters. Perina, Fragola, and the commercial Cuor di Bue cultivars still have g_s quite far from 0 at t_1. While Tondino, Quarantino, Costoluto and the commercial Pearson cultivars still have g_s near to but different from 0 at t_1 (**Figure 2.6**). On the contrary, the remaining cultivars had a value already equal to 0 at t_1. This allowed us to find a first difference in perceiving water shortage as stress. As suggested by Galmes [40], it is valuable to observe the stomatal conductance together with the SWC. A non-vanishing value of g_s at t_1 corresponds to SWC higher than 0.5 in the same cultivars (**Figure 2.7**), probably indicating that water is still available. Therefore, the different perception of water shortage as stress likely corresponds to a better management of the soil water resource in Perina, Fragola, Tondino, Quarantino and the commercial Cuor di Bue cultivars.

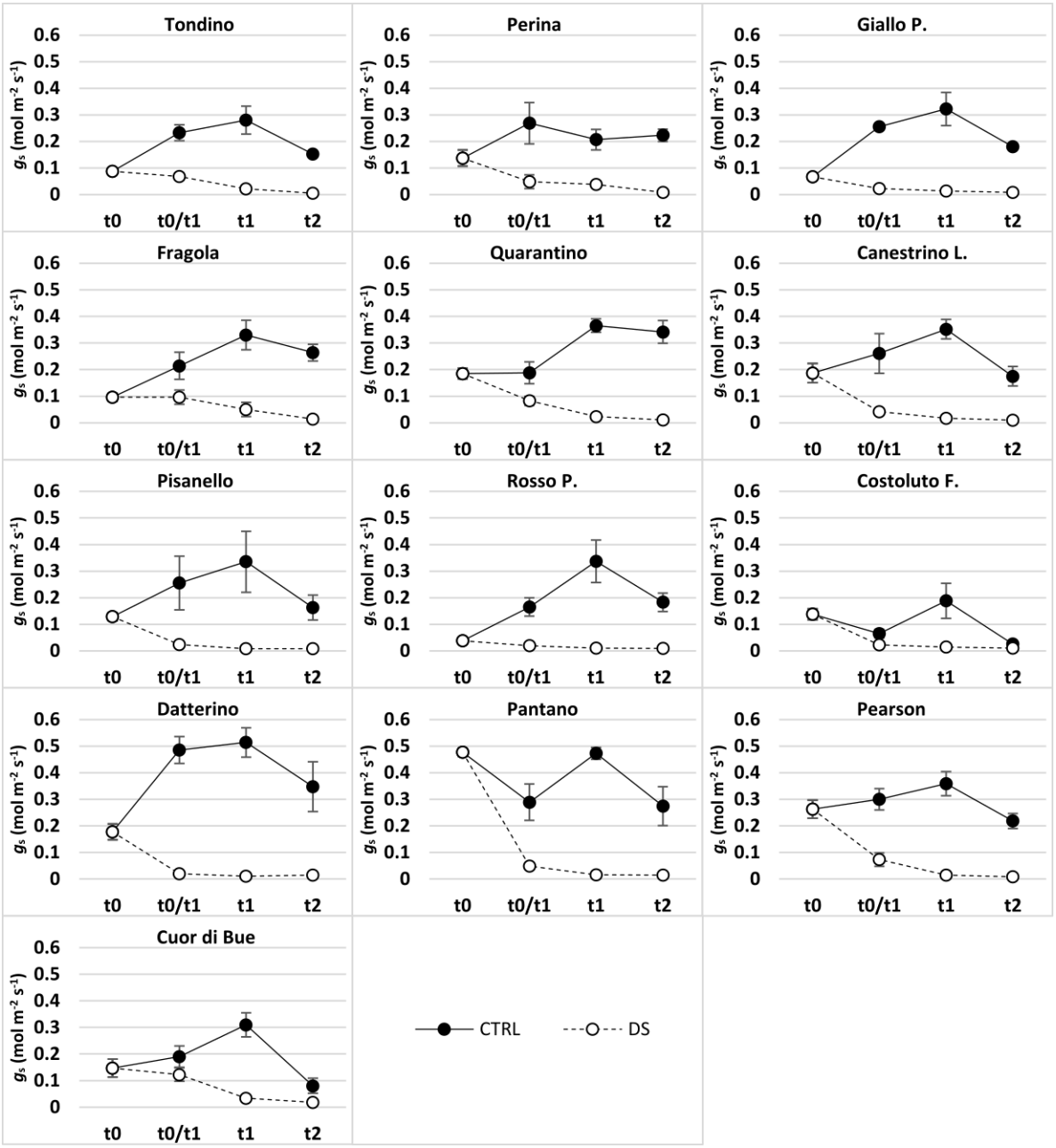


Figure 2.6. Trend of stomatal conductance (g_s) for the vegetative phase. The black straight line indicates the control trend (CTRL) while the dashed line the stress trend (DS). Vertical bars represent standard deviation of averages of the values taken on five plants.

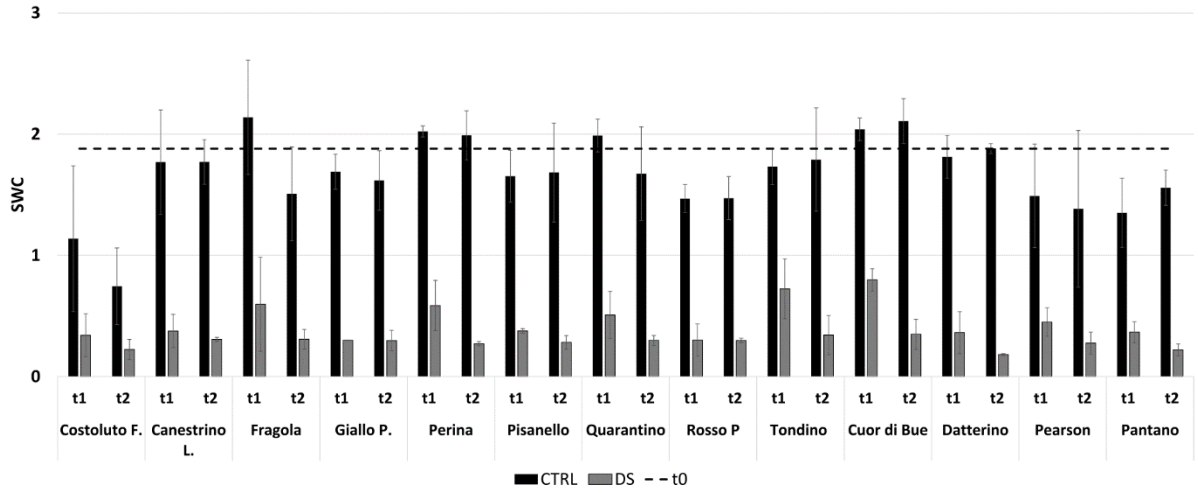


Figure 2.7. Soil Water Content (SWC) at t_1 and t_2 in the stress treatment at the vegetative stage. The dashed line indicates the initial SWC, at t_0. In black are the controls (CTRL), while in stripes the stressed (DS). Vertical bars represent standard deviation of means of the values taken on 5 plants.

The literature reported that photosynthesis is one of the primary physiological targets of water stress [4,40,41]. Considering the values obtained from photosynthesis, Tondino Liscio, Quarantino, Fragola, Perina and Cuor di Bue again have A different from 0 at t_1 (**Figure 2.8**). The parameter A can then provide an indication of the most tolerant genotypes. WUE expresses the ability of a plant to produce biomass through photosynthesis per water consumed [40] and is considered a parameter useful for evaluating the best performing plants in conditions of drought stress [42]. In this study the most promising cultivars are Tondino Liscio, Quarantino, Fragola, Perina and Cuor di Bue (**Figure 2.9**), that can be considered tolerant to drought stress, while all the other cultivars are more susceptible to lack of water.

Among all the cultivars, only four were selected for the next analyses. Combining all the results described so far, Perina and Fragola were chosen as representative of the group of tolerant cultivars. On the contrary, Pisanello was selected to be the most representative of susceptible traits among the local cultivars. Quarantino was selected as the medium cultivar that has both tolerant and susceptible characteristics. First, the stomatal density at t_2 was calculated. As observed in **Figure 2.10**, the DS of Pisanello shows a higher and significantly different density compared to the CTRL, thus confirming a higher sensitivity to the stress [43]. The opposite happens to Perina, which has a lower density in the DS and significantly different from the CTRL, as to indicate an adaptation to drought stress. This result partly justifies the trend of WUE: a lower transpiration allowed a prolonged increase in the Perina compared to t_0, while the increase in stomatal density may have affected the fall of WUE in the Pisanello cultivar. For Quarantino and Fragola the density is almost unchanged between CTRL and DS, indicating a non-susceptibility to stress of this parameter.

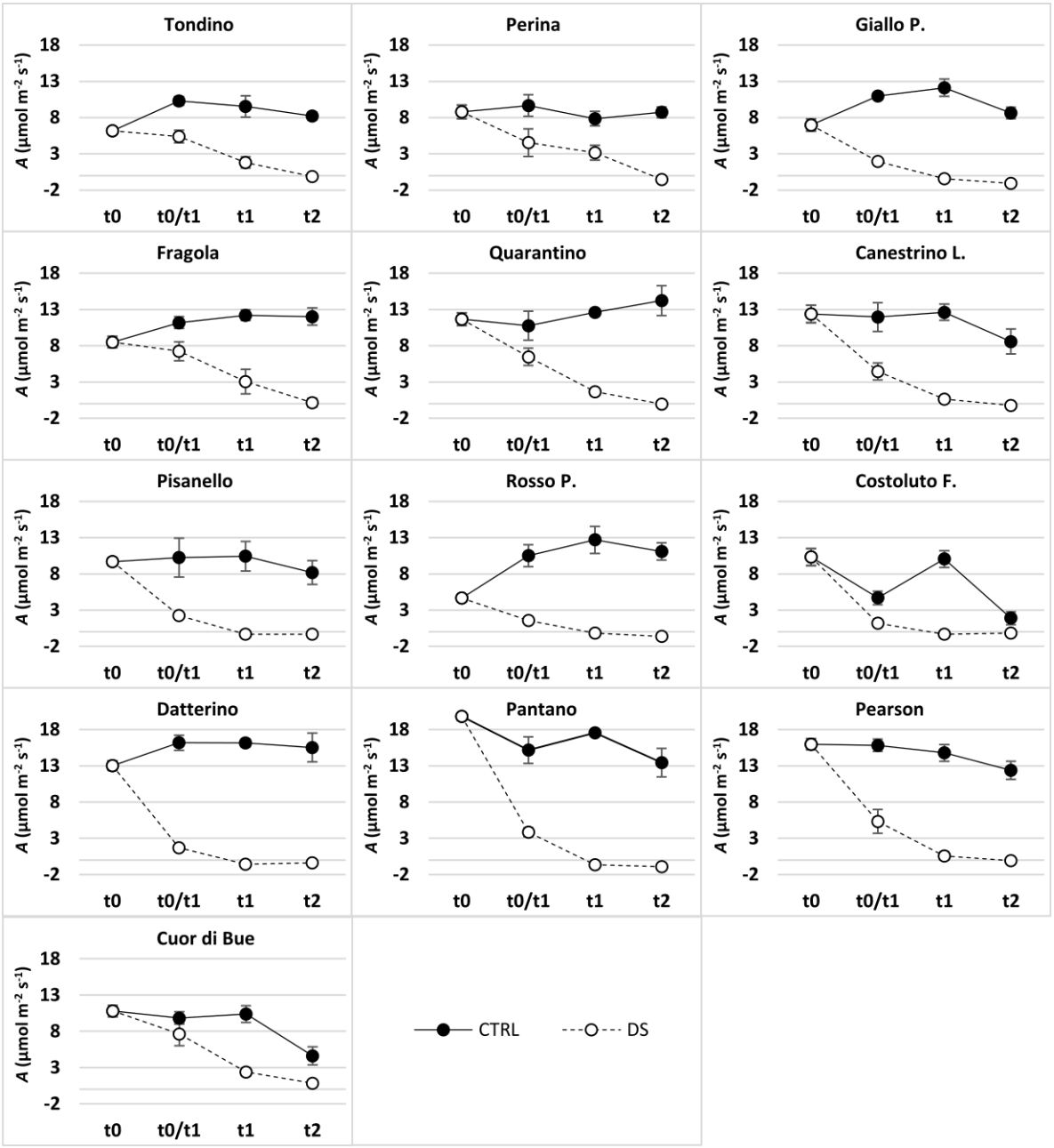


Figure 2.8. Course of photosynthesis (A) for the vegetative phase. The black straight line indicates the control trend (CTRL) while the dashed line the stress trend (DS). Vertical bars represent standard deviation of averages of the values taken on five plants.

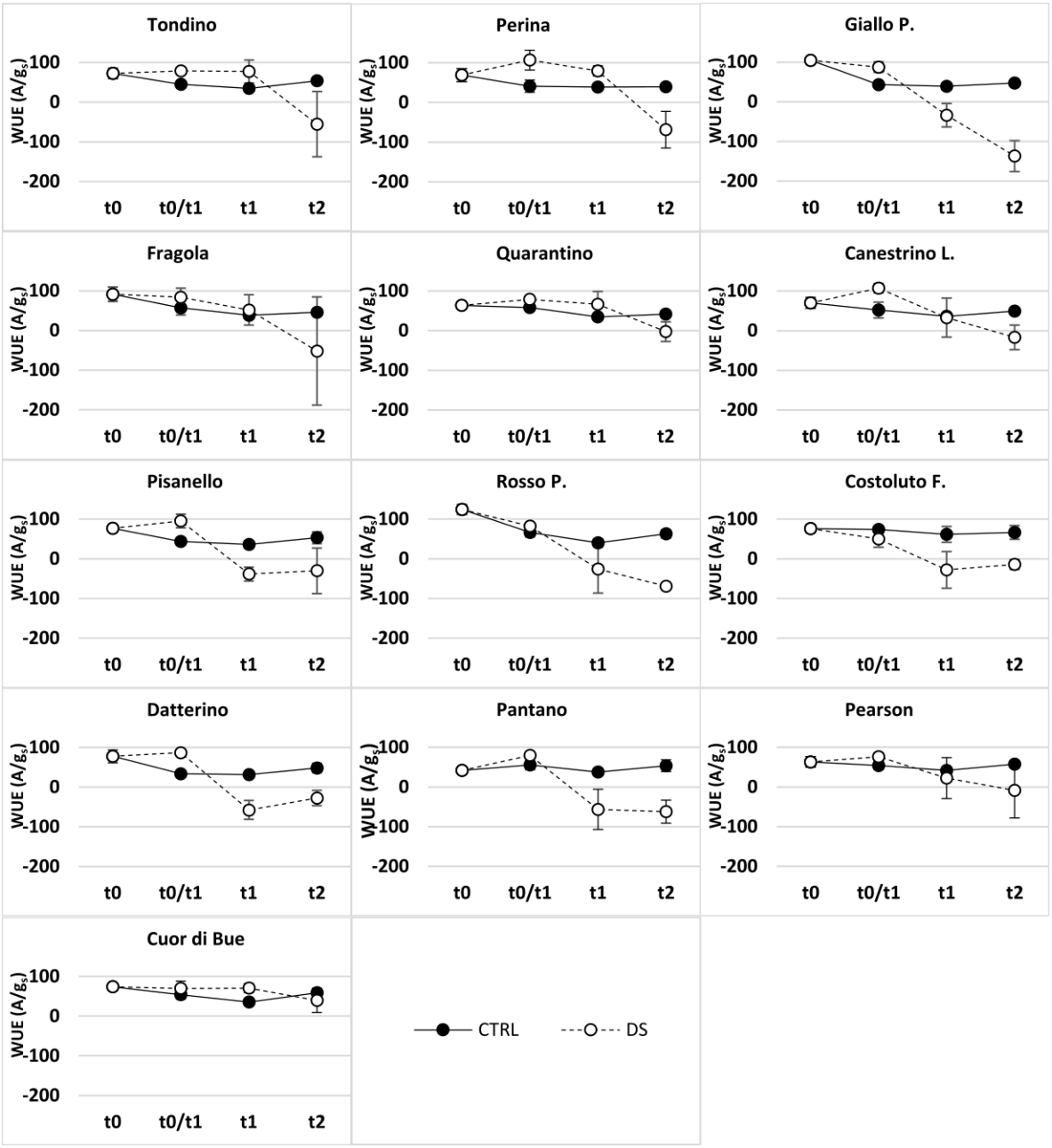


Figure 2.9. Water Use Efficiency (WUE) trend for the vegetative phase. The black straight line indicates the control trend (CTRL) while the dashed line the stress trend (DS). Vertical bars represent standard deviation of averages of the values taken on five plants.

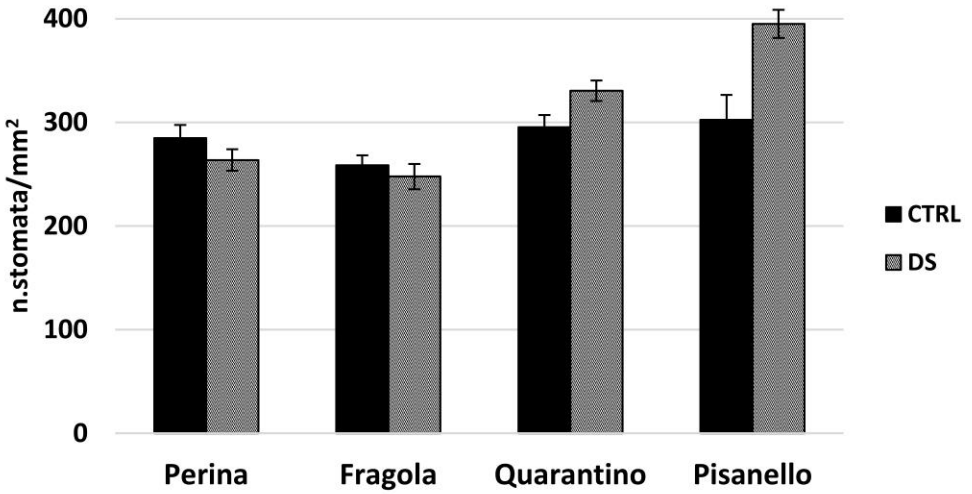


Figure 2.10. Stomatal density at t_2, in the 4 representative cultivars in the stress treatment at the vegetative stage. In black are the controls (CTRL) and in stripes the stressed (DS). Vertical bars represent standard deviation of averages of the values taken on 10 photos for each leaf (three per plant).

The size of leaves plays a key role in the energy and water balance of plants [44–46] as a transpiring and photosynthesizing surface. The leaf area (LA) for the four cultivars at t_0, t_1 and t_2 is shown in **Figure 11**. The stability of LA in Perina during the stress, together with the low stomatal density, confirms its excellent tolerance because it kept the photosynthesizing surface intact while it reduces transpiration. The LA of the DS of Quarantino and Fragola cultivars is also stable while that of Pisanello significantly decreases, differing significantly from the CTRL at t_1. The damage was clearly visible as wilting and yellowing of plants. This confirms a strong sensitivity of Pisanello to drought stress.

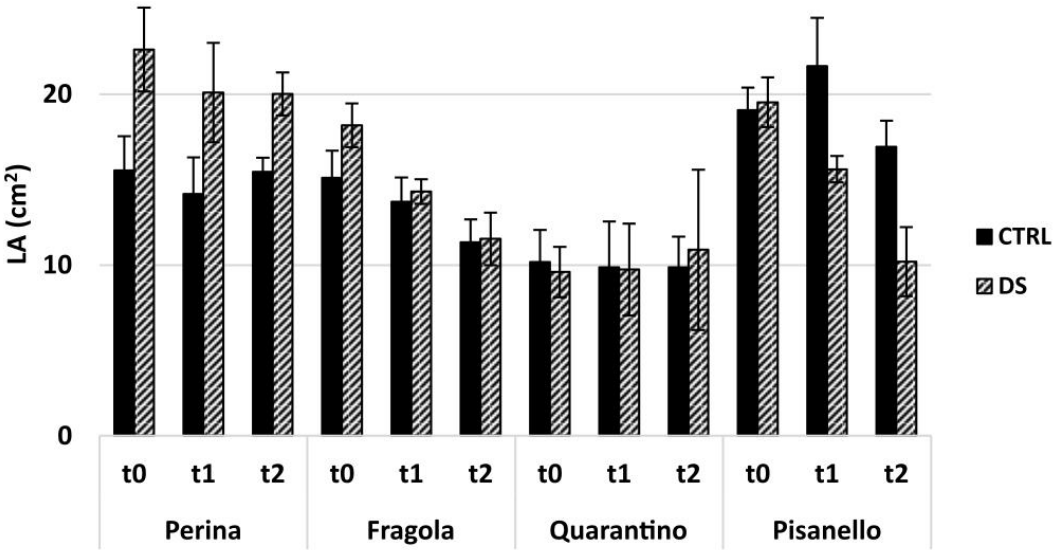


Figure 2.11. Leaf area (LA) of the 4 representative cultivars in the stress treatment at the vegetative stage. In black are the controls (CTRL) and in stripes the stressed one (DS). Vertical bars represent standard deviation of averages of the values taken on 3 leaves per plant.

2.3.2. Reproductive Phase

2.3.2.1. Drought Stress Highlights Differences among Tomato Cultivars

As done for the vegetative phase, also in the reproductive phase a PCA was carried out with the multiple physiological data collected. The complete set of parameters at t_2 was integrated to depict the correlation between the various traits. Photosynthesis (A), conductance (g_s) and soil water content (SWC) have a positive correlation (**Figure 2.12**). There is a similar positive correlation also with water use efficiency (WUE) that is inversely correlated to intercellular concentration of CO_2. The plants' height (h) and the stem's diameter do not show a positive correlation and the same occurs for Fv/Fm and PI. The first factor (PC1), to which A and SWC contribute most, explains 49.5% of the total variance, while the second factor (PC2), to which WUE and Ci contribute most, describes about 16.1% of total variance. Altogether, both PCs explain 65.6% of the total variance for all analyzed variables.

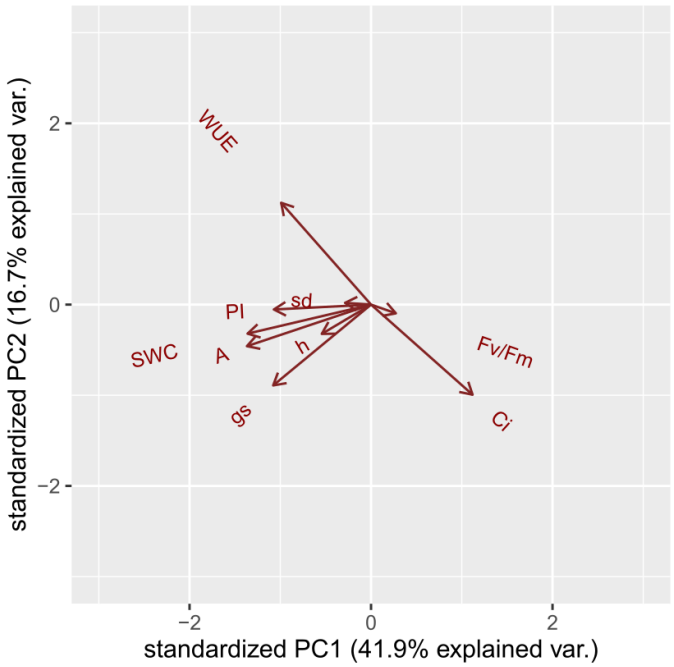


Figure 2.12. Principal component analysis (PCA) for physiological and morphological traits in the stress treatment at the reproductive stage: Water Use Efficiency (WUE), intercellular concentration of CO_2 (Ci), photosynthesis (A), stomatal conductance (g_s), Soil Water Content (SWC), photosynthetic efficiency (Fv/Fm), Performance Index (PI), height (h), stem diameter (sd).

Additionally, it was possible to clearly distinguish the control plants (blue) from the stressed ones (orange) (**Figure 2.13**). However, in the reproductive phase both control and stressed plants are distributed in a relatively large area, with some differences between the cultivars. This indicates that each cultivar has its own physiological behavior at the adult stage. However, drought stress indeed plays an important role since the differentiation is more accentuated in the stressed (orange) group.

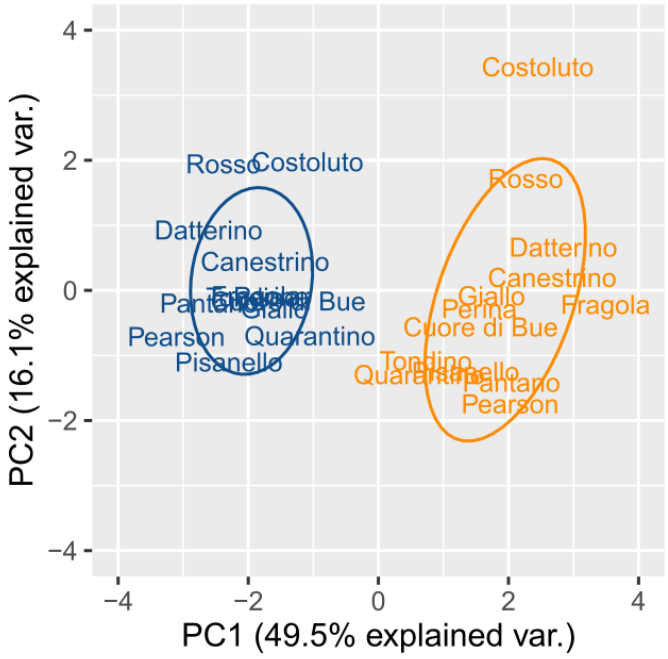


Figure 2.13. Principal component analysis (PCA) for genotypes based on control (blue) and stress (orange) indices calculated for physiological traits at t_2 in the stress treatment at the reproductive stage.

Secondly, to evaluate the behavior of each cultivar, another PCA was performed with each parameter of the stressed plants in relation to their own control (**Figure 2.14**). Following the same subdivision principle used for the vegetative phase, tomato cultivars can be divided into two main groups according to positive or negative values of PC1. In this case, one group consists of Costoluto Fiorentino, Pisanello, Tondino and Quarantino; the other group contains Fragola, Canestrino di Lucca, Giallo di Pitigliano, Rosso di Pitigliano, Datterino, Pearson, Pantano and Cuore di bue. The genotype of Perina is at an intermediate position.

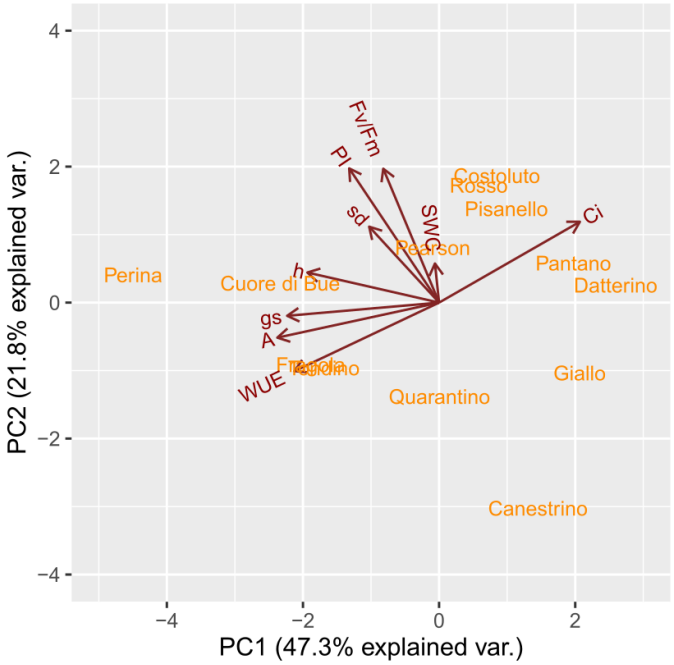


Figure 2.14. Principal component analysis (PCA) for genotypes based on stress indices in relation to control indices calculated for physiological traits at t_2 in the stress treatment at the reproductive stage.

2.3.2.2. Clusterization

A correlogram for each cultivar was constructed (**Figure 2.15**) on the base of nine traits according to their time course. The values related to stressed plants were normalized to their own control (in percentage). Following what was done for the vegetative phase, the correlations of all the traits with respect to WUE were used to construct the dendrogram (**Figure 2.16**). In the reproductive phase two groups (clusters) are visible, but, with respect to the vegetative phase, groups are not too different. One is formed by Fragola, Canestrino di Lucca, Perina, Costoluto Fiorentino and Pisanello; the other is composed of Tondino, Rosso di Pitigliano, Giallo di Pitigliano, Cuor di bue, Pantano, Pearson, Quarantino and Datterino.

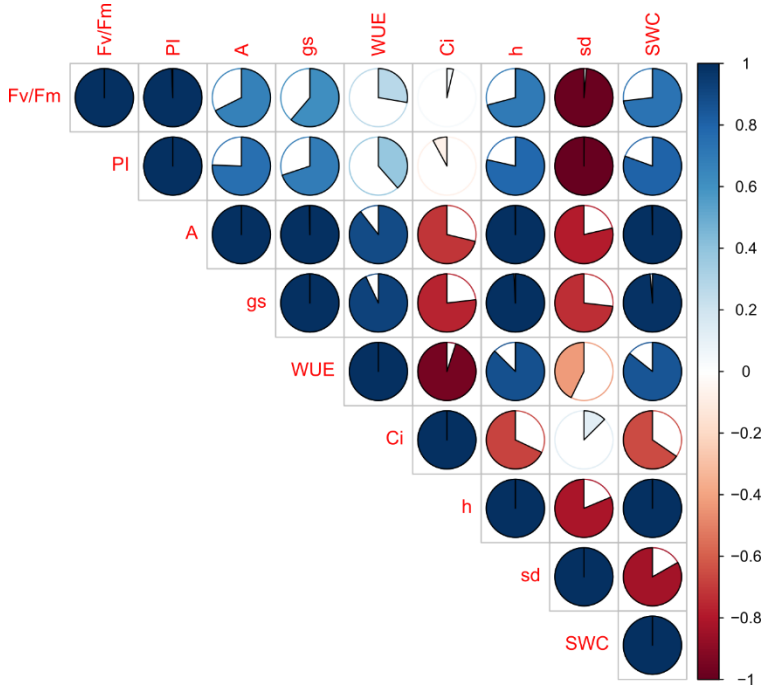


Figure 2.15. Correlogram of 9 physiologic and morphologic traits evaluated in the Perina cultivar during stress treatment at the reproductive stage. Each trait of DS plants is normalized to that of CTRL and then correlated according to the time course (t_0, t_1, t_2). The filling of the cake corresponds to the value of the correlation coefficient (full cake means unit correlation, in absolute value) while the color indicates the sign (blue/red means positive/negative correlation coefficient).

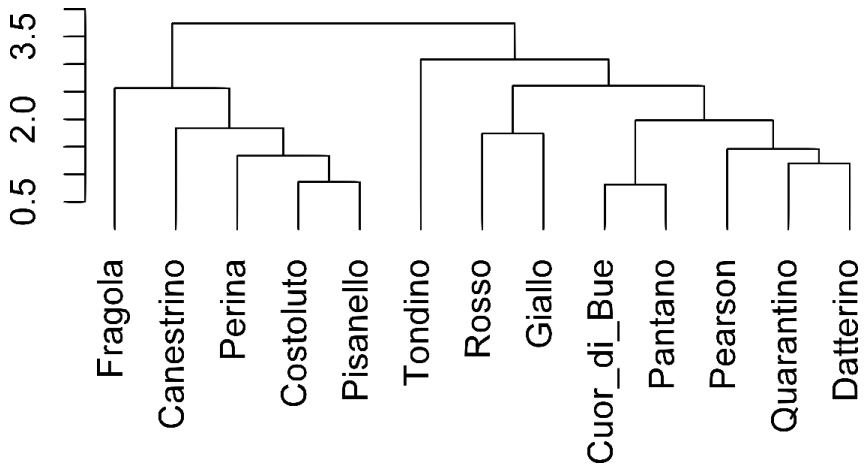


Figure 2.16. Dendrogram built by multivariate cluster analysis using correlation coefficients of all parameters with respect to WUE in the stress treatment at the reproductive stage.

2.3.2.3. Susceptible and Tolerant Cultivars

The analysis of each individual parameter helps to understand the characteristics of cultivars and the differentiation between groups. Regarding stomatal conductance, the Quarantino cultivar has a g_s equal to 0.12 mol $m^{-2}s^{-1}$, which is near to the value of its own control at t_2 (**Figure 2.17**). The Perina, Giallo, Fragola, Canestrino, Rosso and the commercial Datterino, Pearson and Cuor di Bue cultivars have a g_s close to 0 at t_2; in the commercial cultivars, the value of stressed plants differs greatly from their own control. The remaining cultivars have intermediate values between 0.06 and 0.09 mol $m^{-2}s^{-1}$. Like the vegetative phase, there is a correlation with the SWC. In this case, at t_1 the soil of Costoluto, Giallo, Quarantino and Pearson still contained an appreciable amount of water (**Figure 2.18**). Clearly at t_2 the differences between CTRL and DS are amplified without an appreciable varietal difference; only Quarantino maintains a higher SWC than other stressed cultivars. Thus, once again the different perception of water scarcity likely corresponds to better management of the soil water resource in Quarantino.

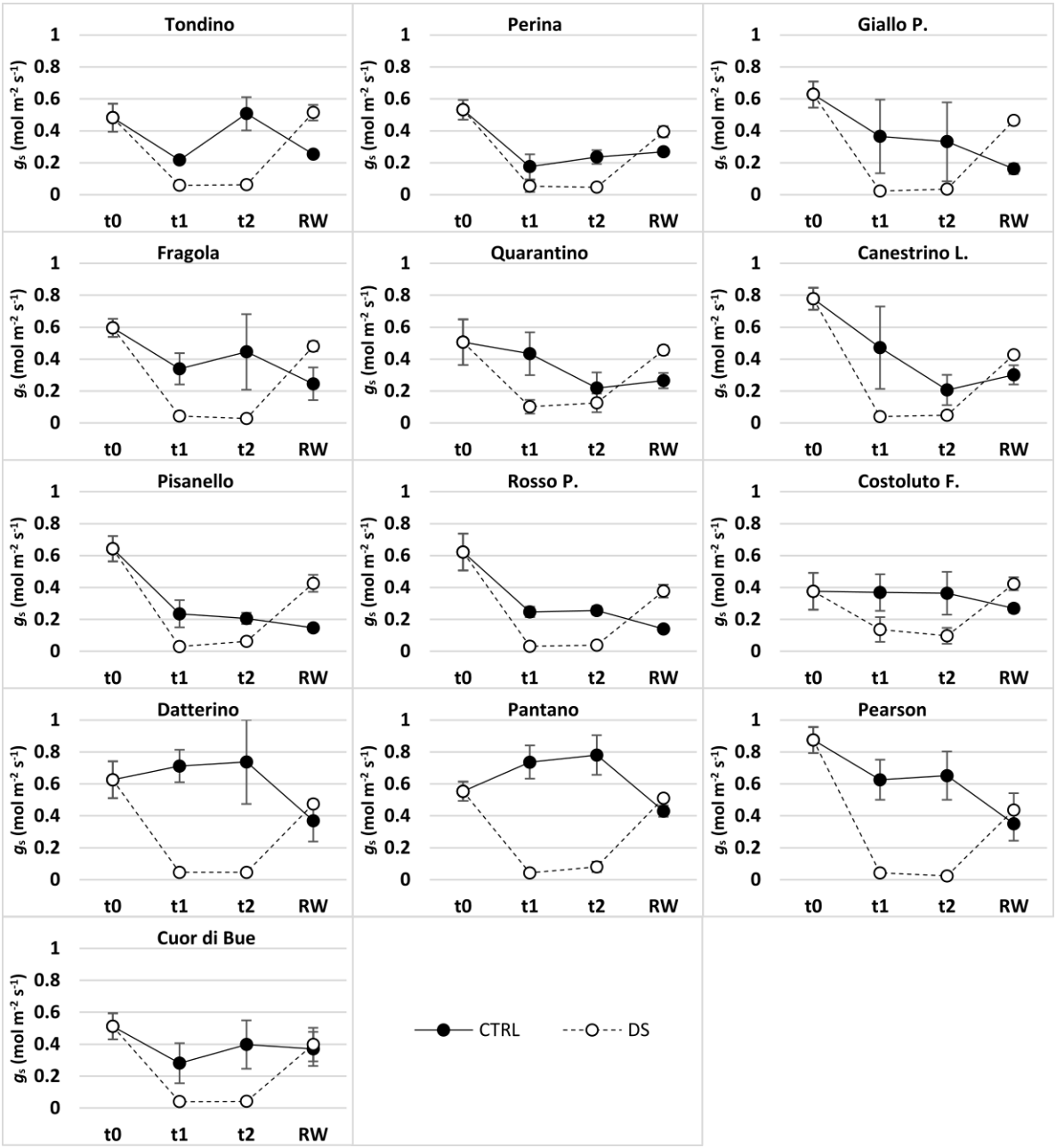


Figure 2.17. Trend of stomatal conductance (g_s) for the reproductive phase. The black straight line indicates the control trend (CTRL) while the dashed line the stress trend (DS). Vertical bars represent standard deviation of averages of the values taken on four plants.

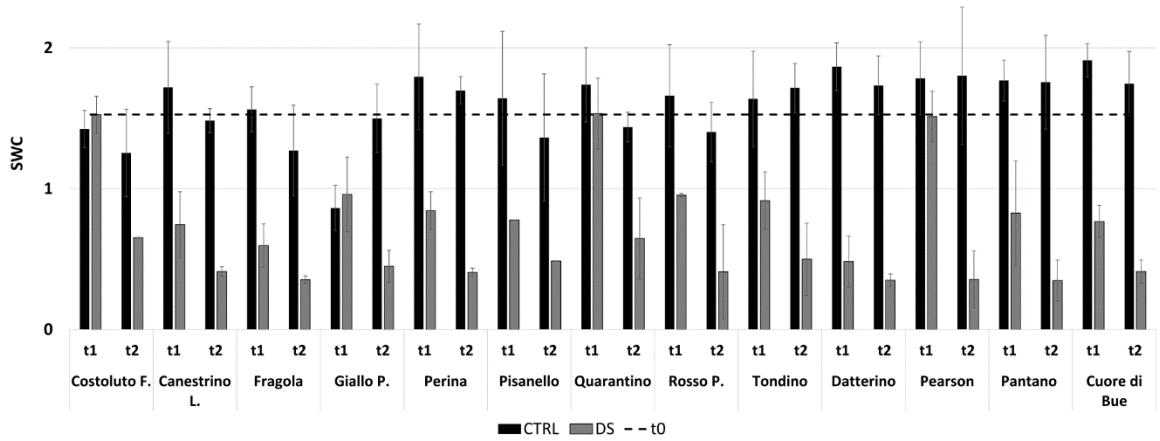


Figure 2.18. Soil Water Content (SWC) at t_1 and t_2 in the stress treatment at the reproductive stage. The dashed line indicates the starting SWC, at t_0. In black are the controls (CTRL) and in stripes the stressed (DS). Vertical bars represent standard deviation of means of the values taken on 4 plants.

The RWC was calculated for the aerial part of the plant. This parameter provides an interpretation of how water stress might affect plants differently [47]. Costoluto, Giallo,Pisanello, Quarantino and Datterino cultivars show a decrease in RWC compared to their own controls (**Figure 2.19**). RWC was established as an indicator of water status balance [48]. The decrease in RWC usually indicates a worse resistance to drought stress [49,50] and the cultivars maintaining RWC values comparable to their control are Canestrino, Fragola, Perina, Rosso, Tondino, Pearson, Pantano and Cuore di Bue.

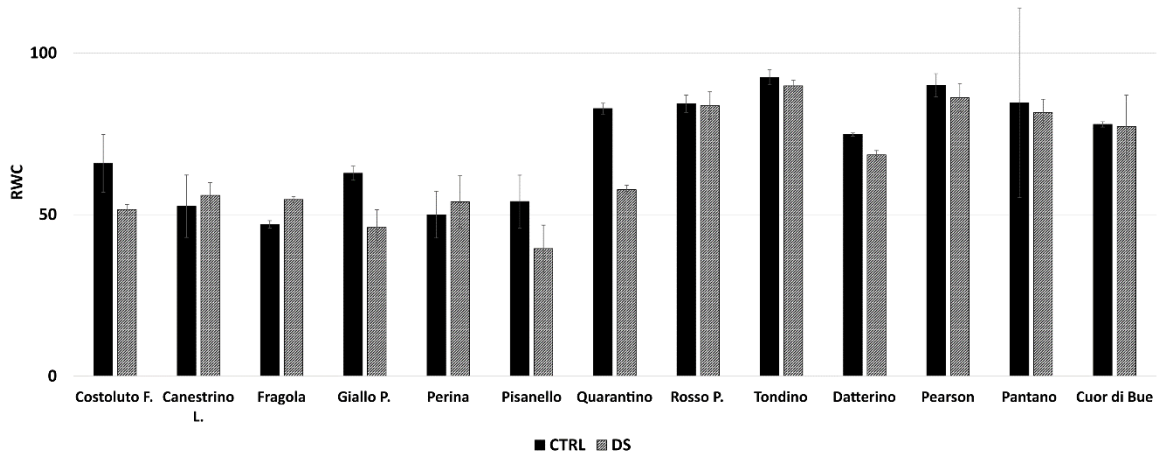


Figure 2.19. Relative Water Content (RWC) at t_2 in the stress treatment at the reproductive stage. In black are the controls (CTRL) and in stripes the stressed (DS). Vertical bars represent standard deviation of means of the values taken on 3 leaves per plant.

As regards photosynthesis in the reproductive phase, the Quarantino cultivar has a value of A equal to 6.3 µmol $m^{-2}s^{-1}$ at t_2, a value like its own control (**Figure 2.20**). The cultivars Tondino, Perina, Pisanello, Costoluto and the commercial Pantano have a positive A greater than 2. However, in the commercial cultivar, the value at t_2 differs particularly from its own control. The other cultivars have an A close to 0 showing that this parameter seems to be

particularly affected by stress. Once again, the WUE in the reproductive phase shows that the Quarantino maintains values comparable to control, indicating that it is not particularly affected by water stress (**Figure 2.21**). Other cultivars with a WUE value close to the control at t_2 are Tondino, Pantano and Cuor di Bue. The cultivars Perina, Pisanello and the commercial Pearson also keep a comparable value. On the contrary, Giallo, Canestrino, Rosso, Costoluto, Datterino, and most of all Fragola are more sensitive to water stress as regards the WUE, as they have an extremely low value at t_2. In general, there is an increase in WUE in all cultivars after a few days from the beginning of the stress (t_1).

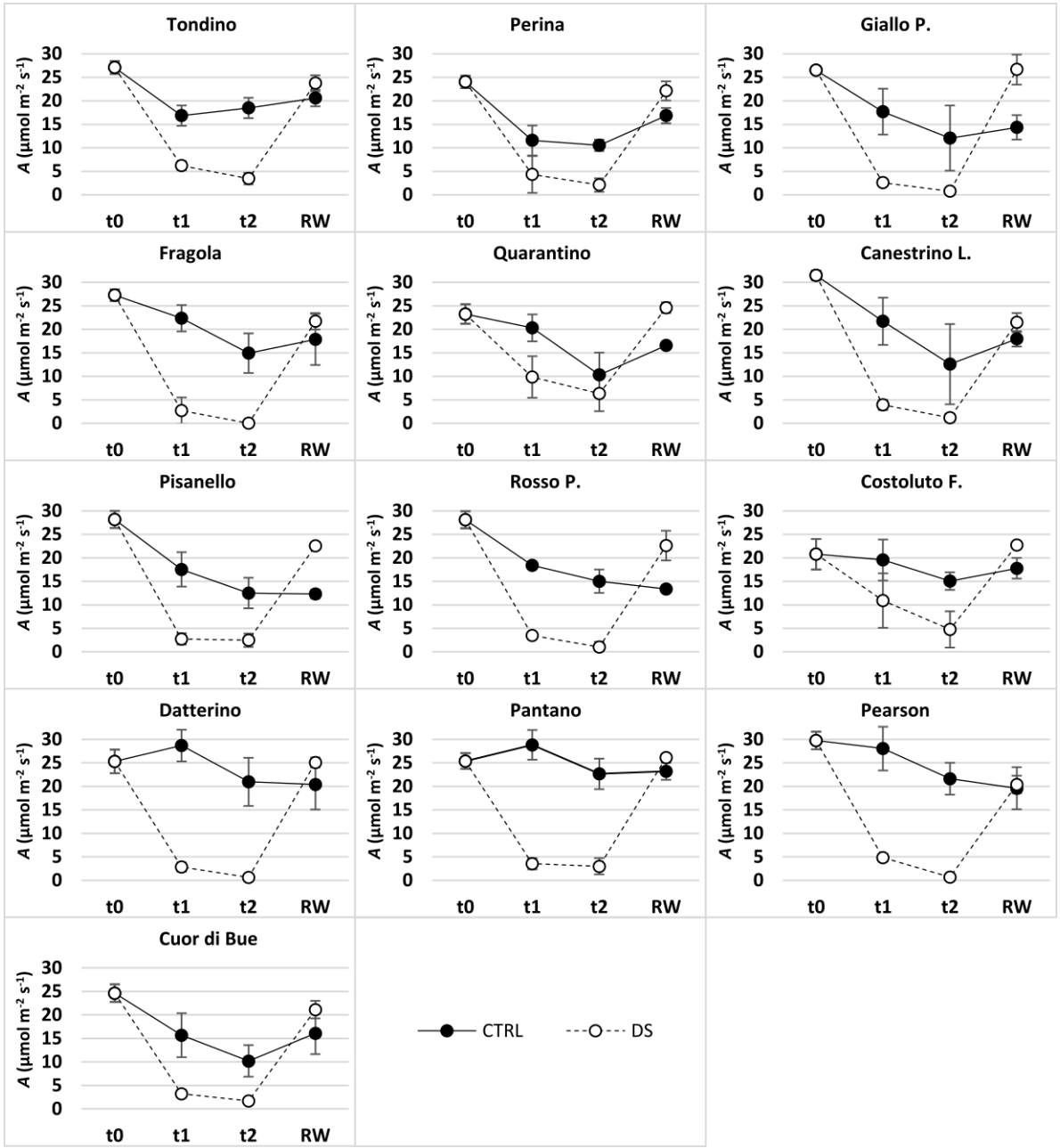


Figure 2.20. Course of photosynthesis (A) for the reproductive phase. The black straight line indicates the control trend (CTRL) while the dashed line the stress trend (DS). Vertical bars represent standard deviation of averages of the values taken on four plants.

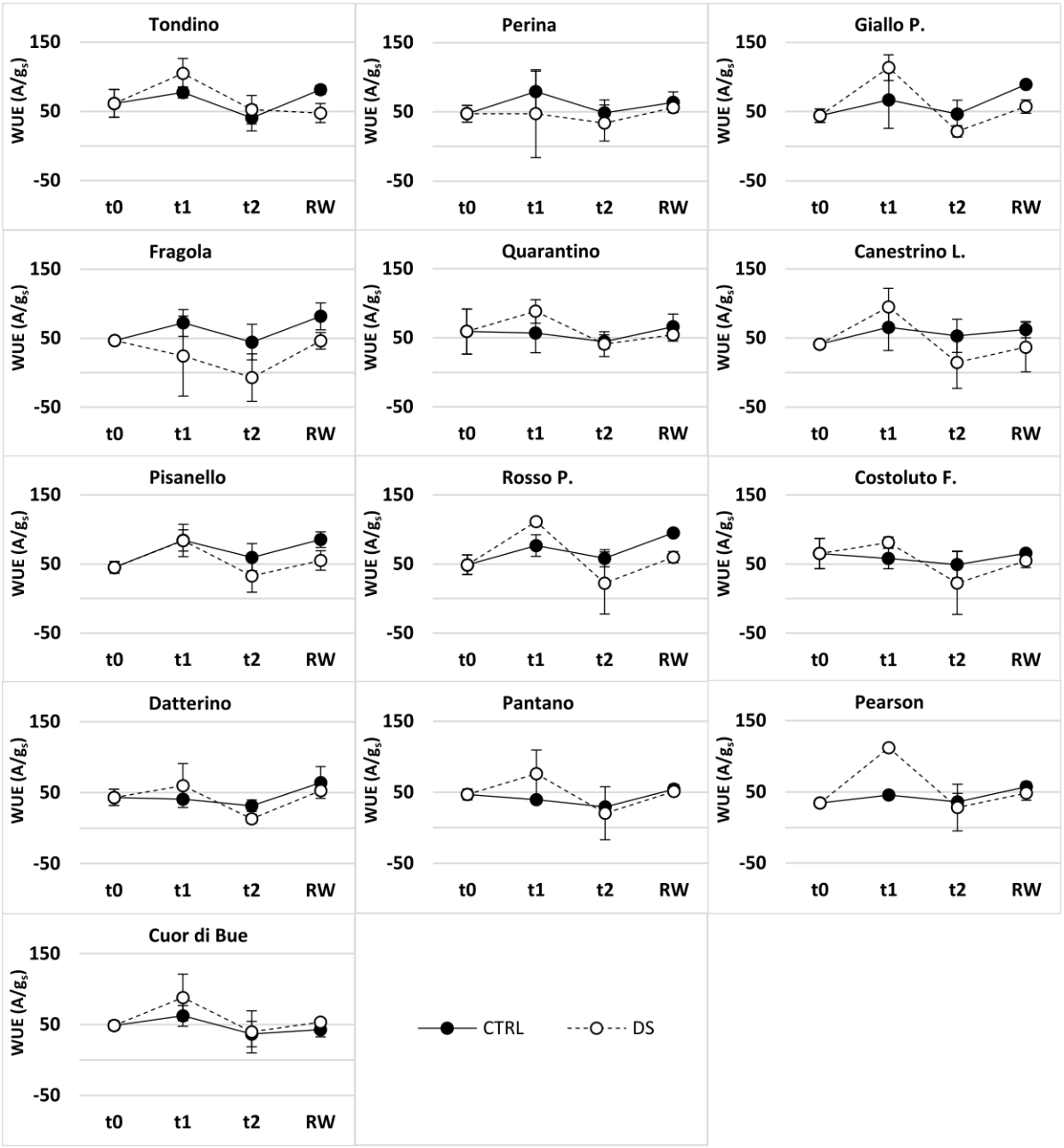


Figure 2.21. Water Use Efficiency (WUE) trend for the reproductive phase. The black straight line indicates the control trend (CTRL) while the dashed line the stress trend (DS). Vertical bars represent standard deviation of averages of the values taken on four plants.

2.4. Discussion

The number and diversity of responses to drought define the ability of a plant species or cultivar to tolerate this abiotic stress [51]. Consequently, lower or higher susceptibility to drought is necessarily related to the plant genotype. Building on these facts, we screened tomato cultivars cataloged in the Regional Germplasm Bank of Tuscany and therefore adapted to the climatic and soil conditions of Tuscany. Plants were analyzed during both the vegetative and reproductive phases; behind that was the question of whether a given cultivar was specifically more tolerant in one phase than the other. This could disclose even more specific mechanisms of tolerance. To obtain the sought information, tomato plants were

evaluated for several physio-morphological parameters that were subsequently integrated and correlated with each other.

In plants, the first perception of water deficit results in the closure of stomata, which leads to the decreasing of stomatal conductance. We found that the g_s of tomato plants is lower in stressed samples than in the corresponding control, suggesting that drought stressed plants strongly perceive stress and consequently adapt [40,49]. Nevertheless, not all tomato cultivars behave the same way. Just to briefly summarize, in the vegetative phase the local cultivars Costoluto Fiorentino, Giallo di Pitigliano, Rosso di Pitigliano and Pisanello as well as the commercial Datterino show g_s close to zero at mid-stress. On the contrary, the cultivars Perina, Fragola, Tondino, Quarantino and the commercial Cuor di Bue are more tolerant, showing a non-varying conductance in the middle and final phase of stress. In the reproductive phase, the situation differs partially because the cultivars Perina, Giallo, Fragola, Canestrino, Rosso and the commercial Datterino, Pearson and Cuor di bue have a gs close to zero at the mid time. The cultivar Quarantino also achieves to maintain an adequate conductance as well as the cultivars Costoluto, Pisanello, Tondino and the commercial Pantano.

Photosynthesis is another physiological target of primary importance for drought [4,40,41]. In the vegetative phase, Tondino, Quarantino, Fragola, Perina and Cuor di Bue show an A value different from zero, while photosynthetic activity is strongly affected at mid-stress in the other cultivars. This suggests that the five cultivars mentioned above are the most tolerant. However, distinctions are present in the reproductive phase because Canestrino, Fragola, Giallo, Rosso, Cuore di Bue, Datterino and Pearson show an A value close to 0, thus a strongly reduced photosynthesis. In contrast, the other cultivars have a positive A; since the A value of Quarantino at t_2 is like the control, this is another indication of its higher drought tolerance. Because there are no studies on the same cultivars in the literature, we can refer to the work of Zhou [49], in which the tomato cultivar Arvento showed an A value different from 0 already at the first-time interval of combined stress (heat and drought) and was the most drought-tolerant cultivar.

In this study, as observed by Mishra [52], none genotype showed differences in photosynthetic efficiency (Fv/Fm and PI) between stressed and control plants after eight days of stress. In an earlier study on Tuscan tomato cultivars under drought conditions, Conti [25] found that photosynthetic efficiency decreased from the fourteenth day of stress. Indeed, a brief period of drought usually does not affect the Fv/Fm parameter [34,52]. This is because the first response to drought (i.e., stomata closure) does not affect the ability of PSII to reduce the first electron transporter, Qa. In fact, the water-water cycle and photorespiration initially

allow stressed plants to accomplish electron transport in a way comparable to control plants, avoiding photodamage to PSII [34]. In contrast, PI is a more drought-sensitive parameter than Fv/Fm [53]. In all stressed genotypes (except Perina, Rosso di Pitigliano, and Tondino Liscio), PI decreased significantly, differing from control values after 16 days of stress in the vegetative stage. In the reproductive phase, PI values show the same course as Fv/Fm. The cultivars Costoluto, Canestrino, Fragola and the commercial cultivar Datterino show a decline of PI already at t_1 with a stronger reduction at t_2. The cultivars Giallo and Quarantino differ from the other cultivars when their performance is compared to the control. On the contrary, the cultivars Perina, Pisanello, Rosso, Tondino and the commercial Pearson and Pantano have a PI that markedly decreases after t_1.

At the vegetative stage, all photosynthetic parameters indicate Perina and Cuor di Bue (followed by Fragola, Quarantino, and Tondino) as the cultivars capable of maintaining photosynthetic activity. The reduction of A value in these cultivars is less significant than in the others and does not correspond to an irreversible damage of photosystems. On the contrary, the photosynthetic system is more compromised in the cultivars Pisanello, Canestrino, Giallo, and commercial Datterino.

In the reproductive phase the situation is slightly different. It is straightforward to establish that the most tolerant cultivar is Quarantino because it shows excellent values for all the photosynthetic parameters. It is also equally simple to recognize the most susceptible cultivar, i.e., Fragola, because all photosynthetic parameters are negative or quite different from the control. The classification of other cultivars, such as Perina, on the base of the photosynthetic parameters is more complicated since in the stressed plants they indicate both better or worse condition compared to control.

Plant growth is clearly linked to photosynthesis as the decrease in photosynthesis rate leads to reduced biosynthesis of carbohydrates that are used for growth [54]. In all tomato cultivars at the vegetative phase, a sharp decrease in growth was observed after eight days of stress ($GI_{(1,0)}$), except for Perina, Canestrino, Quarantino and Cuor di Bue. Significant differences have been found for the commercial Pantano and the cultivars Costoluto, Tondino, Giallo and Pisanello (**Figure 2.22a**). For the $GI_{(2,0)}$, the growth index at the end of stress, a significant decrease was shown for all cultivars except for Quarantino, which is still comparable to its own control (**Figure 2.22b**). An earlier work of our group on a subset of the tomato cultivars showed a difference in growth only after 16 days of stress [25]. In that case, however, the study was carried out in a growth chamber under controlled conditions while in this study plants were grown under natural-like conditions, especially in terms of temperature. We believe this might affect the time plants perceive water deficit. However, the cultivars

whose growth was mostly affected by stress correspond when comparing this study to the earlier one. In the reproductive phase at the middle of stress, the $GI_{(1,0)}$ does not show relevant data and values of most stressed cultivars are similar to their own control, except for Pisanello, Giallo and commercial cultivar Pantano, which show a significant decrease in growth (**Figure 2.23a**). At the end of the stress ($GI_{(2,0)}$) drought significantly affected plant growth. In particular, the cultivars Costoluto, Pisanello, Tondino, Cuor di Bue, Datterino and Pantano suffered the most, with a marked difference in growth between control and stressed plants. On the other hand, the Canestrino, Fragola, Giallo, Perina, Quarantino, Rosso and Pearson cultivars showed a slighter difference in growth, but also high standard deviations like all other cultivars, thus data are difficult to interpret (**Figure 2.23b**). However, in general, plant growth is not particularly affected by cultivar type or stress condition because all data decrease in stressed plants compared to controls.

The WUE parameter (A/g_s) expresses the photosynthetic capacity of plants to produce biomass per unit of water consumed [40] and is considered a useful parameter for evaluating the best performing plants under water deficit conditions [42]. In the vegetative phase, Perina and Fragola maintain a high WUE during the stress period. On the contrary, Pisanello shows an extremely low value of WUE already at mid-term stress. In the reproductive phase, Quarantino shows a high WUE value even at t_2, indicating it as the most tolerant cultivar during this growth period. An adequate WUE value is also achieved by the cultivars Tondino, Perina, Pisanello and by the commercial Cuor di Bue, Pantano and Pearson. However, WUE increases in all cultivars during the first days of water deficit and then gradually decreases. Similar responses (i.e., increase of WUE in the first days of stress) were found for grapevine [54], potatoes [55], where a rapid decrease in WUE occurred at the end of stress, and for tomato cultivars in the Mediterranean area of study [40]. The increase in WUE under moderate drought conditions, such as those in the first days of stress, is due to the slow relative decrease of A in comparison to g_s, which decreases more rapidly; for simplicity, we can assume a higher permeability of plants to incoming CO_2 rather than outgoing H_2O.

One approach to increase WUE is changing the stomatal density: indeed, decrease in stomatal density triggers lower levels of g_s in drought-stressed plants with the same photosynthetic activity [43]. In our work, the Pisanello cultivar shows a higher density of stomata when subjected to drought, confirming a higher susceptibility to stress. Exactly the opposite case occurs for Perina, which has a lower stomatal density under stress, implying an adaptation to water deficit. The stomatal density of Quarantino and Fragola is unchanged between control and stressed plants, indicating less susceptibility to stress. By combining all data, we can discriminate the nine local cultivars into those most susceptible to drought and

those most tolerant. We assume that the difference between susceptible and tolerant cultivars is because of drought tolerant cultivars having more efficient and protective mechanisms [17,56].

The data also allowed us to differentiate cultivars on the basis of vegetative and reproductive stages. We used the PCA tool to identify tolerant and susceptible genotypes; PCA has already proved to be useful in many other studies [51,57,58]. Analysis by PCA and the correlogram data-derived dendrogram confirmed the classification of cultivars into two groups (one tolerant and the other susceptible) at the level of vegetative stage. The cultivars Perina and Fragola are those that perform better to drought stress and can therefore be recognized as the most tolerant; on the other hand, the cultivar Pisanello is the most susceptible to drought, while the cultivar Quarantino shows an intermediate behavior. At the reproductive stage, the situation is different. The first PCA revealed that drought affects and distinguishes controls from stressed plants. The second PCA differentiates two groups, and the detailed analysis of all parameters indicates that Quarantino is the most tolerant cultivar, while Fragola is the most susceptible. Clustering does not reflect the groups obtained by PCA. We hypothesize that cultivars at the reproductive growth stage do not exhibit well-standardized behavior. Because clustering was done by referring to plant behavior during the entire stress period and not just at t_2, this affected the distinction into groups. In the reproductive phase, distinction between genotypes occurs just at the end of stress. For this reason, the cluster division obtained by PCA at t_2 is more relevant than the parameter-based clustering during the entire stress period.

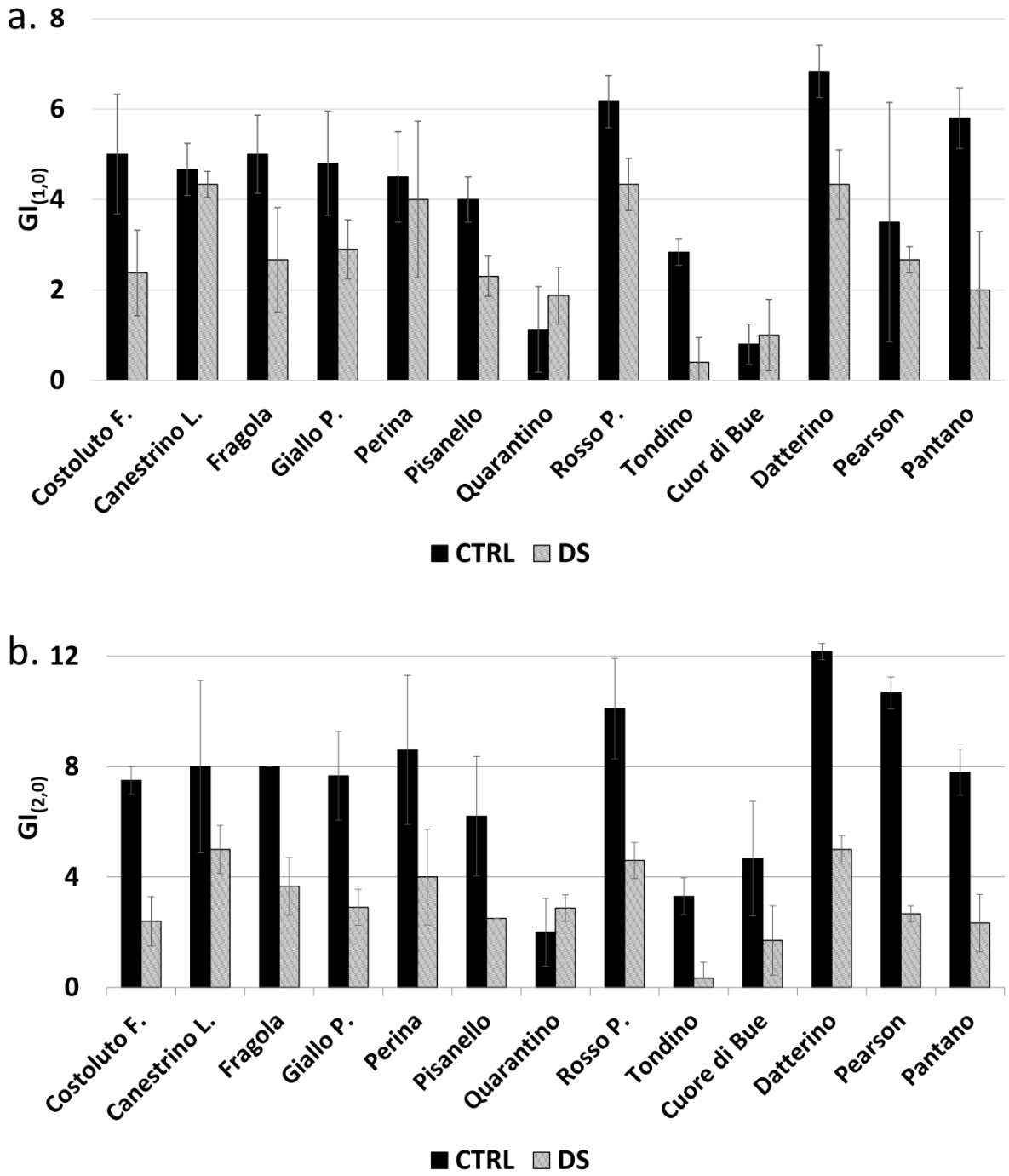


Figure 2.22. Growth Index (GI) for the vegetative phase. Controls (CTRL) are in black while stressed (DS) samples are in stripes. Error bars represent standard deviation of means of values taken on four plants. **(a)** The $GI_{(1,0)}$ indicates the growth between t_0 and t_1. **(b)** The $GI_{(2,0)}$ indicates the growth between t_1 and t_2.

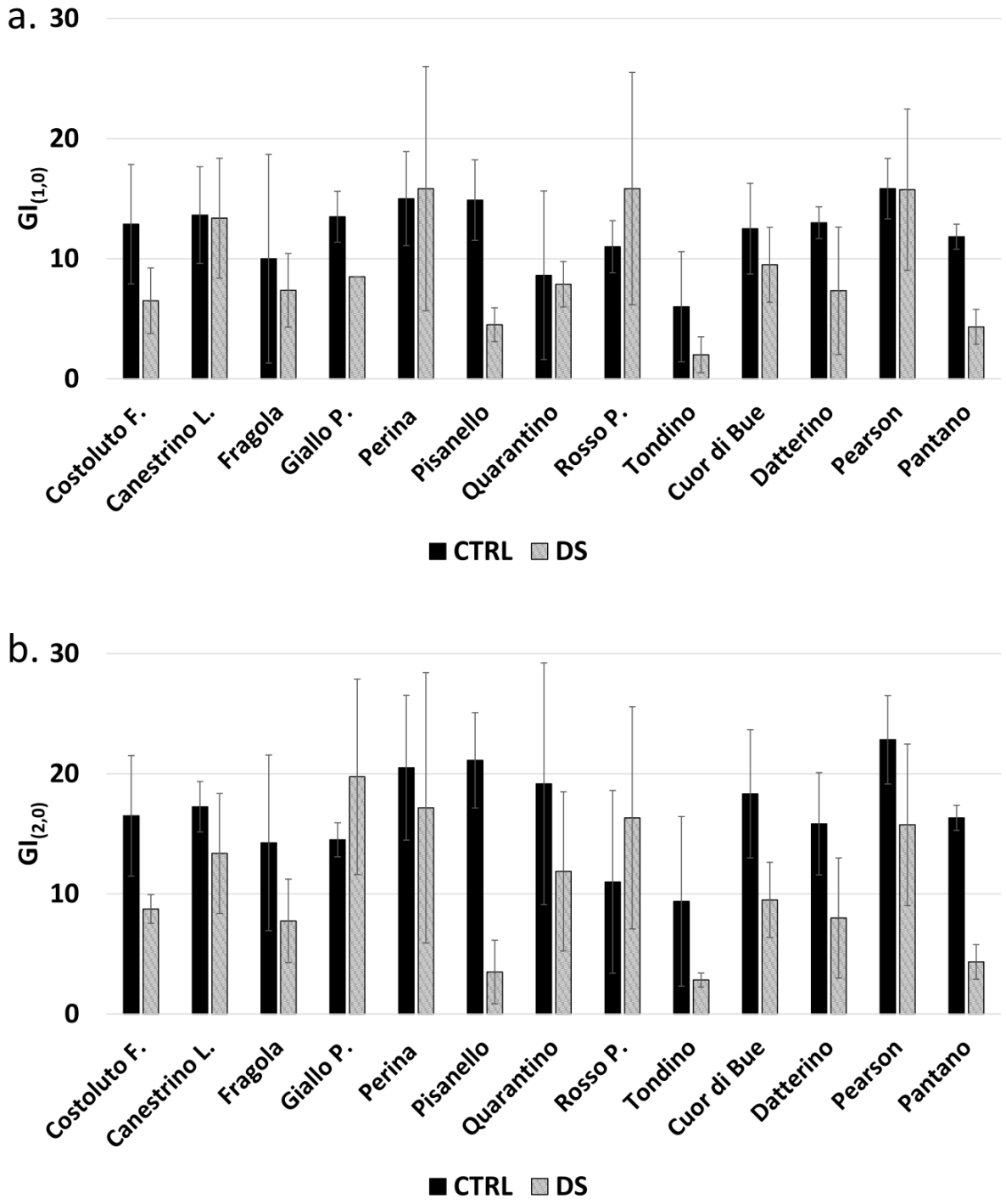
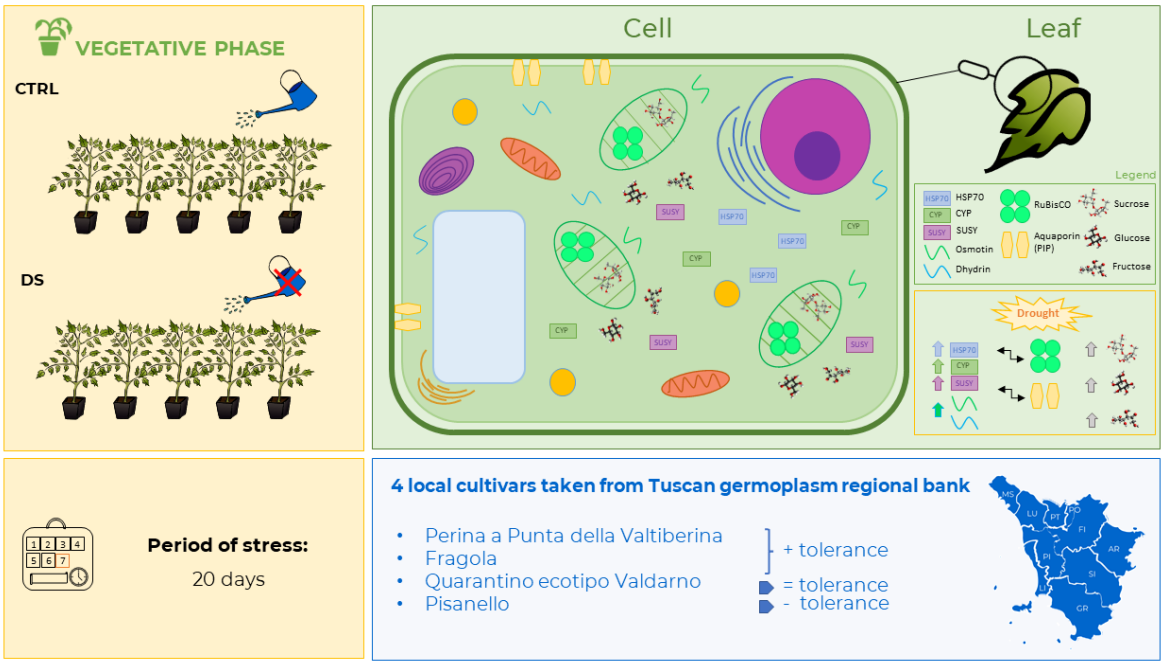


Figure 2.23. Growth Index (GI) for the reproductive phase. Black bars are the control (CTRL) while striped bars are the stressed (DS) samples. Error bars represent standard deviation of means of values taken on four plants. **(a)** The $GI_{(1,0)}$ indicates the growth between t0 and t1. **(b)** The $GI_{(2,0)}$ indicates the growth between t_1 and t_2.

Chapter 3. Distinct tomato cultivars are characterized by a differential pattern of biochemical responses to drought stress

Collaborators: *Marco Romi, Claudio Cantini and Giampiero Cai*



Graphical Abstract: This study aimed to investigate the biochemical mechanisms of plant defense against drought by focusing on specifically involved proteins such as osmotin, dehydrin, and aquaporin (PIP) as well as those involved in the general stress response, such as HSP70 and cyclophilins (CYP). Other proteins involved in sugar metabolism, such as RuBisCO and sucrose synthase (SUSY), were included in our study because it is worth mentioning that sugars also act as osmoprotectants in plant cells. The results of our investigation show crucial differences in biochemical behavior among the selected cultivars and highlight that the more tolerant tomato cultivars adopt quite different biochemical strategies than the more susceptible ones.

3.1. Introduction

For plant organisms, water scarcity is an abiotic stress (drought stress). This adverse condition leads to various damages in plants, including incorrect folding of proteins, alterations in enzymatic functions, and increased production of reactive oxygen species (ROS) [59]. The first response of plants to water deficit consists in the closure of the stomata mediated by abscisic acid (ABA) [30]. ABA plays a key role in the control of ABA-dependent gene transcription, allowing the production of proteins specifically suited to counteract drought stress [60]. In addition to the production of specific hormones able to make plants more tolerant to water stress, other physiological/biochemical defensive activities consist in the production of osmoprotectants (which prevent proteins from denaturing and retain water in the cells) [5] and in enhancing the activity of antioxidant systems, able to reduce the levels of ROS [61].

Among the proteins whose synthesis is induced by ABA are Heat Shock Proteins (HSPs), a class of chaperones involved in protein folding and thus relevant in the defense mechanisms against abiotic stresses [62,63]. HSPs are expressed especially in heat stress conditions, but also in case of drought, salinity stress and pathogen infections [64–66]. HSPs of 70 kDa (HSP70) are more involved in tolerating heat and drought stresses [67].

Cyclophilins (CYPs) is another type of chaperone protein, they are ubiquitous and involved in a wide range of cellular processes [68–70]. CYPs have an enzymatic activity of peptidyl-prolyl cis-trans isomerase by which they catalyze the cis-trans isomerization of the amide bond between a proline residue and the previous amino acid residue, which is essential for the correct folding of proteins [71]. Due to their catalytic activity, CYPs can accelerate the folding of different proteins in response to various biotic and abiotic stresses [72,73].

The synthesis of Late Embryogenesis Abundant (LEA) proteins is also induced by ABA [74]. Dehydrins belong to the family II of LEA proteins and are involved in the plant's response to dehydration and, more generally, to abiotic stresses [75]. They can protect the activity of proteins by preventing their denaturation [76,77]. In addition, they can bind to phospholipids of cell membranes, such as phosphatidic acid, whose level increases in response to ABA [78]. Moreover, in the presence of zinc ions, dehydrins can even bind to DNA, which can therefore be repaired or protected from damage caused by environmental stresses, as observed in Japanese mandarins [79].

Abiotic stresses activate many intracellular signals which lead to the accumulation of osmoprotectants and production of Pathogenesis-related (PR) proteins [80]. Osmotin is a 24 kDa protein belonging to the PR-5 family. In addition to having a significant antifungal and antibacterial activity, it can increase the resistance of plants to various abiotic stresses, such as salt and drought stress [81]. It induces the expression of genes involved in proline biosynthesis, causing its accumulation within cells, thus providing plants with increased tolerance to drought stress [82–85]. Moreover, osmotin can protect chlorophyll and the photosynthetic machinery in conditions of water scarcity [86].

Proteins also important to the efficiency of photosynthesis are the aquaporins. These are known as water and CO_2 transporters [87]. According to their structure and localization, they are classified into five groups, and plasma membrane intrinsic proteins (PIPs) are the ones most involved in CO_2 and H_2O transport. Indeed, some work has shown that overexpression of PIPs in Arabidopsis, rice, or tobacco results in enhanced CO_2 assimilation in leaves [88–90]. Furthermore, overexpression of PIPs in several cultivated plants led to a better response to drought stress [91]. For example, overexpression of a PIP1;2 in bananas

increased tolerance to both drought and salt stress [92]. In tomatoes, overexpression of PIPs also resulted in improved drought stress tolerance [93,94].

Other biochemical adaptations of plants consist of the regulation of the photosynthetic process. Photosynthesis might be limited due to the scarce availability of substrates such as H_2O and CO_2. In the dark phase (the Calvin cycle), the leading enzyme is Ribulose 1,5-Bisphosphate Carboxylase/Oxygenase (RuBisCO) which catalyzes the carboxylation reaction that initiates the fixation of CO_2. The catalytic activity of RuBisCo progressively decreases with increasing duration and severity of the drought conditions [95]. This can be explained by a partial loss of the protein during stress [96]. The degradation of RuBisCO generates fragments of the enzyme, which can be detected by two-dimensional electrophoresis [97]. Furthermore, RuBisCO, through the carboxylation reaction, generates substrates for the synthesis of sucrose, which is crucial for the growth of plants. Any damage to RuBisCo activity or quantity has consequently significant impacts on plant biomass.

At the level of sink tissues, sucrose can enter cells in at least two ways. It can be split by the activity of cell wall invertases into glucose and fructose, which in turn are transported into cells by monosaccharide transporters [98]. Sucrose can also enter cells directly through the activity of sucrose transporters. Once imported into cells, sucrose can be cleaved by both soluble invertase but also by sucrose synthase (SuSy) with energetically different results [99,100]. The SuSy activity is relevant under drought stress conditions because of the splitting of sucrose, which increases the concentration of hexose sugars. The latter are precious osmoprotectants and detoxifying molecules with a key role in plant's protection against oxidative stress [101].

In this study, we analyzed the biochemical response of tomato plants subjected to drought stress conditions. Tomato is a widely grown plant that can suffer dramatically from water stress conditions [24]. Although the genetic response of tomatoes to drought stress is partly known [102–104], the involvement of specific proteins in the defense of water scarcity has yet to be carefully evaluated. It should also be considered that different tomato cultivars may show dissimilar biochemical responses in relation to their specific genetic background. We have already observed that locally adapted Tuscan tomato cultivars may have different responses in physiological terms (Chapter 2.) [25,105], as well as in the content of polyphenols and antioxidants at the fruit level (Chapter 4.) [106]. The aim of this work was to test the hypothesis that drought susceptibility or tolerance in tomato cultivars is based on a different production of metabolic proteins, such as RuBisCO and sucrose synthase (SuSy) that regulate the level of osmoprotective sugars, and a simultaneous change in the content of

proteins more involved in the stress response, such as HSP70, cyclophilins, osmotin, dehydrin and aquaporin.

3.2. Materials and methods

3.2.1 Growth conditions of tomato plants and stress treatment

The plants studied in the present work are a subset of the thirteen tomato cultivars previously analyzed (Chapter 2.) from a morpho-physiological point of view; therefore, plants followed their same growth and drought stress conditions (Chapter 1.) hereby briefly summarized. For each cultivar, 10 plants were studied during the vegetative phase, five plants were used as control (CTRL) and five subjected to drought stress (DS). The stress condition was maintained for 16 days and consisted in complete watering withdrawal; the CTRL group was kept in a fully irrigated regime for the whole period. From the morpho-physiological results it was possible to identify the following four cultivars of interest.

- Perina and Fragola, the most tolerant cultivars,
- Quarantino, the cultivar with medium tolerance,
- Pisanello, the most susceptible.

The analysis in this chapter was carried out on the above four cultivars. Biochemical aspects related to proteins involved in the defense mechanisms against drought stress were investigated. All samples were taken at the final stress phase (after 16 days of drought stress) and were immediately stored at -80 °C.

3.2.2. Protein extraction

Protein extraction was performed as described from Faurobert [107]. Leaves were ground in liquid nitrogen, 1 g of sample was weighed and resuspended in 3 mL of Extraction Buffer (500 mM Tris-HCl, 50 mM EDTA, 700 mM sucrose, 100 mM KCl, 2% ß-mercaptoethanol and 1 mM of protease inhibitors, pH 8.0). Samples were vortexed and incubated on ice for 10 min with gentle agitation to allow for sample resuspension. An equal volume of Tris-buffered phenol (Amresco-Interchim, Biotechnology Grade) was then added, vortexed for 3-5 min and incubated for 10 min at room temperature (RT) with gentle agitation. The mixture was centrifuged at 5500 g for 10 min at 4 °C and the upper phase was taken to which 3 mL of Extraction Buffer were added; samples were vortexed for 3 min and centrifuged at 5500 g for 10 min at 4 ° C. The upper phase was collected and supplemented with four volumes of precipitation solution (0.1 M ammonium acetate in methanol), mixed by inversion, and incubated at -20 °C for at least 4 hours or overnight. The mixture was centrifuged at 5500 g

for 10 min at 4 °C and the supernatant was removed. The pellet was washed with the precipitation solution, centrifuged at 5500 g for 5 min at 4 °C and the supernatant was removed. This last step was repeated twice. The last pellet was washed with cold acetone, centrifuged at 5500 g for 5 min at 4 ° C and the supernatant was removed. Samples were then dried at RT under a fume hood for 10 min. Afterwards, 100 µL of 0.2 M NaOH were added and samples incubated for 2 minutes for more effective solubilization. A volume of 200 µL of LSB1X for 1-D electrophoresis and 200 µL of Rehydration Buffer (RB) for 2-D electrophoresis were added to the samples. Finally, samples were centrifuged for 15 minutes at 10000 g at RT, the supernatants were collected, and the protein concentration was calculated using the 2-D Quant Kit (GE, USA).

3.2.3. 1-D Electrophoresis and immunoblotting

Electrophoresis was conducted on 10% bis-Tris SDS-PAGE [108] at pH 6.5-6.8. Volumes containing 30 µg of protein from the CTRL and DS samples of the four cultivars were loaded into each gel. Electrophoresis was carried out on a Criterion cell (Bio-Rad Laboratories, Segrate, Italy) equipped with a Power Pac BioRad 300 at 200 V for approximately 45 min. XT MOPS (Bio-Rad Laboratories, USA) was used as a running buffer. Transfer of proteins from gels to nitrocellulose or PVDF (for osmotin and dehydrins) membranes was performed using a Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Segrate, Italy) according to the manufacturer's instructions (using the setting for low molecular weight proteins). After blotting, membranes were blocked overnight at 4 °C in 5% ECL Blocking Agent (GE HealthCare Dornstadt, Germany) with 0.1% Tween-20 in TBS (20 mM Tris pH 7.5, 150 mM NaCl). After washing with 1X TBS, membranes were incubated with the primary antibody for 1 h (**Table 3.1**). Subsequently, membranes were washed twice with 1X TBS and then incubated with peroxidase-conjugated secondary antibodies for 1 h (**Table 3.2**). After rinsing the membranes with 1X TBS, the immunological reactions were visualized with ClarityTM Western ECL Substrate (Bio-Rad Laboratories, United State). Images of blots were acquired using a Fluor-S apparatus (Bio-Rad Laboratories, Segrate, Italy) and analyzed with the Quantity One software (Bio-Rad Laboratories, Segrate, Italy). Finally, densitometric analysis was performed with the same software for a relative quantitative evaluation of band intensity (expressed as Integrated density).

Table 3.1. List of primary antibodies used in this work

Name	Source	Antigene	Type	Dilution
Anti-HSP70 (ADI-SPA-820-D)	Enzo Life Sciences	HSP70	Mouse monoclonal	1:5000
Anti-Cyclophilin (CYP)	[109]	CYP	Rabbit polyclonal	1:3000
Anti-Osmotin (AS19 4336)	Agrisera	Osmotin	Rabbit polyclonal	1:1000
Anti-Dehydrin (AS07 206A)	Agrisera	Dehydrin	Rabbit polyclonal	1:1000
Anti-Aquaporins (AS09 489)	Agrisera	Aquaporins	Rabbit polyclonal	1:1000
Anti-RuBisCO	Agrisera	RuBisCO	Rabbit polyclonal	1:10000
K4 anti-SuSy	[110]	SuSy	Rabbit polyclonal	1:1000

Table 3.2. List of secondary antibodies used in this work

Code	Source	Antigene	Type	Dilution
#1706515	Bio-Rad	Anti-rabbit IgG	Polyclonal	1:3000
#1706516	Bio-Rad	Anti-mouse IgG	Polyclonal	1:3000

3.2.4. 2-D electrophoresis and immunoblotting of RuBisCO

Samples were supplemented with 18 mM DTT and 10% IPG Buffer, then brought to the volume of 200 µL with RB to obtain a protein concentration of 1.5 mg/mL. Samples were loaded into the Immobiline DryStrip Reswelling Tray (Pharmacia Biotech) and Readystrip IPG pH 5-8 (Bio-Rad) were placed on top of samples. After 30 minutes, strips were covered with mineral oil (Bio-Rad) and allowed rehydrating for 24 hours. Strips were then positioned on the Focusing Tray (Bio-Rad) and were covered with mineral oil; the tray was positioned in the Protean IEF Cell (Bio-Rad) and run was carried out at 20 °C following an increasing voltage program: from 0 to 500 V in 1 h, 500 V constant for 1 h, from 500 V to 4000 V in 2 h, 4000 V for 2 h, from 4000 V to 8000 V in 2 h, 8000 V constant up to 15000 V/hour, from 8000 V up to 500 V in 30 min and 500 V until strips were removed. For separation in the second dimension, strips were washed with Equilibration Buffer 1 (130 mM DTT, 6 M Urea, 2% SDS, 0.375 M Tris-HCl pH 8.8 and 20% glycerol) for 10 minutes and then with Equilibration Buffer 2 (130 mM Iodoacetamide, 6 M Urea, 2% SDS, 0.375 Tris-HCl pH 8.8 and 20% glycerol) for 10 minutes. At the end, strips were placed in the well of 10% Criterion XT PreCast gel (Bio-Rad) and immobilized with agarose gel. The electrophoretic run was performed in a Criterion Cell (Bio-Rad) at 200 V for 1h using XT MOPS buffer (Bio-Rad). Gels were transferred to nitrocellulose membranes for immunoblotting. The membranes were blocked overnight at 4 °C with 5% ECL Blocking Agent (Bio-Rad) in TBS (20 mM Tris pH 7.5, 150 mM NaCl) plus 0.1% Tween-20. Membranes were incubated for 1 h at RT with a primary anti-RuBisCO antibody, diluted 1:10000 (Agrisera). After washing in 1X TBS, membranes were incubated for 1 h with a secondary goat anti-rabbit antibody, diluted 1:3000 and conjugated to peroxidase. Visualization of the immunological reaction was performed

using ClarityTM Western ECL Substrate (Bio-Rad Laboratories, United State). Images of blots were acquired using a Fluor-S apparatus (Bio-Rad Laboratories, Segrate, Italy) controlled by Quantity One software (Bio-Rad). For the comparison of immunoblots, the PDQuest software (Bio-Rad, version 8.0) was used.

3.2.5. Analysis of soluble sugars

High Pressure Liquid Chromatography (HPLC) was used for the analysis of sugars (pectins, sucrose, fructose, and glucose). Briefly, 100 mg of leaf samples powdered with liquid nitrogen were added to 1 mL of distilled H_2O. Samples were homogenized by Ultra-Turrax® T-25 basic (IKA®-Werke GmbH & Co. KG, Staufen im Breisgau, Germany), centrifuged at 3000 RCF for 5 min; the supernatants were transferred to 2 mL Eppendorf® tubes and then centrifuged again at 12000 RCF for 5 min (Eppendorf® Microcentrifuge 5415D, Hamburg, Germany). Samples were filtered (0.45 µm) and 20 µL of each extract was injected into a Waters Sugar-Pak I ion exchange column (6.5 × 300 mm) at a temperature of 90 °C. The mobile phase consisted of MilliQ H_2O (pH 7) with a flow of 0.3 mL min^{-1}. The overall duration of the separation was 30 min. Identification of components was done using a Waters 2410 refractive index detector by comparing the retention times with those of reference standards. The experiment was conducted in three technical replicates for each sample. Finally, the mean and standard deviation were calculated. To verify the significance of the data obtained, the t-test (* $p \leq 0.05$, ** $p \leq 0.01$) were carried out.

3.2.6. Phosphoprotein profiling

Pro-Q® Diamond Blot Reagent & Buffer (Thermo Fisher) was used to highlight the phosphoprotein patterns. Proteins were separated by electrophoresis and then transferred to PVDF membranes (pre-moistened in methanol). After electroblot, membranes were allowed to dry completely. Proteins were fixed on membranes by dipping it face down in 25 mL of Fix Solution (7% acetic acid, 10% methanol) for 10 minutes. Membranes were washed by immersion in 25 mL of dH_2O for 5 minutes (three times). Proteins were stained by immersing the membrane in 25 mL of the diluted Pro-Q® Diamond Phosphoprotein Blot solution for 15 minutes. Membranes were de-stained by washing them in 30 mL of Destain solution (50 mM sodium acetate, pH 4.0, 20% acetonitrile) for 5 minutes (three times). Fluorescent phosphoproteins could be visualized by the Fluor-S apparatus (Bio-Rad Laboratories, Segrate, Italy) by illuminating membranes with UV light using a 615 nm bandpass filter; exposure times were 10-30 seconds. The resulting electrophoretic lanes were scanned by Quantity One software (Bio-Rad).

3.3. Results and Discussion

In this chapter, we focused on four tomato cultivars characteristic of the Tuscany Region (Italy). This study is based on the previous chapter [105] in which a larger number of cultivars had been selected and analyzed for drought stress tolerance. The previously collected data allowed defining a tolerance/susceptibility profile of the various tomato cultivars. This allowed the identification of a few cultivars of more interest, i.e., the four that are under study in this chapter. In fact, they have been selected to be the most resistant (Perina and Fragola), the most susceptible (Pisanello) and that one with intermediate resistance traits (Quarantino). The choice to focus the analysis on four tomato cultivars as opposed to the 13 previously studied should not be seen as reducing the value of this thesis, but as an attempt to focus on cultivars with distinct characteristics. The protein analysis, which is in addition to the previously performed studies, helps to define the tolerance/susceptibility profile of the four selected cultivars. We chose to analyze proteins involved in general stress responses (such as HSP70 and cyclophilin), in drought resistance (such as dehydrins, osmotin, aquaporins), and in more strictly metabolic aspects (RuBisCO and sucrose synthase).

3.3.1. Levels of HSP70 increase after drought stress

The antibody used in this study was directed against HSP70 purified from human HeLa cells. It recognizes protein homologues in plants and its efficiency has been confirmed in citrus and pepper plants [111] as well as in leaves of olive trees [112]. **Figure 3.1A** shows the blotting with anti-HSP70 antibody. Expression of the protein is detectable in each of the 8 samples examined. This result shows constitutive basal-level expression of HSP70 even in samples from plants that were irrigated normally. **Figure 3.1B** shows the result of densitometric analysis carried out on the HSP70 bands detected after immunoblotting. It can be observed how HSP70 levels increase in stressed plants compared with controls for each cultivar. This finding is not surprising because many studies have reported that under abiotic stress conditions the content of HSP70 increases to protect the structure of proteins and cell membranes as well as to counteract the increase in ROS levels [63,66]. In addition, a direct correlation has been observed between drought stress and the accumulation of HSP70 (Augustine et al.,2015).

Comparing the expression of HSP70 in DS samples of the four cultivars, the increase in protein levels from the most tolerant cultivar to the most susceptible cultivar is evident. The cultivar Perina showed lower expression levels than the others, especially compared to Pisanello. It can be observed that even in the CTRL samples, the expression levels of HSP70 increased from the most tolerant cultivar to the most susceptible one. Furthermore, in the

control samples, basal levels of HSP70 in Perina are lower than in Quarantino or Pisanello. Therefore, results cannot be interpreted only in terms of quantity, but it is necessary to focus on the increase of these proteins from CTRL to DS. Both the cultivars Perina and Fragola increase HSP70 expression more than 200 % in DS plants compared to those in the CTRL group, whereas in Quarantino, HSP70 levels increase about 50 %. In contrast, Pisanello, while having a more abundant basal expression of HSP70, shows a smaller increase than the other cultivars. Therefore, it is inferred that the expression of HSP70 in Pisanello under drought stress is the lowest among all cultivars analyzed.

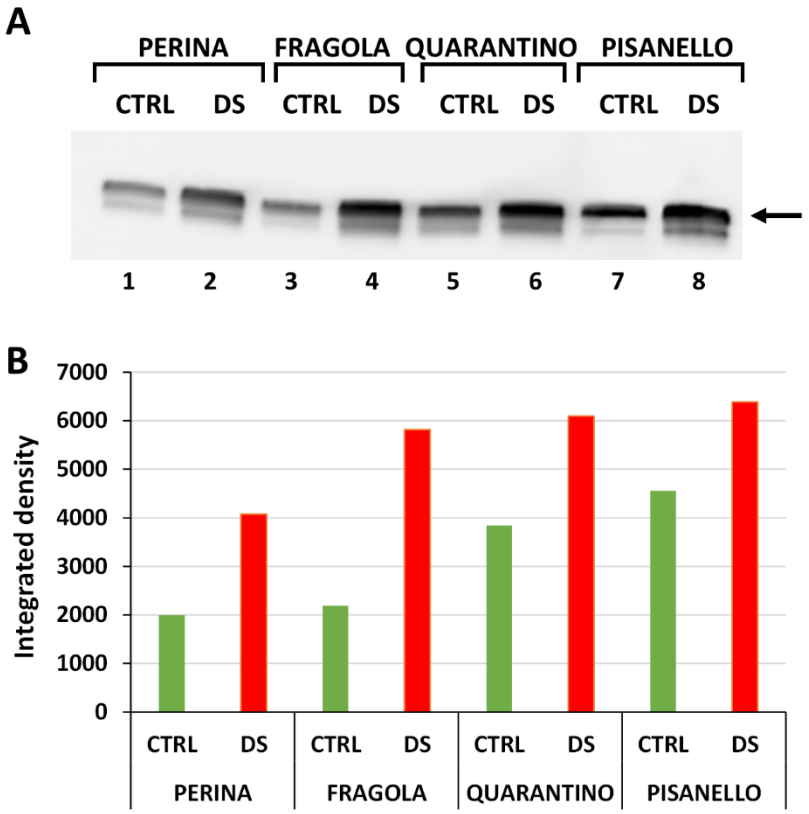


Figure 3.1. Content of HSP70 in leaves of the four tomato cultivars from both control (CTRL) and stressed (DS) samples. **(A)** Immunoblotting with anti-HSP70 antibody. The arrow indicates the band with molecular weight between 70-75 kDa. Lane 1, Perina CTRL; lane 2, Perina DS; lane 3 Fragola CTRL; lane 4, Fragola DS; lane 5, Quarantino CTRL; lane 6 Quarantino DS; lane 7, Pisanello CTRL; lane 8, Pisanello DS. Here and in all subsequent gels an equal amount of protein (40 **µ**g) was loaded into all lanes. **(B)** Quantitation of the relative content of individual bands in different samples. Green bars indicate control samples, red bars those that are drought stressed.

Consistent with morpho-physiological analysis (Chapter 2.) [105], the cultivars Fragola and Perina experience much less lack of water as stress due to a more efficient molecular response. An opposite situation occurs for Pisanello, which shows unstable and ineffective responses to drought stress from the first days of treatment. These results confirm the protective role of HSP70 in drought and osmotic stress, demonstrating how the most

tolerant tomato cultivars respond with increased expression of this protein to restore cell function and recover from drought stress. An increase in this protein has been repeatedly observed under abiotic stress conditions, for example in tomatoes subjected to high temperatures [113] and in sugarcane under drought stress conditions [67]. Although the increase in HSP70 under abiotic stress is not actually surprising, the difference observed in the analysis between several tomato cultivars is noteworthy; this again highlights how different genotypes can exhibit different responses in terms of chaperone proteins.

3.3.2. Cyclophilin levels also trend upward in drought-stressed cultivars

The antibody against cyclophilins (CYPs) was raised against a 172-residue polypeptide of *Solanum sogarandinum* O. [114]. Our workgroup has also successfully tested it on *Pyrus* L. pollen [109]. **Figure 3.2A** shows that the anti-cyclophilin antibody cross-reacts with at least three polypeptides (at 25, 23 and 15 kDa) in the stressed and control plant samples of each cultivar.

As in the previous case, blottings were subjected to densitometric analysis (**Figure 3.2B**). This analysis shows that cyclophilin levels increased in drought-stressed samples compared to controls. The levels of all three cyclophilin bands expressed by the DS group of the cultivar Perina increased relative to CTRL. The same occurs for the cultivar Fragola, although less pronounced. In contrast, in the cultivar Quarantino the intensity of bands in the DS sample decreases relative to CTRL. Finally, the cultivar Pisanello behaves in a peculiar way, drastically decreasing the amount of the 25 kDa and 23 kDa bands but increasing the amount of the 15 kDa band.

The cultivars Perina and Fragola were selected in previous studies based on their tolerance to drought stress; however, the cultivar Quarantino showed both traits of tolerance and susceptibility [25,105]. The higher expression of cyclophilins in drought-stressed cultivars Perina and Fragola compared to levels in Quarantino is consistent with the study by Barik [72]. A correlation between cyclophilins and resistance to abiotic stresses has been described in several cases; for example, in Arabidopsis, cyclophilin encoded by the ROC3 gene is positively correlated with resistance to drought stress, as cyclophilin Roc3 appears to regulate the levels of reactive oxygen species and stomatal opening [115]. A correlation between drought stress and increased expression of specific cyclophilins was also found in wheat [116].

The role of cyclophilins in resistance to drought, as well as to other abiotic stresses, is further supported by the overexpression of a pigeon pea gene in transgenic *Arabidopsis* plants, which consequently acquired increased tolerance to abiotic stresses [117]. Also in

sorghum, application of drought stress induced expression of a 20-kDa cyclophilin in a cultivar-dependent manner [118]. In rice, drought and salt stress induce considerable expression of a specific cyclophilin; furthermore, overexpression of this protein in transgenic rice and Arabidopsis plants increased drought tolerance [119]. Therefore, it can be hypothesized that the tolerance of the tomato cultivars is due to a high expression of cyclophilins, which, through their enzymatic activity, accelerate the process of protein folding under stress conditions. The Pisanello cultivar has been reported to be the most susceptible to drought stress. It is likely that the low stress tolerance could be due to down-regulation of transcription of the 25- and 23-kDa cyclophilin genes, which could be important protective factors.

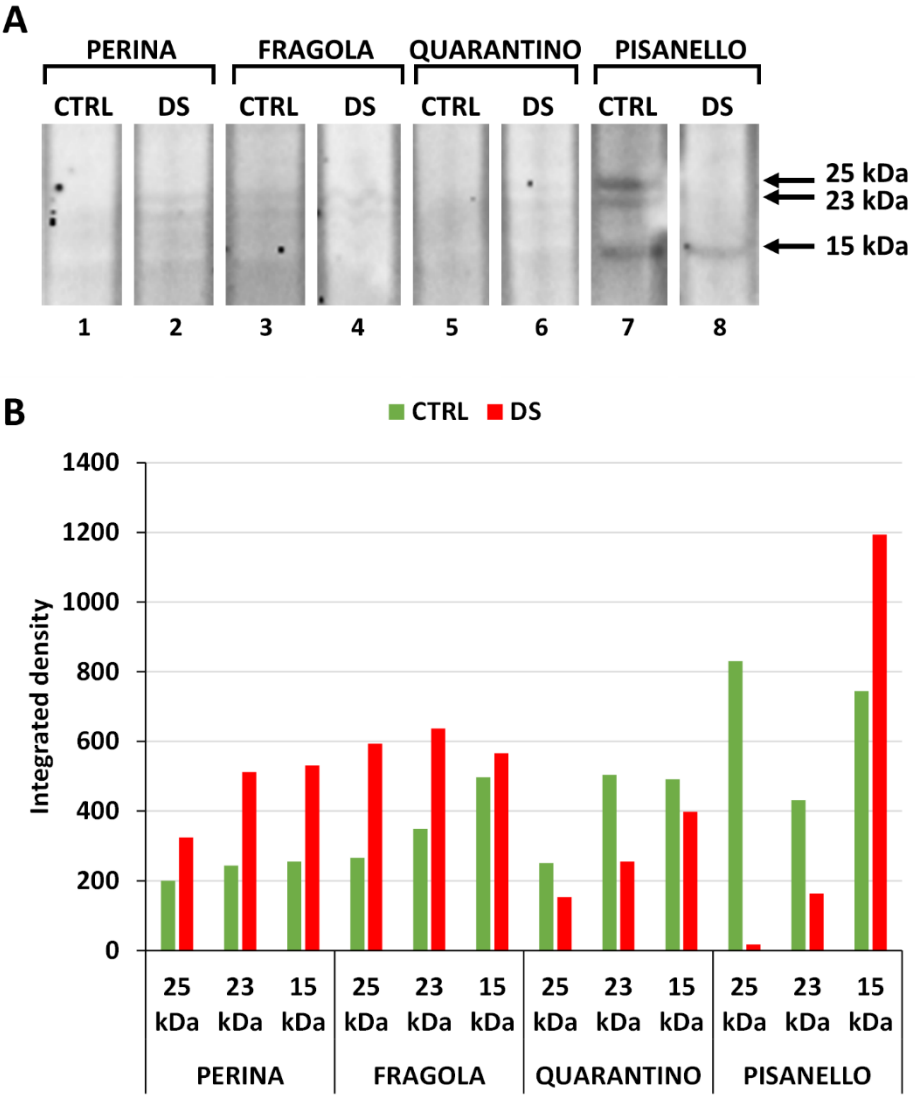


Figure 3.2. Content of the cyclophilin family in the four tomato cultivars in both control (CTRL) and drought-stressed (DS) samples. **(A)** Immunoblot analysis of cyclophilin with the three bands identified at 25, 23, and 15 kDa. Lane 1, Perina CTRL; lane 2, Perina DS; lane 3 Fragola CTRL; lane 4, Fragola DS; lane 5, Quarantino CTRL; lane 6 Quarantino DS; lane 7, Pisanello CTRL; lane 8, Pisanello DS. **(B)** Quantitative analysis of the relative content of the three cyclophilin bands in the different samples. In green the control samples, in red the stressed samples.

3.3.3. Cultivars under drought stress exhibit a significant increase in dehydrin levels

The antibody used binds to the dehydrin family, which are proteins involved in protective reactions against dehydration. Specifically, the antibody binds to the k-segment peptide sequence (TGEKKGIMDKIKEKLPGQH) conserved in a wide range of different plant species. The reactivity of this antibody has also been confirmed in *Solanum lycopersicon* L., as well as in other species such as *Pistacia vera* L. and *Cucumis sativus* L. [120–122].

Figure 3.3A shows the blotting performed with the anti-dehydrin antibody; five polypeptide bands corresponding to the molecular weights of 33.7, 30, 22, 17.8, and 15 kDa can be highlighted. The presence of numerous bands was predictable because dehydrins are a family of proteins classified into at least five different structural types based on the number and order of the three conserved distinctive motifs, the K, Y, and S segments [123]. The blot shows that expression increases significantly under drought stress conditions. This result is expected and consistent with findings by Borovskii [124] who demonstrated the relationship between dehydration and increased dehydrin levels.

Furthermore, it is evident that the increase in dehydrin expression in stressed samples is inversely proportional to the tolerance of the cultivar toward drought stress (**Figure 3.3B**). The intensity of bands increases significantly from the cultivar Perina (identified as the most tolerant in previous studies) to the cultivar Pisanello, which on the contrary was the most susceptible to stress [105]. This result can be compared with that obtained by Velasco-Conde [125], in which a drought-resistant variety of pine (*Pinus pinaster*) was shown to express higher amounts of dehydrins when subjected to drought stress. Dehydrins are proteins traditionally associated with resistance against drought and other stressful conditions. In fact, they are capable of increasing water retention capacity, have positive effects on chlorophyll content and preserve the photosynthetic machinery, as well as increasing detoxification of reactive oxygen species and promoting the accumulation of compatible solutes [126]. Supporting data were also obtained in soybean; dehydrins of 28 and 32 kDa were found after water deprivation in developing seeds, but not in seeds from well-watered plants [127]. A case comparable to our findings in tomato was described in wheat, where analysis of several cultivars revealed that a specific 24-kDa dehydrin accumulated in distinct cultivars under water stress, whereas no accumulation was detected in control wheat plants [128]. Different soybean varieties under drought stress also showed distinct accumulation of specific antibody-detected dehydrins, again emphasizing that varietal response can be quite distinctive [129]. In the case of tomato cultivars, Pisanello (the most susceptible) appears to require higher amounts of dehydrins to cope with stress damage. On the contrary, the most tolerant genotypes do not suffer particularly severe damages and produce less dehydrins than the most

susceptible cultivars. Most notably in the case of Perina and Fragola, an increase in the 33.7 and 30 kDa bands is noted, supposedly the dehydrins most used to control drought stress.

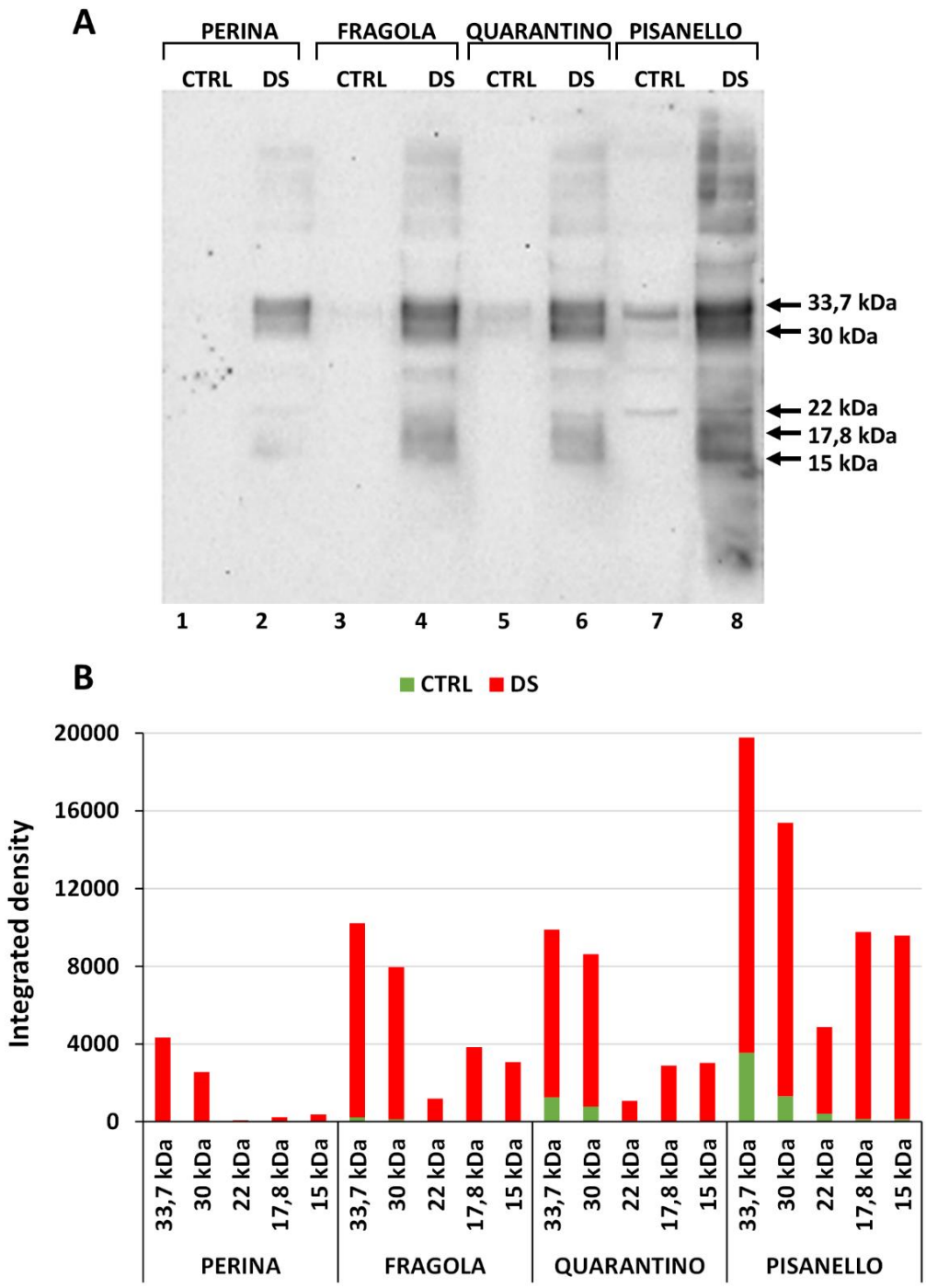


Figure 3.3. Content of dehydrins in both control and stressed plants of the four tomato cultivars. **(A)** Immunoblotting in leaves of the four tomato varieties analyzed. Lane 1, Perina CTRL; lane 2, Perina DS; lane 3 Fragola CTRL; lane 4, Fragola DS; lane 5, Quarantino CTRL; lane 6 Quarantino DS; lane 7, Pisanello CTRL; lane 8, Pisanello DS. The major dehydrins identified have molecular weights of 33.7, 30, 22, 17.8, and 15 kDa. **(B)** Relative content of dehydrins in the four tomato cultivars in both control (CTRL, in green) and drought-stressed (DS, in red) samples. Please note that in this graph the green bars of control samples are superimposed on the red bars of stressed samples.

3.3.4. Osmotin levels increased only in the Pisanello cultivar under drought stress

To cope with various abiotic stresses, plants possess several defense mechanisms including the protein osmotin, which belongs to the PR-5 family of pathogenesis-related (PR) proteins [130]. The antibody to osmotin is derived from the *Nicotiana tabacum* L. protein sequence, ranging from amino acid 22 to 246. The predicted reactivity is also on *Solanum lycopersicum* L.

The blot in **Figure 3.4A** shows that only the cultivar Pisanello had detectable levels of osmotin in both the CTRL and DS samples. The other three cultivars, namely Perina, Fragola, and Quarantino, which exhibited higher drought tolerance, did not show immunoreactive bands. It is likely that the Pisanello cultivar, being the most susceptible to drought and the most damaged in terms of photosynthetic apparatus, is the only cultivar to need the expression of osmotin. Indeed, this protein has a protective activity against chlorophyll and the entire photosynthetic apparatus as damaged, for example, by osmotic stress [86].

Densitometric analysis in **Figure 3.4B** showed that the osmotin levels detected in DS samples of the Pisanello cultivar are significantly higher than in CTRL samples. This data is consistent and confirms what has been demonstrated by previous studies [131,132], where osmotin production in tomatoes was found to be induced by endogenous levels of ABA and therefore by severe drought. The importance of osmotin is also demonstrated by transgenesis experiments in which the tobacco osmotin gene was expressed in tomato plants. The results showed increased tolerance to salt and drought stresses in transgenic plants, with higher relative water content, higher chlorophyll, and proline content [81]. The same protective effect of overexpressing the tobacco osmotin gene in tomato plants was also observed in response to cold treatment, suggesting that osmotin is important in all conditions related to a lack of water uptake [133]. The results show that osmotin is a highly discriminating protein for selected tomato cultivars, especially regarding Pisanello, the most susceptible one. It is not clear why only the Pisanello cultivar should express osmotin in a stress-dependent manner. The only reasonable conclusion is that the other more tolerant cultivars do not need to implement this protective mechanism. Only the cultivar Pisanello, which is defective in other responses to drought stress, therefore induces an increased expression of osmotin to counteract the deleterious effects of stress.

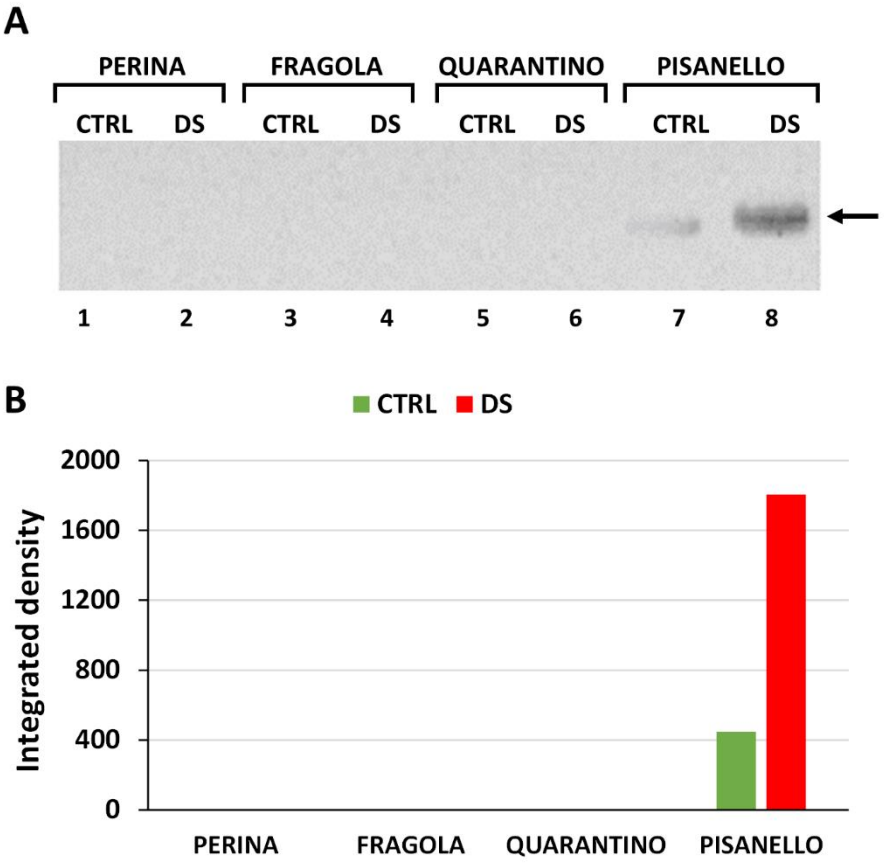


Figure 3.4. Content of osmotin in the leaves of the four tomato cultivars. **(A)** Immunoblotting with the anti-osmotin antibody. Lane 1, Perina CTRL; lane 2, Perina DS; lane 3 Fragola CTRL; lane 4, Fragola DS; lane 5, Quarantino CTRL; lane 6 Quarantino DS; lane 7, Pisanello CTRL; lane 8, Pisanello DS. The arrow indicates the position of the only immunoreactive band. **(B)** Relative quantization of the immunoblotting signal in controls (CTRL, green bar) and stressed samples (DS, red bar).

3.3.5. Aquaporins

Aquaporins are proteins located in the plasma and intracellular membrane and are well known transporters of H_2O and CO_2, the two important substrates for photosynthesis [87]. The immunogen for aquaporin antibody is a KLH-conjugated synthetic peptide derived from N terminus of *Raphanus sativus* L. The peptide is conserved in PIP1;1, PIP1;2, PIP1;3 N-terminus of *Raphanus sativus* L. and in all 5 isoforms (PIP1;1, PIP1;2, PIP1;3, PIP1;4, PIP1;5) of *Arabidopsis thaliana* L. The reactivity in *Solanum lycopersicum* L. is not confirmed but predicted.

The blot in **Figure 3.5A** shows quite different protein expression between cultivars. Three major immunoreactive bands were identified, at 50, 37, and 25 kDa. Bands were not present in all samples but showed an extremely specific distribution with respect to both individual cultivars and molecular weights. Densitometric analysis in **Figure 3.5B** shows a clear increase of the 50-kDa aquaporins in the Perina DS cultivar compared to its control. On the contrary, the cultivar Fragola is characterized by a decrease of both 50- and 37-kDa

aquaporins in stressed samples. Finally, in Quarantino and Pisanello no relevant difference between the CTRL and DS samples is evident.

The role of aquaporins in plants under drought conditions has not yet been fully investigated. Aquaporins were placed in relation to salt stress in tomatoes [134], also in relation to plant-fungus interactions in mycorrhizae [135]. The expression of aquaporins in tomato seeds was also related to the specific irradiation light and the presence of metals such as mercury [136]. However, given their role as water transporters, aquaporins are likely involved in several physiological processes, such as the movement of water and solutes that results in the subsequent control of stomata opening and the maintenance of hydraulic conductance between roots, stems, and leaves [137]. Consequently, a correlation between the expression of aquaporins and plant susceptibility or resistance to drought stress is expected [138]. This is also confirmed by genetic analyses revealing that the expression of aquaporins can be related to a high tolerance to drought stress in tomatoes [139].

Forty-seven genes encoding for aquaporins have been identified in tomatoes. Regarding the family of plasma membrane intrinsic proteins (PIPs), among the five forms recognized by the antibody only one is present in mature tomato leaves, namely PIP1;3. The PIP1;1 protein is strongly expressed in roots and fruits, PIP1;2 in the young leaf and root, PIP1;5 only during fruit development, and PIP1;4 is not present in tomatoes [140]. In addition to the role as water carriers, PIP family members facilitate the diffusion of CO_2 in the mesophyll [88,90]. Considering the data and results obtained, it can be concluded that the higher expression of PIPs in the Perina cultivar might allow for higher stress tolerance; this is likely related to increase of both CO_2 and H_2O transport and more efficient photosynthesis. This hypothesis is also strengthened by work on transgenic tomato plants expressing a drought-inducible aquaporin gene PIP1;3, which derived from *Malus domestica* Borkh [94]. These plants exhibited a slower rate of water loss than the wild type and stomata closed faster to respond to drought.

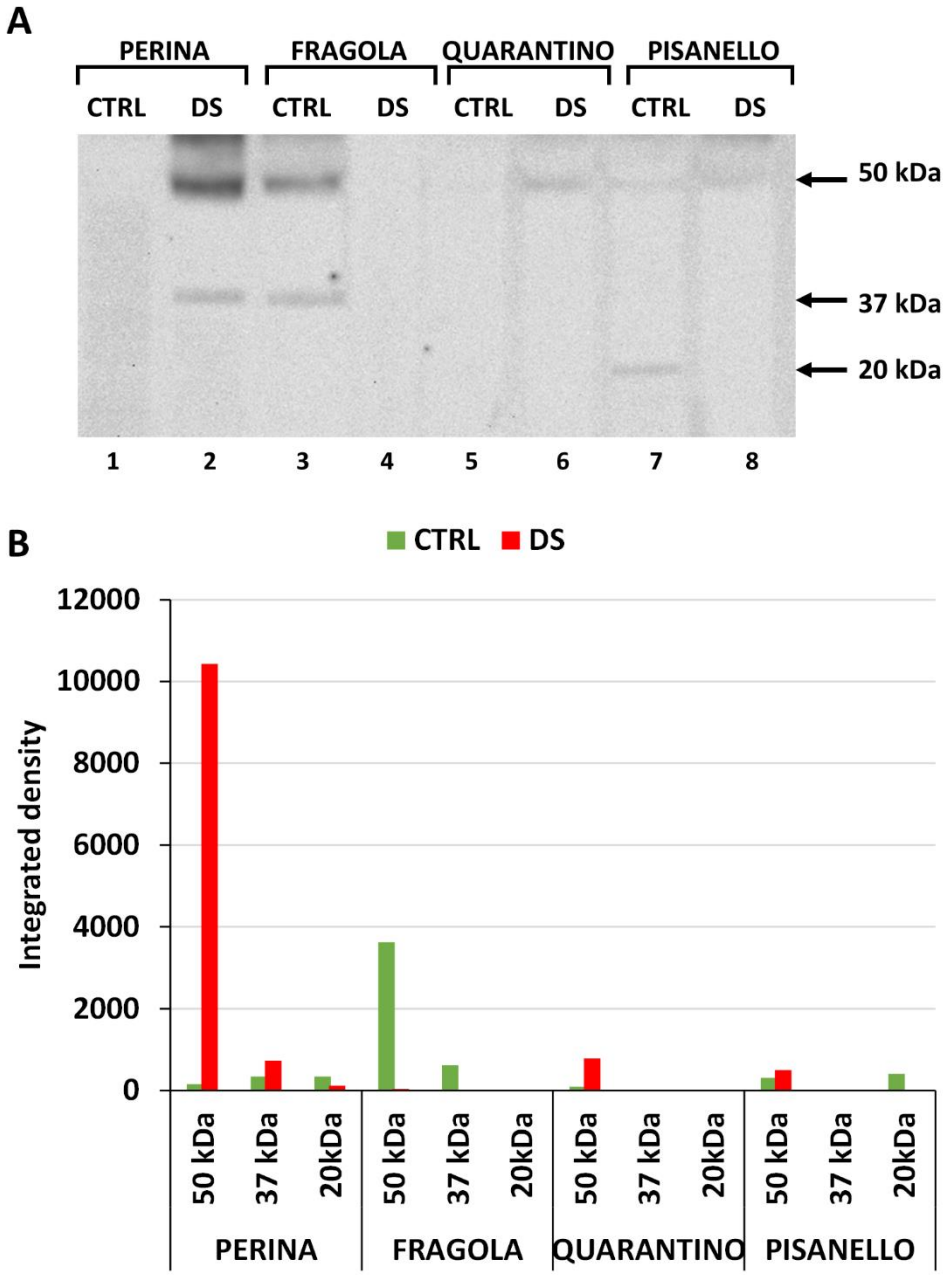


Figure 3.5. Aquaporin content in leaves of the four tomato cultivars under both control and stressed conditions. **(A)** Immunoblotting with anti-aquaporin antibodies in the four cultivars, control samples (CTRL), and stressed samples (DS). On the right, molecular weights of the three main bands identified. **(B)** Relative quantization of blotting expressed as integrated density (y-axis). Green bars indicate control samples, red bars indicate drought-stressed samples.

3.3.6. RuBisCO levels decrease significantly in the Pisanello cultivar while the four cultivars make differential use of RuBisCO isoforms

The immunogen for the RuBisCO antibody was a synthetic KLH-conjugated peptide preserved in all known plant, algal and cyanobacterial protein sequences. Reactivity was confirmed and predicted on several plant species but not on *Solanum lycopersicum* L. However, the reactivity against tomato was evaluated in a previous work on the Micro-Tom cultivar [141].

Ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is the first enzyme in the Calvin-Benson cycle. It catalyzes the carboxylation reaction that initiates CO_2 fixation. This enzyme can account for up to 50% of the protein content in a leaf. There is no need to emphasize that it is an extremely critical enzyme in the metabolism and life of plants and that any alteration in the levels or activity of this enzyme has major consequences in the production of plant biomass.

Figure 3.6A shows the RuBisCO content in the leaves of the four cultivars as revealed by the antibody reaction. RuBisCO levels seem to increase or only slightly decrease in the most tolerant cultivars. On the contrary, they decrease drastically in the most sensitive cultivar, Pisanello. **Figure 3.6B** confirms the visual data and highlights the drastic drop (about 80%) of RuBisCO in the Pisanello cultivar. As a term of comparison, the study by Hasanagić [142] showed that RuBisCO decreases in tomato leaves after an extended period of drought stress. At the same time, the decrease in intracellular CO_2 concentration caused by stomata closure induces an increase in the oxygenase activity of RuBisCO [4]. To initiate the process of photorespiration, the enzyme uses O_2, which helps to keep the light phase of photosynthesis active. Under drought stress conditions, decreased transcription of genes encoding for minor subunits may occur, thus leading to the loss of enzyme stability [95]. In addition, the catalytic activity of RuBisCo progressively decreases with increasing duration and severity of drought conditions [95], and this may be due to a partial loss of the protein during stress.

Figure 3.6C, on the other hand, is the master blot (or virtual blot) obtained from the sum of all spots present in the four cultivars under both control and drought stress conditions. The virtual blot provides a complete picture of all isoforms in the leaves of the tomato cultivars. The graphs below (**Figure 3.6D-E-F-G**) compare quantitatively the isoforms of RuBisCO in the four cultivars. The analysis revealed eight protein isoforms, but they differed among tomato cultivars. Loss of RuBisCO isoforms is associated with plant susceptibility to drought stress as reported, for example, in wheat [97] and sunflower varieties [143]. This may be explained by the degradation of the most susceptible isoforms or by the fact that plants modulate biosynthetic activity by using the isoforms most adapted to drought conditions. Either of these explanations would imply that RuBisCO isoforms in DS groups correspond to those more resistant to water stress conditions.

Analysis by 2D electrophoresis and immunoblotting on RuBisCO revealed a series of spots, whose total number is shown in the virtual master blot in **Figure 3.6C**. The 8 spots identified are more or less present in every case analyzed, at cultivar level as in the comparison between control and stressed sample. However, some substantial differences

emerge. From a qualitative point of view, the differences between cultivars are minimal. Some cultivars are characterized by spots present only in stressed samples. For example, the cultivar Perina expresses the isoform 9905 only in the stressed sample. Even the cultivar Fragola shows a typical expression with the isoforms 9904 and 9905 represented only in the stressed samples. Others, such as the cultivar Quarantino, lack a specific spot, in this case 9906. Pisanello differs in the fact that some spots, such as 9904, are present only in the stressed sample, while 9905 is represented only in the control sample. Apart from the qualitative aspect, it is also noteworthy that the four cultivars differ in terms of the quality (usage) of individual spots. In the cultivar Perina some isoforms are represented in a corresponding manner between the control and stressed samples while others are more typical of the control sample, such as spot 9902, while 9905 is typical of stressed samples. The cultivar Fragola differs substantially from Perina because spots can be categorized in two ways, those almost exclusive to the control sample (such as 9901, 9902, 9906, 9907) and those exclusive to the stressed sample, such as 9904, 9905 and 9908. The cultivar Quarantino has a behavior similar to the cultivar Perina, while the cultivar Pisanello is similar to the case of the cultivar Fragola with spots almost exclusive to the control sample and two spots (9904 and 9906) exclusive to the stressed sample. It is worth noting, in the case of Pisanello, the absence of spot 9908.

In summary, the data indicate that the 9905 RuBisCO isoform is typical of the most resistant cultivars (Perina and Fragola) and is therefore preferentially used; this isoform is partially expressed in Quarantino and is completely absent in the most susceptible cultivar (Pisanello). RubisCO is an enzyme characterized by several potential co-/post-translational modification sites [144]. It is assumed that upon stress, modifications can generate RubisCO isoforms that are better suited to cope with a demanding situation. In support of this hypothesis, similar work on olive leaves subjected to UV-B stress [112] and a paper on Micro-Tom leaves subjected to heat stress [141] can be cited. In both cases, the stress treatment altered the profile of RuBisCO isoforms resulting in a more targeted use of isoforms, those most capable of functioning in the altered environmental conditions.

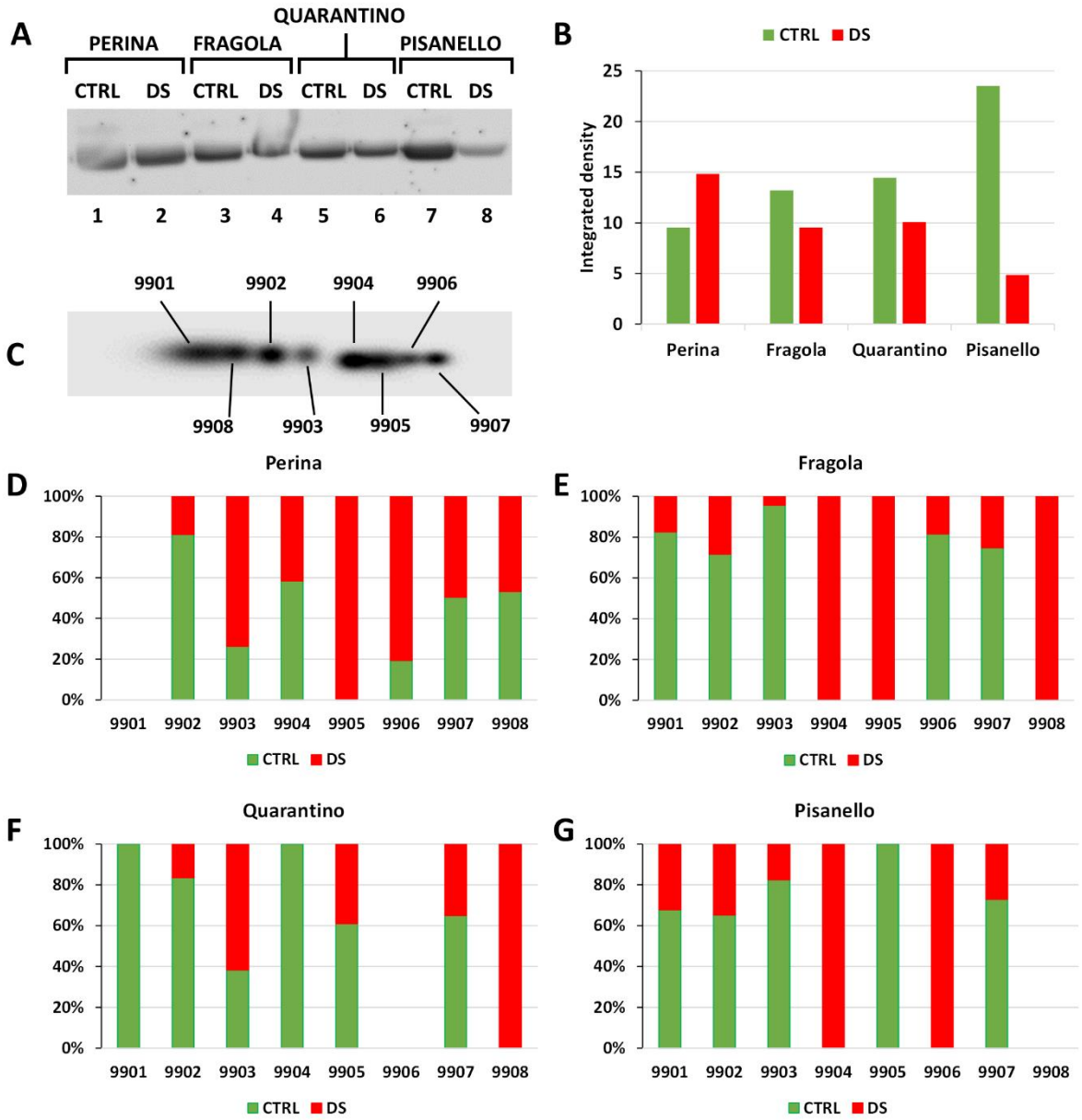


Figure 3.6. Content and isoform composition of RuBisCO in control and stressed plants of the four tomato cultivars. **(A)** 1D immunoblotting of RuBisCO in the four cultivars analyzed. Lane 1, Perina CTRL; lane 2, Perina DS; lane 3, Fragola CTRL; lane 4, Fragola DS; lane 5, Quarantino CTRL; lane 6 Quarantino DS; lane 7, Pisanello CTRL; lane 8, Pisanello DS. **(B)** Relative quantitative analysis of 1D immunoblotting in both control (green bar) and stressed samples (red bar). **(C)** Master (virtual) blot of RuBisCO isoforms after 2D electrophoresis. Each sample contained 300 µg of protein. The blot contains all the spots detected by the anti-RuBisCO antibody, which are numbered automatically by the PDQuest software. Relative percentage content of RuBisCO isoforms in both control and stressed samples of Perina **(D)**, Fragola **(E)**, Quarantino **(F)** and Pisanello **(G)** cultivars. Again, green bars represent control samples and red bars indicate stressed samples.

3.3.7. Pisanello cultivar exhibits the most consistent increase in sucrose synthase

The antibody against SuSy was made in the *Zea mays* L. on the complete protein [145]. SuSy is a key enzyme in sucrose metabolism as it cleaves sucrose producing UDP-glucose and fructose. While fructose can be directed toward respiration, UDP-glucose provides a

conservative form of energy that can be redirected toward both intracellular metabolic processes and in the building of cell wall polysaccharides [146]. Thus, a consistent change in the amount or activity of SuSy impacts multiple aspects of cellular physiology.

As can be seen in **Figure 3.7A**, the expression of SuSy increases in plants subjected to water deprivation, compared to plants treated with normal irrigation. In addition, the signal intensity of SuSy (**Figure 3.7B**) as detected in drought-stressed plant samples increased significantly in the cultivar most susceptible to drought stress (Pisanello) than in those more tolerant. These results are consistent with findings in the literature; because SuSy catalyzes the cleavage of sucrose into its hexose monomers (UDP-glucose and fructose), more susceptible varieties may have a more pressing need to positively regulate SuSy expression to achieve increased levels of free sugars, which act as osmoprotectants under osmotic stress conditions [101]. A direct correlation between water deficiency and sucrose synthase was also observed in selected species of the genus Populus. Although levels of soluble sugars did not show a direct correlation with increased sucrose synthase, it was evident that sucrose synthase increased in response to a water-deficient condition [147].

Indeed, the accumulation of Susy can also be attributed to an increased production of fructose that accumulates in plants under drought stress, as in the case of wheat [148]. However, it is worth noting that drought stress does not always result in an increase in sucrose synthase. For example, in wheat seedlings undergoing water shortage both invertase and sucrose phosphate synthase increase in response to drought, whereas sucrose synthase levels are unaffected between tolerant and susceptible plants [149]. An increase in sugar content and enzyme activity (such as sucrose phosphate synthase, sucrose synthase, and acid invertase) was also observed in soybean cultivars subjected to drought stress. Simultaneously, a decrease in starch, fructose, and glucose content and a parallel increase in sucrose content were found. This supports the evidence that an increase in enzymes that metabolize sucrose does not necessarily result in a subsequent reduction in levels of the disaccharide [150]. Although in the roots (thus not in the leaves) tomato plants can compensate for reduced energy production by targeting the sucrose synthase pathway, which is more energy conservative [151]. Consideration can also be given to the hypothesis that increased sucrose cleavage by SuSy results in higher levels of UDP-glucose, which in turn can be directed toward the synthesis of trehalose, a much-studied component in abiotic stress resistance [152,153].

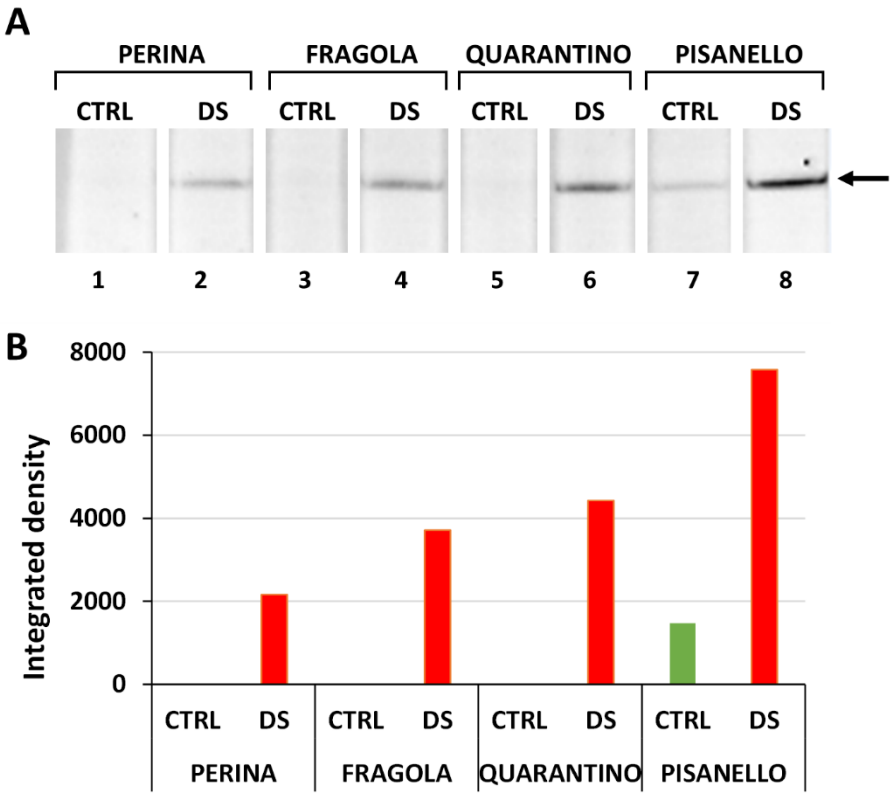


Figure 3.7. Content of sucrose synthase (SuSy) in the leaves of the four cultivars, both in control (CTRL) and in stressed samples (DS). **(A)** Immunoblotting; the arrow indicates the position of the cross-reactive SuSy. Lane 1, Perina CTRL; lane 2, Perina DS; lane 3 Fragola CTRL; lane 4, Fragola DS; lane 5, Quarantino CTRL; lane 6 Quarantino DS; lane 7, Pisanello CTRL; lane 8, Pisanello DS. **(B)** Quantitative analysis of immunoblotting to SuSy. It should be noted that the level of SuSy in the control samples of Perina, Fragola and Quarantino was extremely low, almost indistinguishable from the background.

SuSy is just one of the many enzymes regulated by phosphorylation events [154]. In general, phosphorylation/dephosphorylation mechanisms mediated by kinases and phosphatases control numerous metabolic enzymes and proteins involved in signal transduction. Indeed, metabolic adaptations are very delicate processes that must be finely regulated [155]. It follows that the activity of proteins examined in this work might depend on their regulation by phosphorylation in addition to their concentration. For this reason, we carried out a preliminary analysis by determining the changes in protein phosphorylation in the leaves of the four tomato cultivars (**Figure 3.8**). Phosphorylation levels were analyzed on proteins separated on gels, transferred to membranes, and stained with a phosphoamino acid specific dye. We found differences in the phosphorylation levels of proteins expressed in plants subjected to water stress compared to controls. The result is consistent with what was found by Raghavendra [155] in tomatoes where protein phosphorylation levels change under drought stress conditions. The Perina cultivar is characterized by slight changes in protein phosphorylation levels after drought stress; this would confirm that the Perina cultivar is the most tolerant to drought stress not requiring major post-translational protein modifications to

increase drought tolerance. In contrast, the Fragola and Quarantino cultivars show significant changes in protein phosphorylation levels in the water-deprived sample compared to control. Finally, in the Pisanello cultivar phosphorylation levels are drastically reduced in samples subjected to drought stress compared to control samples. This may suggest that the most efficient responses against drought stress involve protein phosphorylation mechanisms and that the Pisanello cultivar is not capable of implementing adequate phosphorylation mechanisms.

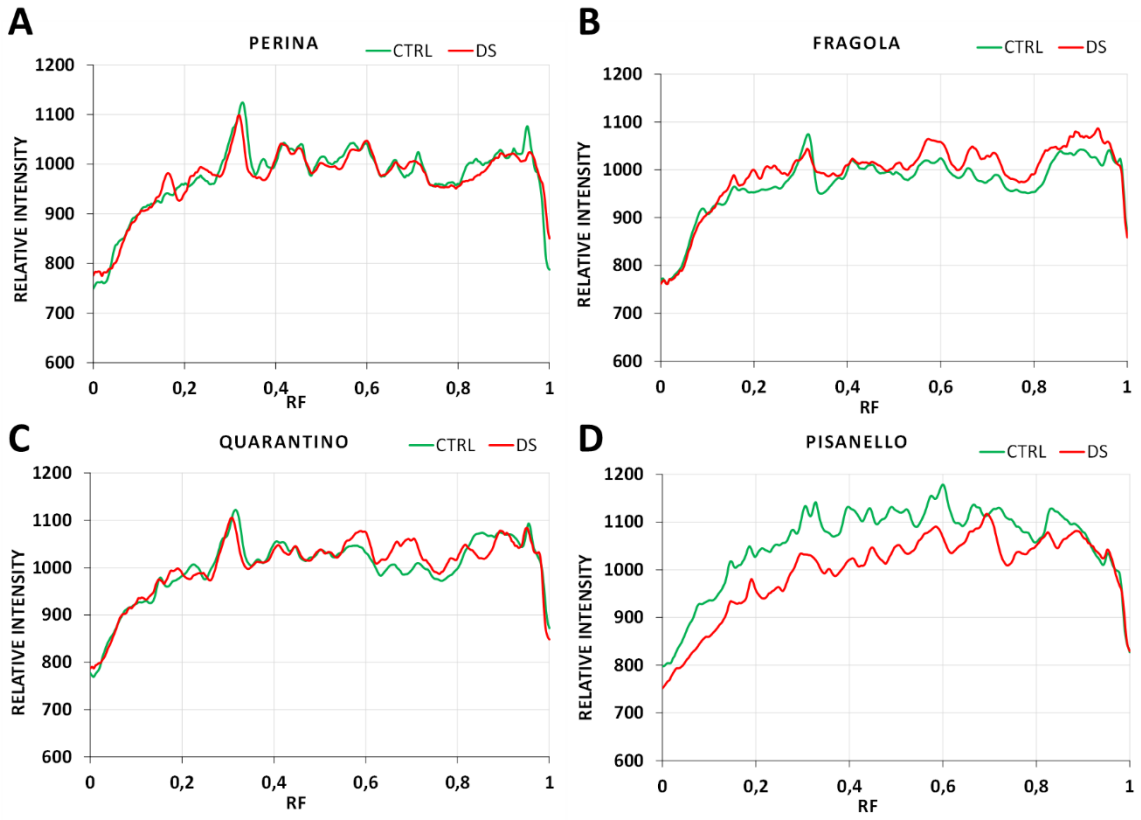


Figure 3.8. Profiling of phosphorylated proteins extracted from tomato leaves of the four cultivars, separated by 1D electrophoresis and labeled for phosphoamino acids in both control (CTRL, in green) and drought-stressed (DS, in red) samples. Intensity is reported as integrated density. The x-axis reports relative protein movement expressed as Rf. **(A)** Perine; **(B)** Fragola; **(C)** Quarantine; **(D)** Pisanello.

3.3.8. Sucrose, glucose, and fructose increase differentially in drought-stressed cultivars

Carbohydrates produced by photosynthesis in plant leaves provide energy and building blocks for growth and productivity. In addition to their energetic action, soluble carbohydrates (e.g., sucrose, fructose, glucose) are known to act as important osmoregulatory substances capable of maintaining cell turgor under conditions of osmotic stress such as that caused by drought and salt stress [156]. Therefore, the regulation of soluble carbohydrate concentrations in plant cells is an important adaptation of plants to water deficit.

Sucrose is the main product resulting from reactions involving 3-carbon sugars generated by photosynthesis; it represents a form of energy storage and transport [100].

Figure 3.9A shows an increase in this sugar in DS cultivars as well as a higher amount of sucrose in the Pisanello cultivar compared with Perina or Fragola. In contrast, dissimilar data were obtained for glucose (**Figure 3.9B**) and fructose (**Figure 3.9C**), respectively. Again, we observed an increase in these two carbohydrates in drought-stressed plants, but unlike sucrose, Pisanello was the cultivar with the lowest amount of glucose and fructose. In contrast, the cultivars Perina, Fragola, and Quarantino showed a significant increase in both sugars.

In addition to being cleaved into glucose and fructose, sucrose can be cleaved into fructose and UDP-glucose by sucrose synthase (SuSy) [100]. In leaves, sucrose levels are also influenced by biosynthesis activity. Therefore, a direct correlation between drought stress tolerance and sucrose level is not straightforward. As described above, sucrose cleavage by SuSy has the advantage of conserving some of the energy of sucrose, which has significant implications in recovery from stress conditions [149]. It should also be considered that most stress conditions (especially drought) result in carbohydrate accumulation in leaves, which may play a key role in osmoprotection and osmotic adaptation [157]. Thus, in this case, it can be assumed that the more tolerant cultivars attempt to break down sucrose to have more available osmoprotectants. In contrast, the tolerance mechanism of the Pisanello cultivar is not as efficient because Pisanello continues to produce sucrose while also hypothetically reducing the synthesis of osmoprotectants, at the same time gaining less energy to counteract the effects of stress.

Because the content of sucrose and related sugars in leaves is the result of different metabolic pathways, the data on sugar content do not perfectly match the expression of SuSy (**Figure 3.7**). In particular, Pisanello, while showing an increase in SuSy, does not exhibit a comparable increase in free sugars, such as fructose and glucose, which would also be excellent osmoprotectants. Consequently, the increased content of SuSy does not always correspond to a direct cleavage of sucrose. We can speculate that the increase in SuSy does not imply higher enzyme activity. This could be related to the lower levels of phosphorylation observed in Pisanello.

The analysis of soluble sugars in tomato cultivars was also extended to water-soluble pectins (**Figure 3.9D**). Generally, drought stress conditions can impact cell wall composition. Although it is challenging to draw a general picture, water deficiency induces cell wall strengthening through increased production of hemicelluloses and reduced activity of pectin-degrading enzymes such as polygalacturonases [158]. The latter finding is not constant; indeed, in cucumber conditions of water stress induce an increase in the expression of the polygalacturonase gene and therefore probably a higher degradation of pectins [159].

Strengthening of the cell wall could allow the cells to counteract the loss of water and to maintain an adequate level of turgidity even at low water potential. This is coupled with increased pectin biosynthesis, sometimes even increased branching resulting in enhanced binding to water molecules, as well as improved cross-linking with other polysaccharides [160]. Nor can it be excluded that a remodeling of pectins can be perceived as a signal of stress conditions and initiate response mechanisms [161].

Figure 3.9D shows an increase in water-soluble pectins in the cultivars Fragola, Quarantino, and Pisanello, but not in the cultivar Perina. However, in both Fragola and Quarantino differences are not statistically significant. First, this suggests that each cultivar performs differently in terms of soluble pectin production. In addition, the finding implies that the most tolerant cultivars (Fragola, Perina, and Quarantino) do not need to increase the level of water-soluble pectins, a fact that could contribute to its tolerance to drought stress. However, a significant increase in water-soluble pectins is only found in the Pisanello cultivar, the most susceptible among those examined. This might suggest that the damage observed in the Pisanello cultivar is also due to excessive production and release of water-soluble pectins.

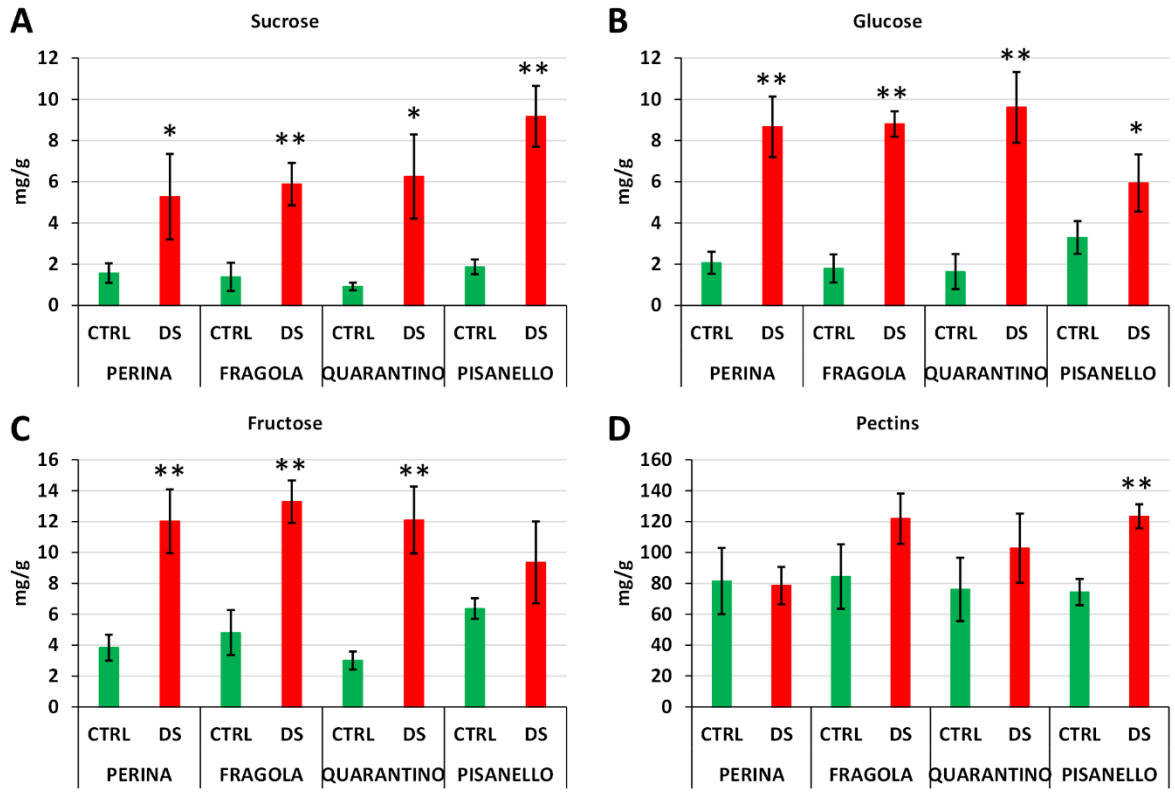
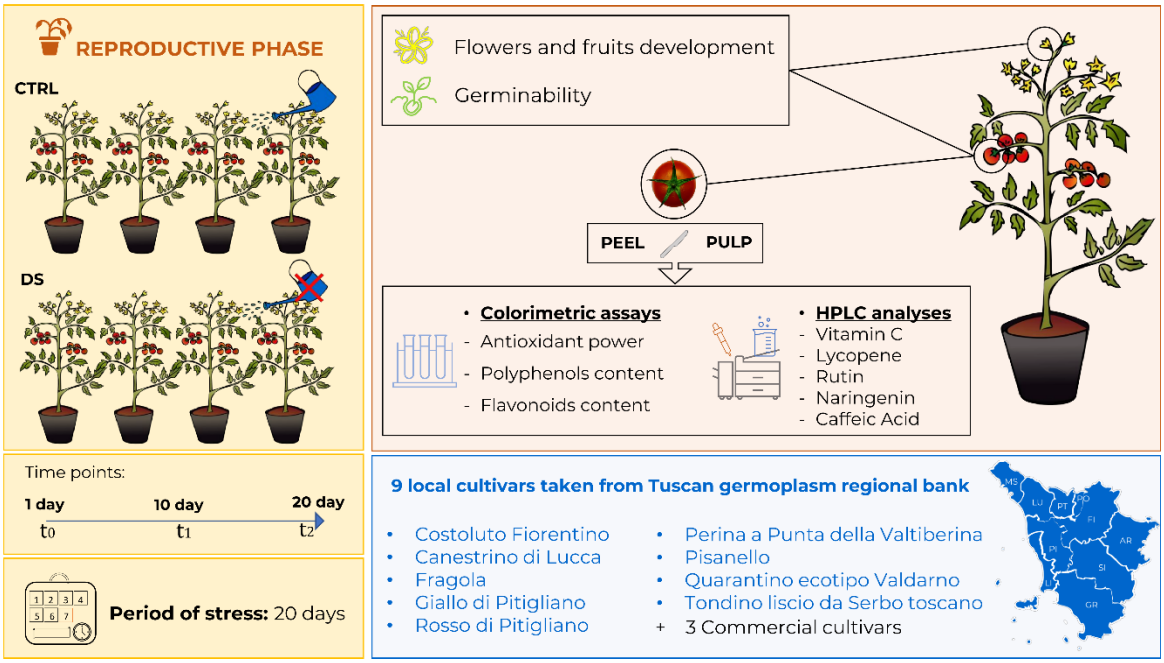


Figure 3.9. Content in mg per g of sucrose **(A)**, glucose **(B)**, fructose **(C)** and water-soluble pectins **(D)** in leaves sampled from control (green bars) and drought stressed (red bars) plants belonging to four Tuscan tomato cultivars. Asterisk indicates significant difference between control and stressed plants with $p \leq 0.05$ (*) or $p \leq 0.01$(**).

Chapter 4. Pulp and Peel of Italian Tomato Cultivars Show Different Content of Bioactives under Drought Stress

Collaborators: *Marco Romi, Massimo Guarnieri, Claudio Cantini and Giampiero Cai*



Graphical Abstract: The present study analyzes drought stress as a tool to increase the content of secondary metabolites and thus to improve the quality of tomato fruits. The nutraceutical characterization of fruits was performed by analyzing the content of antioxidants, polyphenols, flavonoids, lycopene, ascorbic acid (vitamin C), rutin, caffeic acid and naringenin. At the same time, the susceptibility of plants to stress during the reproductive phase was monitored in terms of flower abscission, fruit drop and seed germination. Perina turns out to be the tomato cultivar with the best nutraceutical properties in the absence of stress while the Quarantino cultivar is for the content of flavonoids (control plants) and the content of lycopene and vitamin C (stressed plants). Perina has the highest concentrations of bioactives and, together with Quarantino, is included in the cultivars with the best response to drought. Quarantino responds more effectively to stress in the reproductive phase. Data confirm that drought stress increases bioactive production in some local tomato cultivars, which produce higher quality fruits.

4.1. Introduction

Tomato fruits have good nutritional qualities as they contain active biomolecules and elements beneficial to human health, for example vitamin C, potassium, folic acid, carotenoids [162,163], polyphenols such as hydroxycinnamic acids (caffeic acid, chlorogenic acid) and flavonoids such as rutin, quercetin, and naringenin [164,165]. Indeed, many studies have linked the dietary consumption of tomatoes to the prevention and lower risk of cardiovascular and coronary heart disease, as well as cancer [166]. This protective action is attributed to secondary metabolites such as antioxidants, polyphenols, flavonoids, and anthocyanins [167].

Genetic factors, ripeness, and environmental conditions lead to differences in the bio-metabolic and nutraceutical characteristics of tomatoes [168]. Differences in biomolecule

content have often been found between the exocarp, mesocarp, and endocarp of tomato fruits. Examples can be found in the cultivar Camone [165], where the peel contains the highest concentration of polyphenols while the mesocarp contains about four times less. The most abundant flavonoid in Camone is rutin, present in the peel. In another study, three commercial New Zealand tomatoes were shown to contain higher levels of polyphenols, flavonoids, lycopene, and ascorbic acid in the fruit peel than in the pulp and seeds [169].

Various stress conditions (including drought) can induce a significant increase in bioactive molecules. For plants, these molecules are of critical importance in the defense against abiotic stress [9]. In the case of drought, production of reactive oxygen species (ROS) or free radicals is a consequence of stress and leads to oxidative damage to proteins, DNA, and lipids[35,162]. Antioxidants have the function of scavenging free radicals, and they include flavonoids, ascorbate, glutathione, carotenoids, and tocopherols [170]. However, the exposure to drought stress causes morphological, anatomical, physiological, and biochemical changes and, consequently, affects the growth and development of organs. Drought (as well as heat stress) damages the reproductive stage, leading to pollen sterility and reduced flower development with consequent decrease in seed and fruit production [23,24,171]. When drought stress occurs during seed formation, this leads to reduced seedling vigor and germination [7]. In crops, drought drastically reduces production and thus commercial performance [29]. Just to name a few examples, drought stress in sunflowers during germination compromises yield before the seeds even germinate [172,173]; in wheat, drought stress prior to flowering causes a decrease in grain number and size [174]. In tomato, drought stress significantly affects yield [175,176] as well as fruit volume, diameter, and composition in nutrients and biomolecules [177]. The tomato plant is sensitive to lack of water during reproductive development, especially during flowering and fruit growth [103]. Under drought stress conditions, tomato plants exhibit reduced leaf area and growth, flower drop, mineral deficiency, reduced fruit size, fruit breakage, and calcium deficiency-related physiological disorders such as flower rot and poor seed viability [178].

Today, a more sustainable agriculture, which requires fewer water resources, must take into account genetic biodiversity as a fundamental factor for improving yield and quality of crops, as well as resistance to biotic and abiotic stress. In the long term, this would allow farmers to sustain productivity even in drastic environmental conditions. This requires the identification and use of species/cultivars best adapted to their growing area.

Indeed, local cultivars are a source of unique genetic traits derived from adaptation to their area of origin, are often more resistant to biotic and abiotic stresses, and have high content of phytochemicals beneficial to human health [179–181]. In Italy, several tomato

cultivars are present, adapted to growing environments and selected for agronomic traits of interest, such as productivity, transportation durability, and marketability [182]. Italy, and specifically the Tuscany region, is also characterized by locally adapted cultivars that show marked genetic variability (compared to commercial cultivars) [25]. In previous chapters [25,105], we have analyzed locally adapted Tuscan tomato cultivars to prove their tolerance to water deficiency while identifying the most tolerant and susceptible. The previous data at morphological and physiological levels have allowed us to catalog the Tuscan cultivars based on their resistance to drought. However, analyses stopped at the vegetative and reproductive phase without considering the phytochemical content of fruits. The content of bioactive compounds in fruit pulp and peel was correlated with seed set and the development of flowers and fruits, to get an indication of the susceptibility to drought stress and to highlight the most promising cultivars (both under stress and non-stress conditions) in terms of nutraceutical compounds. The starting hypothesis is that tomato peel extracts represent a reliable source of bioactive molecules that can protect human health from oxidative stress [183]. In this chapter, we evaluated whether locally adapted (and drought stressed) Tuscan tomato cultivars can biosynthesize more antioxidant compounds in fruits. Thus, the tomato defense mechanism could be exploited to increase the production of secondary antioxidant metabolites useful for human health.

4.2. Materials and Methods

4.2.1. Plant Growth and Drought Conditions

The plants studied in the present chapter are previously analyzed (Chapter 2) from a morpho-physiological point of view; therefore, plants followed their same growth and drought stress conditions (Chapter 1). For each cultivar, eight plants were studied during the reproductive growth phase. Plants were divided into two groups: four plants were subjected to drought stress (DS) while four were the controls (CTRL) [184]. All plants were positioned in the greenhouse according to a randomization plan. The CTRL group was irrigated regularly, while the DS group was subjected to a total lack of water for 20 days. The DS treatment was based on existing literature [26,27]. Fruits, when fully ripe (total red fruit), were sampled and stored at −80 °C.

4.2.2. Development of Flowers and Fruits

To study flower development during drought stress, at the beginning of stress (t_0) plants of each cultivar were marked with differently colored strings (pink for open flowers, blue for fertilized flowers, green for small green tomatoes (period of cell division), red for large green

unripe tomatoes (period of cell expansion close to the ripening period), which are shown in **Figure 4.1**, these phases-marking steps were taken from Azzi [185] and Mazzucato [186]. The temporal development of each flower and fruit was monitored by counting them at the middle (10 days, t_1) and end (20 days, t_2) of drought stress. For each marker, counts made at t_1 and t_2 were reported as a percentage of those at t_0.

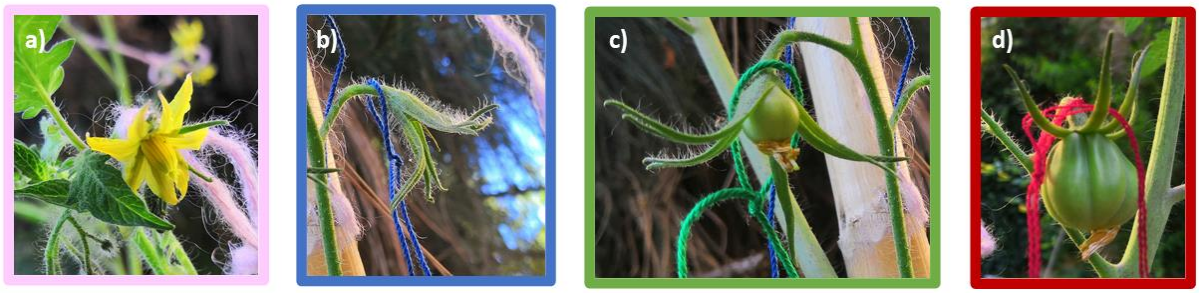


Figure 4.1. An example of marking made at various developmental stages on the same plant: **(a)** pink for opening flowers, **(b)** blue for fertilized flowers, **(c)** green for small green tomatoes, and **(d)** red for large unripe green tomatoes

4.2.3. Germination of Seeds

Seeds were removed from three fruits of each cultivar, washed with water, dried on tissue paper, and then stored in polyethylene bags at room temperature. The germination test was performed by placing 100 seeds on two layers of moist filter paper in Petri dishes. Seed germination was calculated daily for eight days. A seed was considered germinated when a 3–4 cm long rootlet was visible outside the seed coating [7]. Percentage of germination and shoot length were recorded.

4.2.4. Preparation of Samples for Colorimetric Analysis

For each tomato cultivar, five fruits were selected randomly and chopped. Then, 1 g of peel and 1 g of pulp were weighed and 6 mL (for peel) and 3 mL (for pulp) of 70% acetone were immediately added. Samples were homogenized by Turrax (UltraTurrax® T25 based IKA, Saint Louis, MO, USA) for 5 min, then placed in a sonicator (Elma Transsonic T 460/H, Wezikon, Switzerland) for 15 min and then homogenized again by Turrax. Samples were then centrifuged at 4000×g for 5 min (Eppendorf® 5415D centrifuge, Hamburg, Germany). Finally, supernatants (i.e., the extract) were transferred to 2 mL Eppendorf tubes.

4.2.5. Determination of the Antioxidant Power

The total antioxidant potential of tomato peel and pulp extracts was determined using the FRAP (ferric reducing antioxidant power) assay reported by Benzie and Strain [187]. The test is based on reduction of Fe^{3+}-2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) to a blue Fe^{2+}-TPTZ. The absorbance was read at 593 nm (Perkin Elmer spectrophotometer, Lamba 25, Waltham, MA,

USA). The FRAP value of extracts, expressed as µmol Fe^{2+}/g of fresh weight (FW), was determined using a standard curve of ferrous sulphate. The experiment was conducted in three technical replicates for each sample. Finally, the mean and standard deviation were calculated. To verify the significance of the data obtained, the t-test (* $p \leq 0.05$, ** $p \leq 0.01$) were carried out.

4.2.6. Determination of Phenolic Content

The total polyphenol content (TPC) of tomato peel and pulp extracts was determined in fruits by the spectrophotometric method of Folin–Ciocâlteu [188]. This assay is based on electron transfer in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes, which are read at 765 nm. Results were expressed in gallic acid equivalent (GAE), a universally accepted standard for polyphenols, to determine the value of TPC in mg/100 g of fresh weight (FW). Actually, the reagent used in the Folin–Ciocâlteu method is not strictly specific to phenolics and can react with other substances. Therefore, the results of the assay should more generally be interpreted as an estimate of the reducing capacity. The experiment was conducted in three technical replicates for each sample. Finally, the mean and standard deviation were calculated. To verify the significance of the data obtained, the t-test (* $p \leq 0.05$, ** $p \leq 0.01$) were carried out.

4.2.7. Determination of Flavonoid Content

Flavonoids are determined by the aluminum chloride assay. Complexes of aluminum chloride with flavonoids cause the solution to turn yellow, which is read by a spectrophotometer at 415 nm [189]. The data obtained were compared to a calibration curve obtained with the quercetin standard. Values were expressed as mg of total flavonoids in 100 g of fresh weight (FW). The experiment was conducted in three technical replicates for each sample. Finally, the mean and standard deviation were calculated. To verify the significance of the data obtained, t-tests (* $p \leq 0.05$, ** $p \leq 0.01$) were carried out.

4.2.8. Determination of Lycopene

Extraction of lycopene was made according to Barba [190]; 0.3 g of tomato peel and pulp (taken from the pull described previously in Section 4.2.4) were added to 10 mL of a solvent solution made by hexane/acetone/ethanol (50:25:25 v/v/v) and homogenized with Ultra-Turrax (IKA®). Subsequently, 1.5 mL of distilled water was added, and the samples were vortexed. The upper layer (1 mL) was dried under vacuum and the dry extract was resuspended in 0.4 mL of tetrahydrofuran (THF)/acetonitrile (ACN)/methanol (15:30:55

v/v/v). The mobile phase for HPLC (Perkin Elmer Nelson 3200 Series) analysis consisted of methanol/ACN (90:10 v/v) and 9 mM triethanolamine (TEA) at a flow rate of 0.9 mL/min, using a RP-C18 column (SUPELCO Kromasil 100A-5u-C18 4.6 mm × 250 mm); the absorbance was set at 475 nm and the run time was 20 min. Quantification was carried out using a standard calibration curve consisting of five points at increasing concentrations (6.25, 12.5, 25, 50, and 100 µg/mL) of lycopene standard (Sigma Chemical, St. Louis, MO, USA). The experiment was conducted in three technical replicates for each sample. Finally, the mean and standard deviation were calculated. To verify the significance of the data obtained, t-tests (* $p \leq 0.05$, ** $p \leq 0.01$) were carried out.

4.2.9. Determination of Vitamin C

Extraction of ascorbic acid was carried out using 1 g of both tomato peel and pulp (taken from the pull described previously in Section 4.2.4) in 2 mL of distilled water; samples were homogenized with Ultra-Turrax (IKA®), then filtered through a 0.45-µm membrane filter [191]. For HPLC analysis, an RP-C18 column (SUPELCO Kromasil 100A-5u-C18 4.6 mm × 250 mm) was used. The mobile phase consisted of 0.01 mol/l KH_2PO_4 buffer solution (pH = 2.6 with o-phosphoric acid), with a flow rate of 0.5 mL/min and an absorbance set at 250 nm. The quantification was carried out using a standard calibration curve consisting of five points at increasing concentrations (6.25, 12.5, 25, 50, and 100 µg/mL) of ascorbic acid standard (Sigma Chemical, St. Louis, MO, USA). The experiment was conducted in three technical replicates for each sample. Finally, the mean and standard deviation were calculated. To verify the significance of the data obtained, t-tests (* $p \leq 0.05$, ** $p \leq 0.01$) were carried out.

4.2.10. Determination of Rutin, Quercetin, Naringenin, and Caffeic Acid

Determination of rutin, quercetin, naringenin, and caffeic acid was performed with an RP-C18 column (SUPELCO Kromasil 100A-5u-C18 4.6 mm × 250 mm). Sample extraction was performed according to Tokusoglu [192], with some modifications. Samples of peel and pulp fruit (1 g, taken from the pull described previously in Section 4.2.4) were added to 1 mL of 70% acetone containing 1% (v/v) HCl and 0.02 mg/mL TBHQ (tert-Butylhydroquinone). The mixture was then homogenized by Ultra-Turrax (IKA®) and 0.2 mL of 1.2 M HCl was added. The mixture was incubated at 90 °C for 2 h under continuous stirring. Samples were then cooled at room temperature and sonicated for 3 min. Finally, extracts were centrifuged for 5 min at 3000× g and filtered through a 0.45-µm membrane filter. The HPLC method was performed according to Kumar [193], with slight modifications. The mobile phase was water (phase A) and acetonitrile with 0.02% trifluoroacetic acid (TFA) (phase B); elution was

performed with a linear gradient of 80% A and 20% B (0–5 min), 60% A and 40% B (5–8 min), 50% A and 50% B (8–12 min), 60% A and 40% B (12–17 min), 80% A and 20% B (17–21 min). The flow rate was 1 mL/min, and the absorbance was set at 365 nm for rutin and quercetin, 325 nm for caffeic acid, and 280 nm for naringenin; the run time was 21 min. Quantification was carried out using standard calibration curves consisting of five points from 5 to 80 µg/mL using standards of rutin, quercetin, naringenin, and caffeic acid (Sigma Chemical, St. Louis, MO, USA). The experiment was conducted in three technical replicates for each sample. Finally, the mean and standard deviation were calculated. To verify the significance of the data obtained, t-tests (* $p \leq 0.05$, ** $p \leq 0.01$) were carried out.

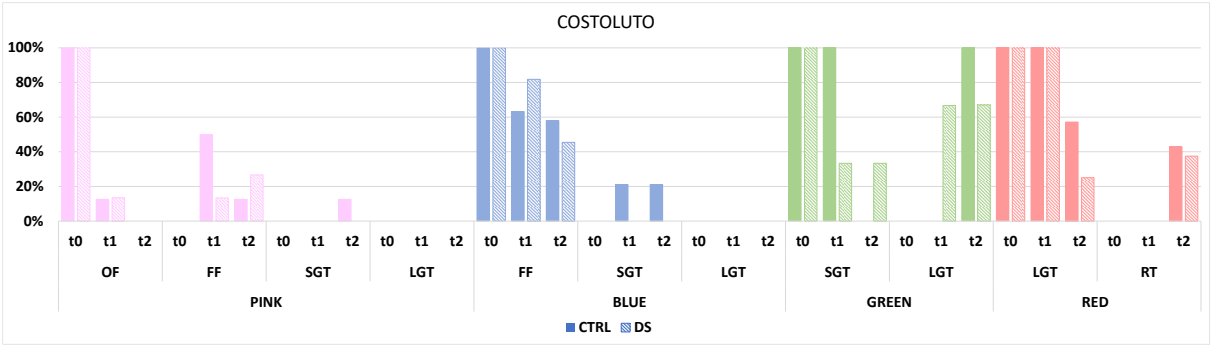
4.3. Results

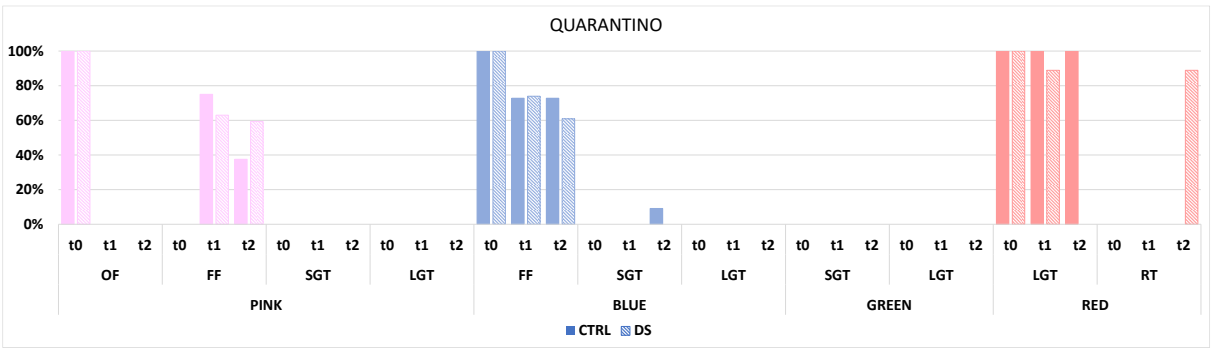
4.3.1. Development of Flowers and Fruits

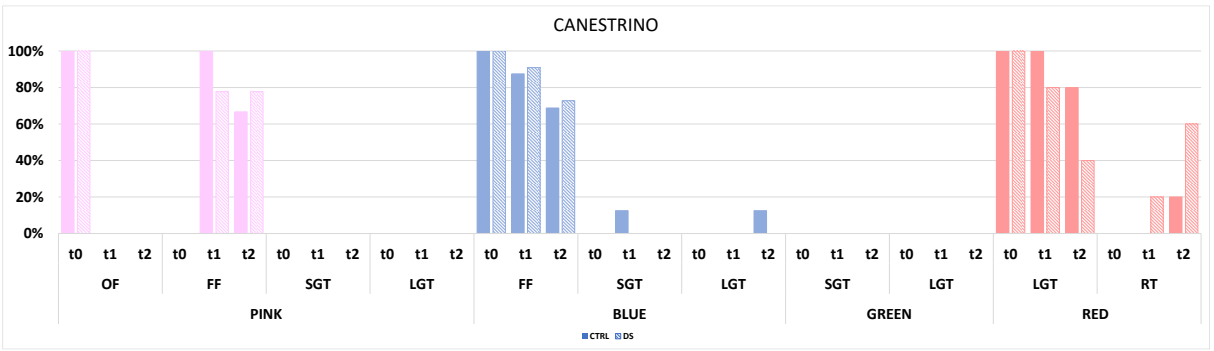
Analysis of flower and fruit development throughout the drought stress period provided an indication of the reproductive (and thus productive) performance of plants [194]. In general, drought stress induces early flowering, which could be due to rapid phenological development aimed at completing the life cycle under unfavorable environmental conditions.

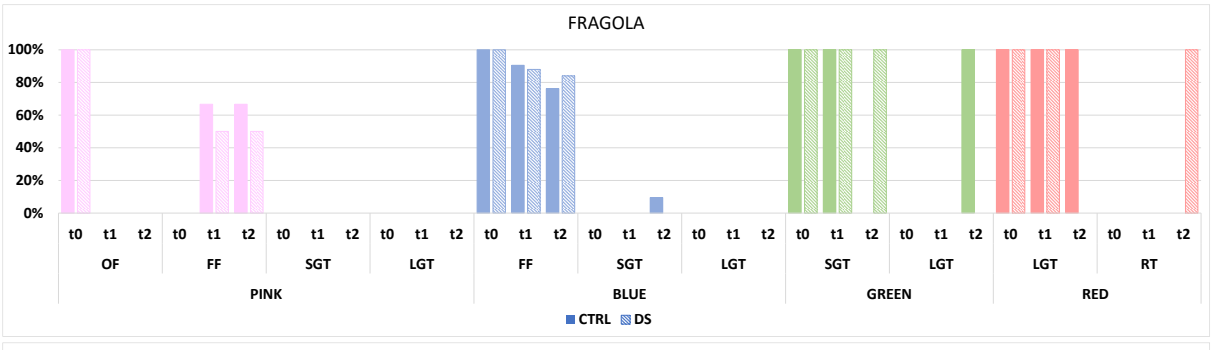
The results obtained on the development of open flowers (pink thread) reveal that in most cultivars the loss of open flowers is less than 50% both for the CTRL and DS groups (**Figure 4.2**). Major differences are found between CTRL and DS in cultivars such as Giallo, Perina, and Pisanello, where loss is higher in DS samples with 57% total loss compared to 10% in the CTRL group. By contrast, in cultivars such as Tondino and Rosso the loss in the DS group is less than the CTRL group or even no loss at all. Lower ratio of abscised flowers in tolerant genotypes could also be due to maintenance of efficient photosynthesis [177]. Indeed, reduced photosynthesis decreases the availability of sugars and their contribution to floral organ development leading to their abscission [195,196]. Development of fertilized flowers (blue thread) shows a general delay in the DS group compared to the CTRL, and loss is always higher in the DS. An exception is the cultivar Perina for which both loss and development time are comparable to the CTRL group. In contrast, in the cultivars Rosso, Datterino, and Pantano flowers in the DS group develop earlier, whereas for Datterino there is substantial loss in the DS group compared to CTRL. In contrast, there is no fruit drop in the stages marked with green wires, i.e., those that monitored small green fruits.

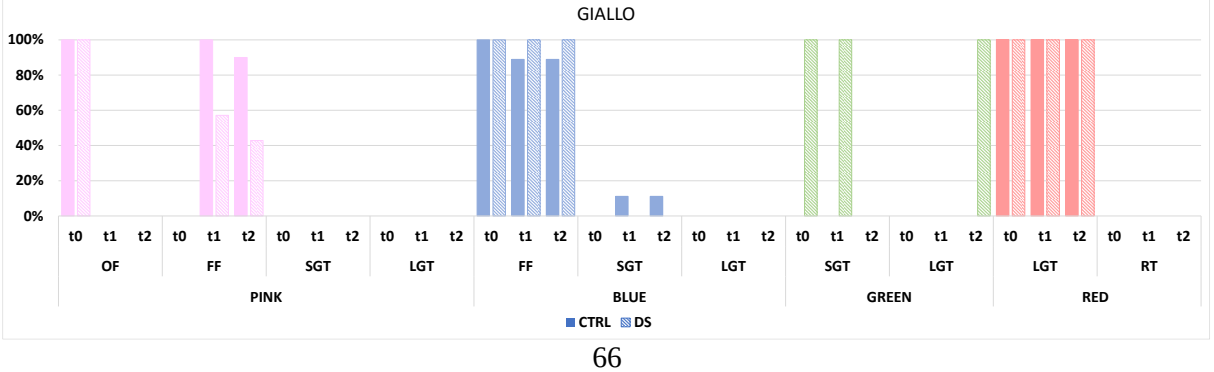
Legend:	
OF = Opening Flowers	
FF = Fertilized Flowers	PINK = Development of OF
SGT = Small Green Tomatoes	BLUE = Development of FF
LGT= Large Green Tomatoes	GREEN = Development of SGT
RT = Red Tomatoes	RED = Development of LGT

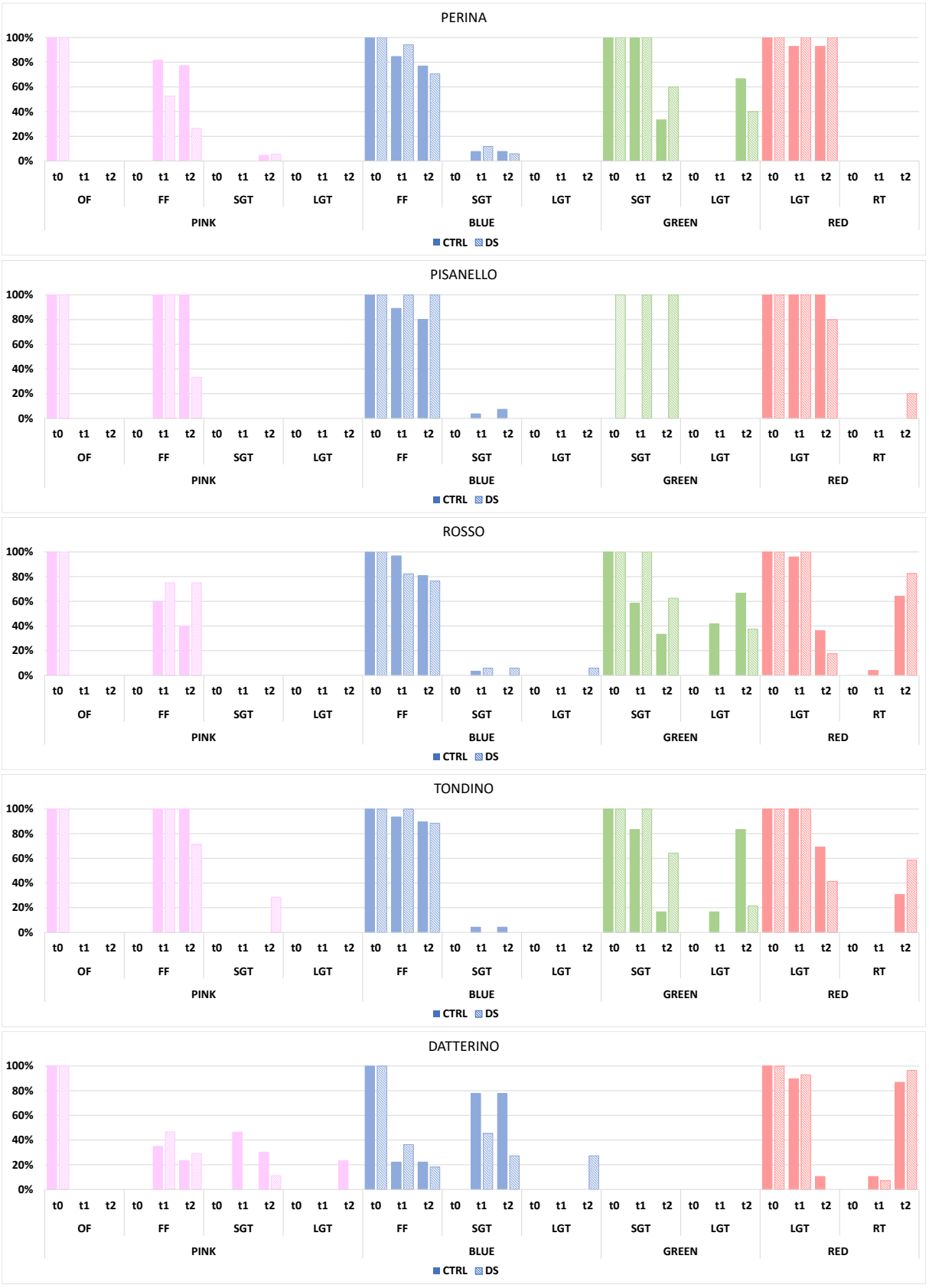











PERINA
PISANELLO
ROSSO
TONDINO
DATTERINO
100%
80%
60%
40%
20%
0%
t0 t1 t2 t0 t1 t2 t0 t1 t2 t0 t1 t2 t0 t1 t2 t0 t1 t2 t0 t1 t2 t0 t1 t2 t0 t1 t2 t0 t1 t2 t0 t1 t2 t0 t1 t2
OF FF SGT LGT FF SGT LGT SGT LGT LGT RT
PINK BLUE GREEN RED
CTRL DS

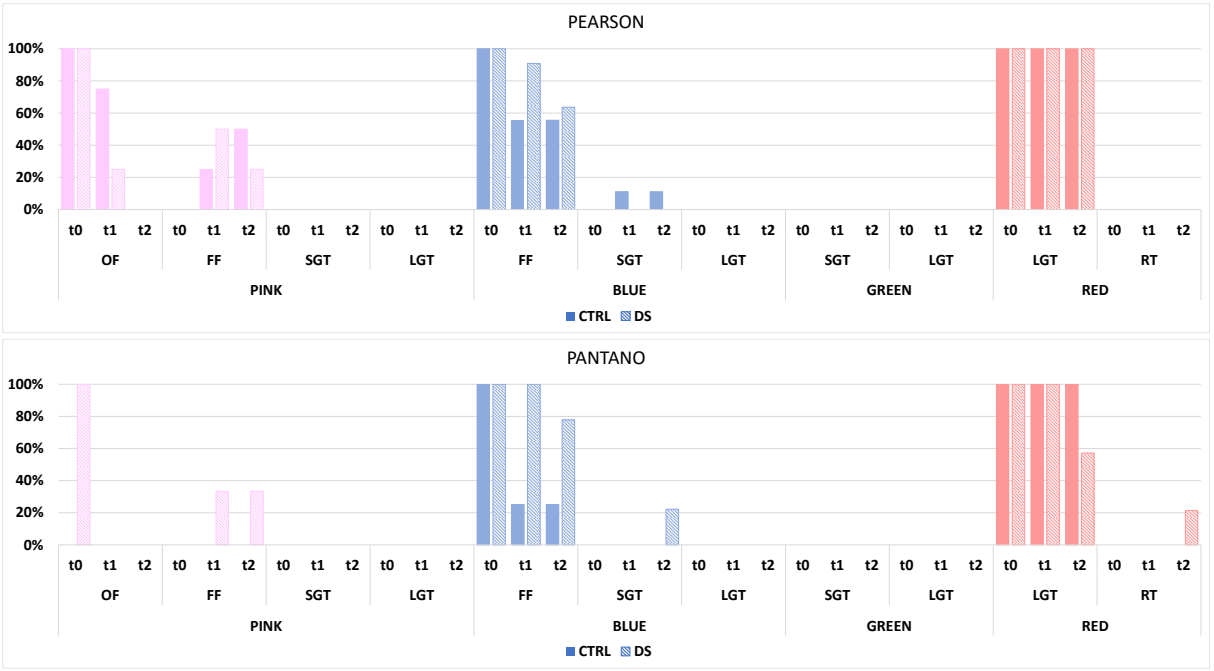


Figure 4.2. The bar chart illustrates the flowers and fruits development during drought stress. The colors of the bars in the graphics correspond to the different colored strings with which the plants of each cultivar were marked at the beginning of stress (t0): PINK for open flowers (OF), BLUE for fertilized flowers (FF), GREEN for small green tomatoes (SGT), RED for large green tomatoes (LGT). The heights of the bars correspond to the number of flowers and fruits at each developmental stage t_0, t_1 (10 days) and t_2 (20 days). The values are expressed as a percentage of the total number at t_0.

However, for the cultivars Rosso, Tondino, and Fragola, development is delayed in the DS groups compared to the CTRL; the cultivar Costoluto shows early development in the DS group while the cultivar Perina exhibits development similar to the CTRL group. Red thread (marking the growth and ripening of large green fruits) does not reveal major losses in plants subjected to drought stress. In most cultivars there is early ripening in the DS group, but for the cultivars Costoluto, Giallo, Perina, Datterino, and Pearson, development times in the DS group are remarkably similar to the CTRL group.

4.3.2. Seed Germination

Seed germinability is an index of the productivity and reproductive efficiency of plants. This aspect was tested to monitor the effect of drought stress on the production capacity of the cultivars under examination.

Differences between the cultivars were already visible after 4 days (**Figure 4.3A**). The Datterino, Pearson, Fragola, and Pisanello cultivars show a clear progress in the germination of seeds from tomatoes that suffered stress. This does not occur in the case of seeds of control tomatoes. The Pantano, Canestrino di Lucca, Rosso, and Tondino cultivars show a progress of germination in seeds from stressed plants, but the performance remains similar to that of controls. In the Quarantino cultivar, after 4 days no differences in the germination rate

between control seeds and seeds of stressed plants are observed. On the contrary, the germination rate of the Costoluto Fiorentino and Giallo cultivars is low for both CTRL and DS. The Perina cultivar shows no differences because after 4 days neither the CTRL seeds nor the DS seeds are germinated.

After 8 days, the germination rate is adequate for all cultivars but with some differences (**Figure 4.3B**). Datterino, Pearson, Pantano, Fragola, and Pisanello cultivars exhibit a fair percentage of germination in both the CTRL and DS; for the latter the percentage is slightly higher, probably because of early germination of seeds. The opposite occurs for Canestrino di Lucca, Costoluto Fiorentino, and Perina in which the percentage of germinated seeds of the DS group is lower than the CTRL. The highest germination rate in CTRL is found in Perina. Rosso, Giallo, Tondino, and Quarantino cultivars show a nearly equal germination rate between CTRL and DS.

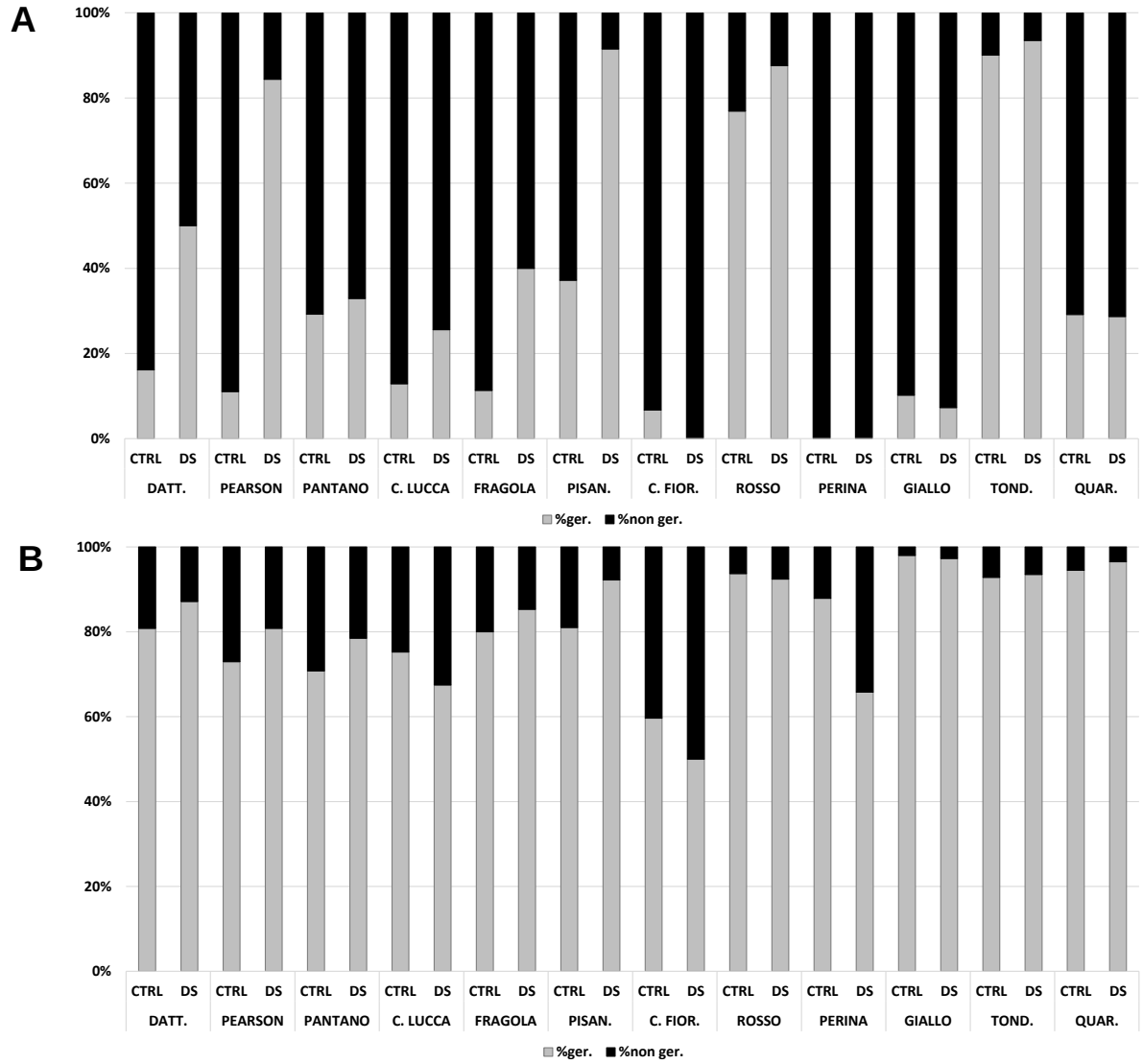


Figure 4.3. (A) Seed germination after 4 days expressed in percentage. **(B)** Seed germination after 8 days expressed in percentage. In black the percentage of non-germinated seeds and in gray the percentage of germinated seeds. CTRL indicates the control group of tomato, and DS the drought-stressed group.

4.3.3. Antioxidant Power in Peel and Pulp

Analysis of the peel (**Figure 4.4**) showed that stressed plants of the three commercial cultivars exhibit a decrease in antioxidant power compared to controls. The three commercial cultivars have similar values: in stressed plants the antioxidant power is around 20 µmol/g, while in the control group it is around 25 µmol/g. More precisely, the stressed Pearson cultivar has the lowest value (17.86 µmol/g) and undergoes a drastic decrease compared to the control (27.17 µmol/g). Among local cultivars, Perina has the highest antioxidant power for both the CTRL group (48.19 µmol/g) and the DS group (53.30 µmol/g). In this case, the antioxidant capacity in the peel of stressed plants is higher than in control plants. The cultivar Quarantino, on the other hand, has the lowest value under drought stress (12.10 µmol/g), a value below that of the CTRL group (18.32 µmol/g). Finally, the cultivar that most clearly increases the antioxidant content in the peel under drought stress is Giallo di Pitigliano, which shows an extremely low content in the control group (11.37 µmol/g) but it doubles in the stressed group (22.54 µmol/g). The overall picture in the pulp (**Figure 4.5**) remains the same as in the peel, with antioxidant contents being significantly lower in the pulp. The cultivar Perina has the highest antioxidant power in both the control (12.85 µmol/g) and the stressed group (14.65 µmol/g). A difference from the peel is observed for the cultivars Quarantino and Datterino, which have higher antioxidant content in the pulp in the CTRL group than in the DS.

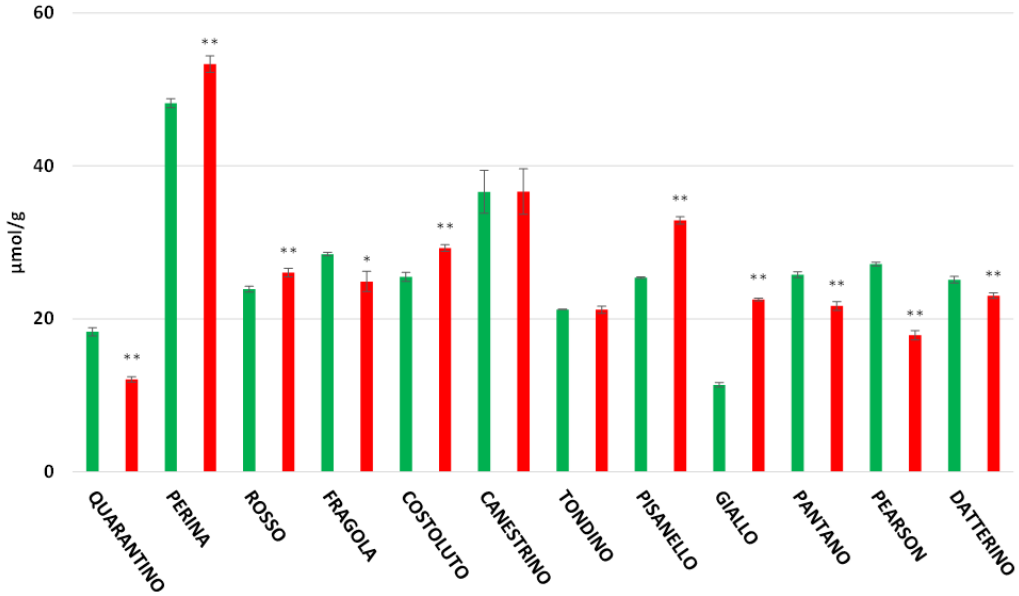


Figure 4.4. Total antioxidant (expressed as µmol/g of FW) in tomato peel of 9 Italian cultivars and three commercial cultivars. Controls (CTRL) in green and drought-stressed group (DS) in red. The bars indicate the standard deviation. A significantly difference is shown between CTRL and DS of each cultivar by * for a $p \leq 0.05$ and ** for $p \leq 0.01$.

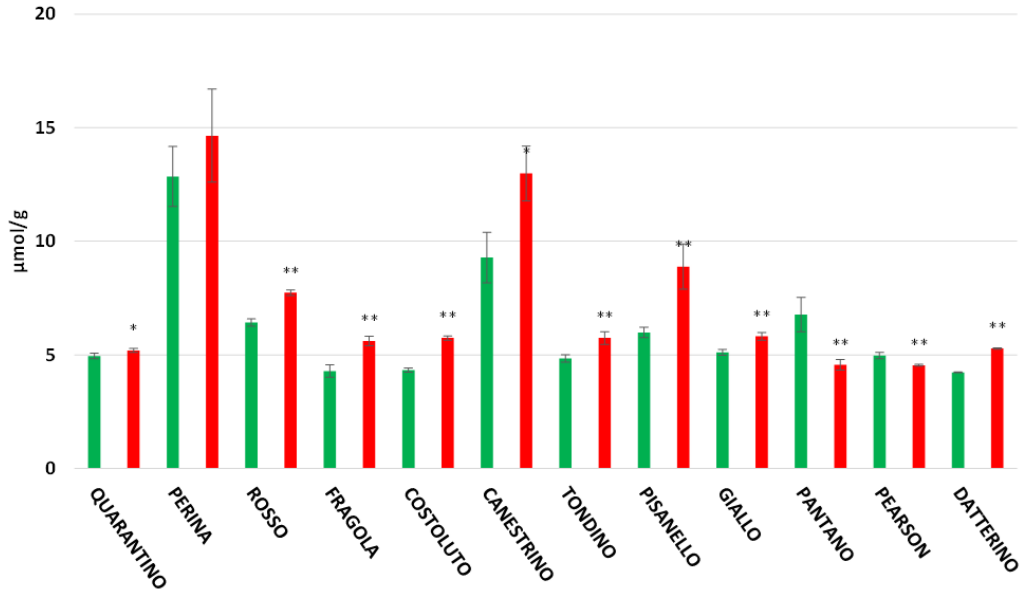


Figure 4.5. Total antioxidant (expressed as µmol/g of FW) in tomato pulp of 9 Italian cultivars and three commercial cultivars. Controls (CTRL) in green and drought-stressed group (DS) in red. The bars indicate the standard deviation. A significantly difference is shown between CTRL and DS of each cultivar by * for a $p \leq 0.05$ and ** for $p \leq 0.01$.

4.3.4. Polyphenol Content

In the peel (**Figure 4.6**), the highest polyphenol content is found in the cultivar Perina (709.44 mg/100 g in the DS group and 477.77 mg/100 g for the CTRL group). The lowest content among stressed plants is found in the cultivar Quarantino (116.56 mg/100 g), where the amount of polyphenols is reduced if compared to when plants are hydrated (172.85 mg/100 g). The opposite situation occurs for Rosso di Pitigliano, which significantly increases polyphenol content under drought stress (361.33 mg/100 g) compared to the control (152.47 mg/100 g). In the pulp (**Figure 4.7**), the cultivar with the highest polyphenol content is Rosso di Pitigliano (80.88 mg/100 g for DS group plants and 67.46 mg/100 g for CTRL). The cultivar Perina maintains high values in both DS and CTRL groups.

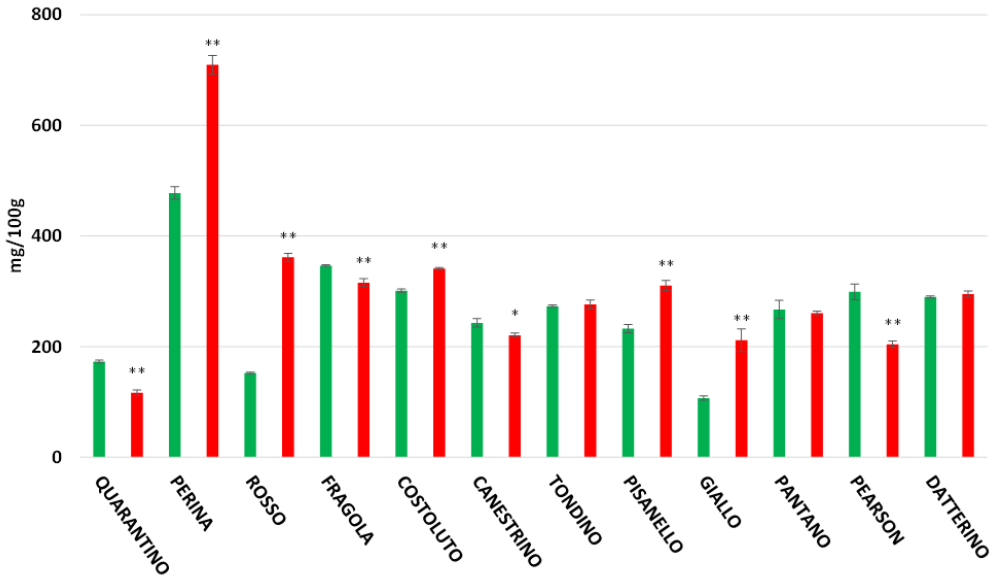


Figure 4.6. Total polyphenol content (expressed as mg/100 g of FW) in tomato peel of 9 Italian cultivars and three commercial cultivars. Controls (CTRL) in green and drought-stressed group (DS) in red. The bars indicate the standard deviation. A significant difference is shown between CTRL and DS of each cultivar by * for a $p \leq 0.05$ and ** for $p \leq 0.01$.

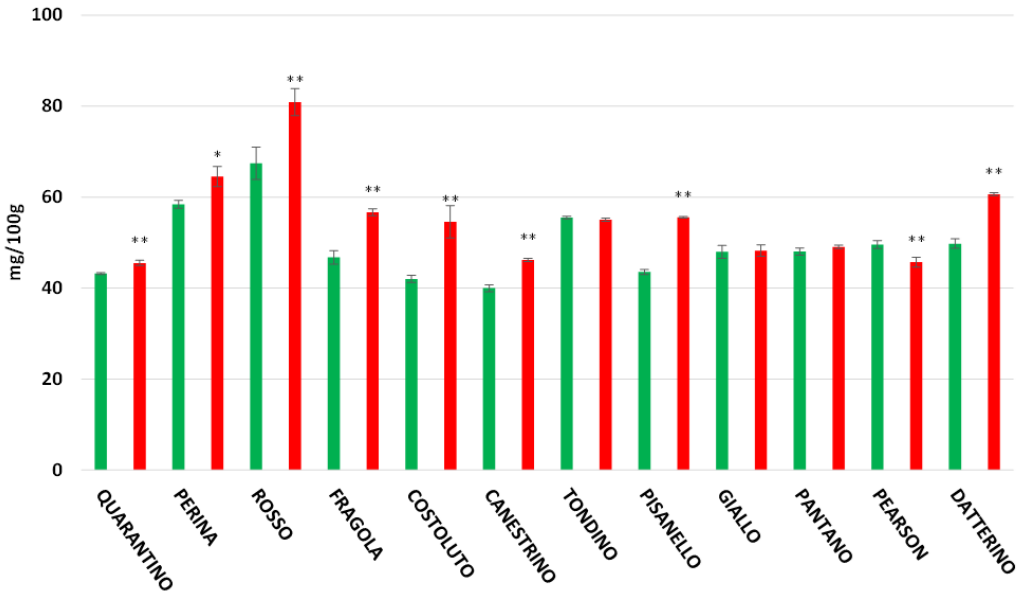


Figure 4.7. Total polyphenol content (expressed as mg/100 g of FW) in tomato pulp of 9 Italian cultivars and three commercial cultivars. Controls (CTRL) in green and drought-stressed group (DS) in red. The bars indicate the standard deviation. A significantly difference is shown between CTRL and DS of each cultivar by * for a $p \leq 0.05$ and ** for $p \leq 0.01$.

4.3.5. Flavonoids Content

The highest flavonoid content recorded in the peel is found in the CTRL group of the cultivar Quarantino with 193.98 mg/100 g (**Figure 4.8**), a value that far exceeds the content of the corresponding stressed group (59.09 mg/100 g). The cultivars Costoluto, Canestrino, Fragola, and the commercial Pantano follow the same trend. In contrast, the cultivars Giallo, Perina, Pisanello, Rosso, Datterino, and Pearson showed an increase in drought-stressed plants. The

highest flavonoid content for stressed plants was found in the cultivars Perina and Datterino, with about 140 mg/100 g. The stressed Perina cultivar had a particularly high flavonoid content compared to the control (48.67 mg/100 g). For the pulp, results are different (**Figure 4.9**). Stressed cultivars such as Costoluto, Canestrino, Giallo, Rosso, Datterino, and Pearson show increased flavonoid content compared to the control. Pisanello, Tondino, and Pantano cultivars show no clear differences between plants in the CTRL and DS groups. Fragola and Perina are the only two cultivars showing a decrease in stressed plants compared to controls.

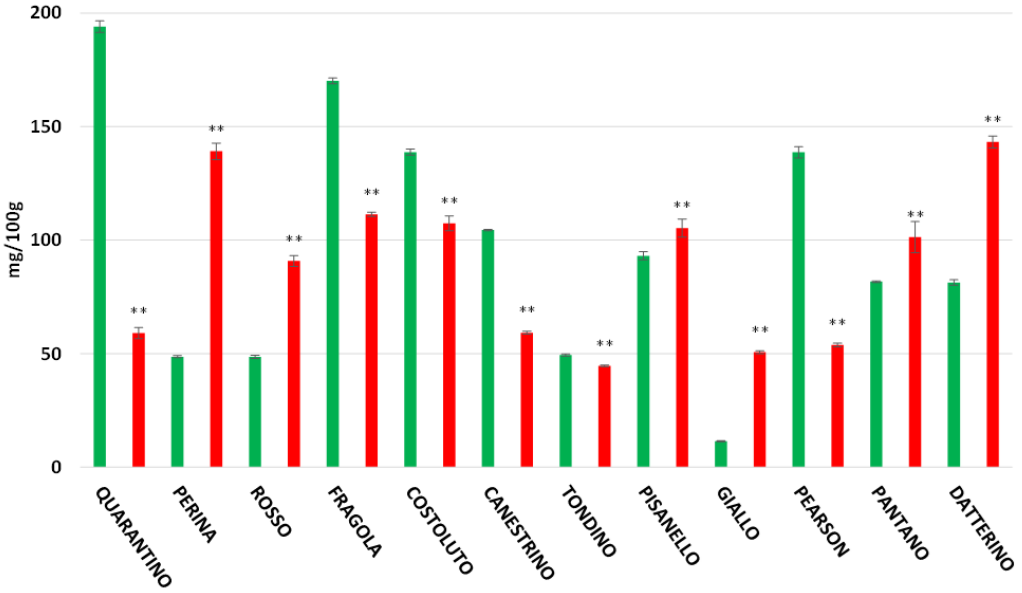


Figure 4.8. Total flavonoids content (expressed as mg/100 g of FW) in tomato peel of 9 Italian cultivars and three commercial cultivars. Controls (CTRL) in green and drought-stressed group (DS) in red. The bars indicate the standard deviation. A significantly difference is shown between CTRL and DS of each cultivar by ** for $p \leq 0.01$.

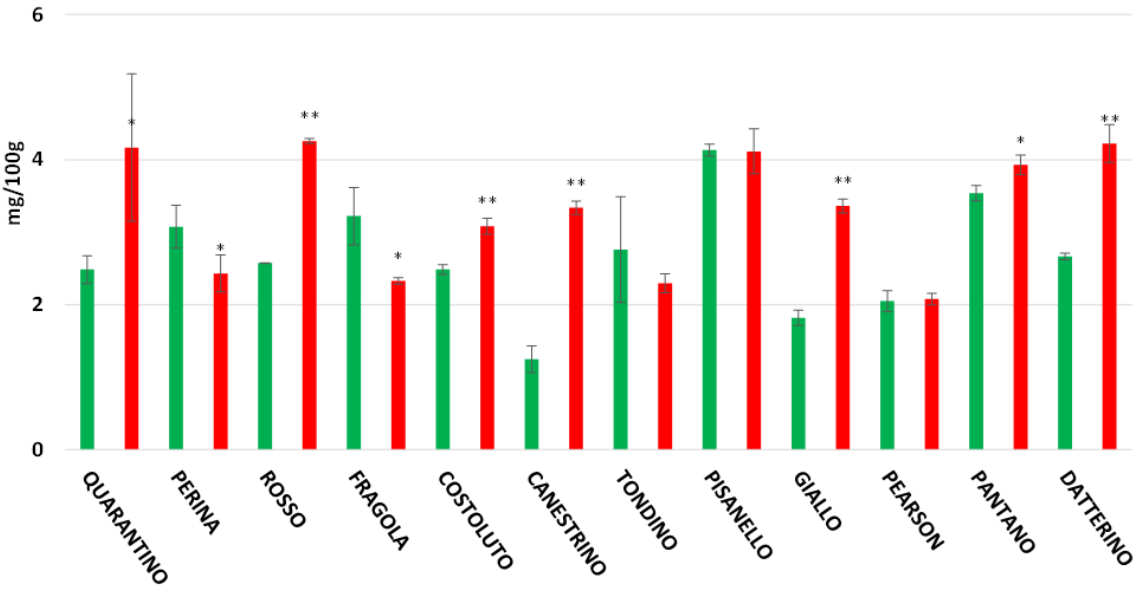


Figure 4.9. Total flavonoids content (expressed as mg/100 g of FW) in tomato pulp of 9 Italian cultivars and three commercial cultivars. Controls (CTRL) in green and drought-stressed group (DS) in red. The bars indicate the standard deviation. A significantly difference is shown between CTRL and DS of each cultivar by * for a $p \leq 0.05$ and ** for $p \leq 0.01$.

4.3.6. Vitamin C

The content of ascorbic acid in the skin of different cultivars is shown in **Figure 4.10**. For most genotypes, stressed fruits have lower vitamin C content than controls. This is particularly evident for Fragola, Pisanello, Giallo, Pantano, and Pearson. For other cultivars, such as Quarantino, Perina, Tondino, and Datterino, the ascorbic acid content of stressed fruits is similar to that of controls. On the other hand, the cultivar Rosso has a slightly higher content in stressed fruits than the control. In contrast, a few differences are found in the pulp (**Figure 4.11**). The stressed Rosso cultivar increases the content of vitamin C, as in the peel, and the Datterino cultivar behaves similarly. Giallo, Pantano, and Pearson decrease the ascorbic acid content in the pulp of stressed fruits just as in the peel. The concentration of vitamin C in the control of Giallo cultivar differs because the value in the pulp is also comparable to those in the peel.

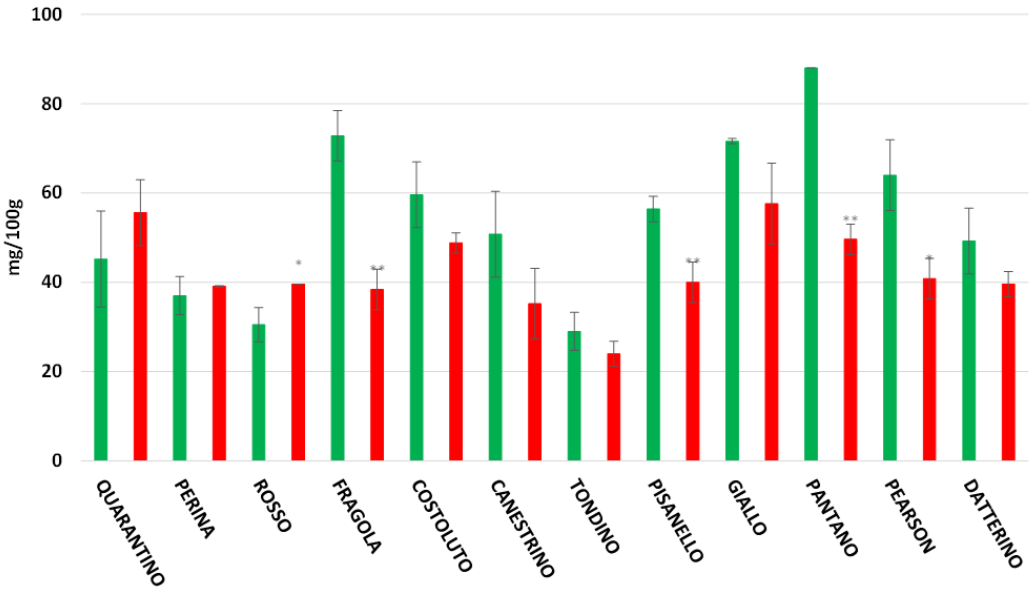


Figure 4.10. Vitamin C (ascorbic acid) content in tomato peel of 9 Italian cultivars and three commercial cultivars. Controls (CTRL) in green and drought-stressed group (DS) in red. The bars indicate the standard deviation. A significantly difference is shown between CTRL and DS of each cultivar by * for a $p \leq 0.05$ and ** for $p \leq 0.01$.

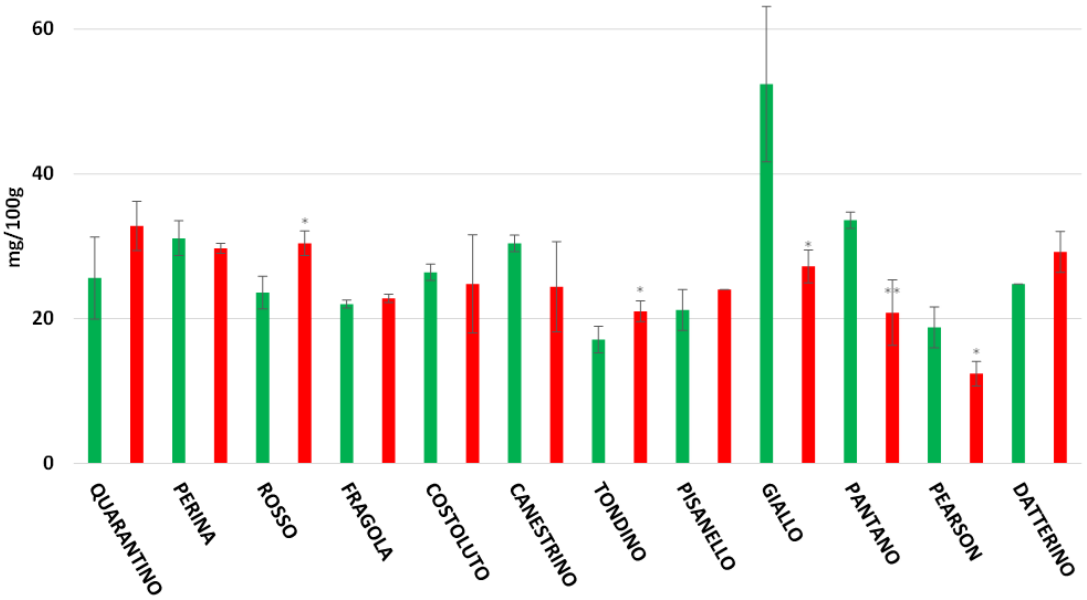


Figure 4.11. Vitamin C (ascorbic acid) content in tomato pulp of 9 Italian cultivars and three commercial cultivars. Controls (CTRL) in green and drought-stressed group (DS) in red. The bars indicate the standard deviation. A significantly difference is shown between CTRL and DS of each cultivar by * for a $p \leq 0.05$ and ** for $p \leq 0.01$.

4.3.7. Lycopene

Lycopene is the most common carotenoid present in tomatoes. In the peel (**Figure 4.12**) the concentration is extremely high for all genotypes except for the Giallo cultivar. This was already inferred from the yellow color of its fruits, since higher amounts of lycopene provide a reddish color. The cultivars Quarantino, Tondino, Pantano, and Datterino show an increase in lycopene concentration in stressed fruits compared to controls. The opposite occurs for Perina, Rosso, and Costoluto. In the other cultivars, there are no significant differences between CTRL and DS. In the pulp, lycopene concentration is generally lower than in the peel, except for the cultivar Giallo, which conversely shows a higher content for both CTRL and DS (**Figure 4.13**). The cultivars Quarantino, Tondino, Pantano, and Datterino show an increase in lycopene in the pulp of stressed fruits as well as in the peel. The opposite occurs for Perina, Rosso, and Fragola. In all other cultivars there are no differences in lycopene concentration between CTRL and DS.

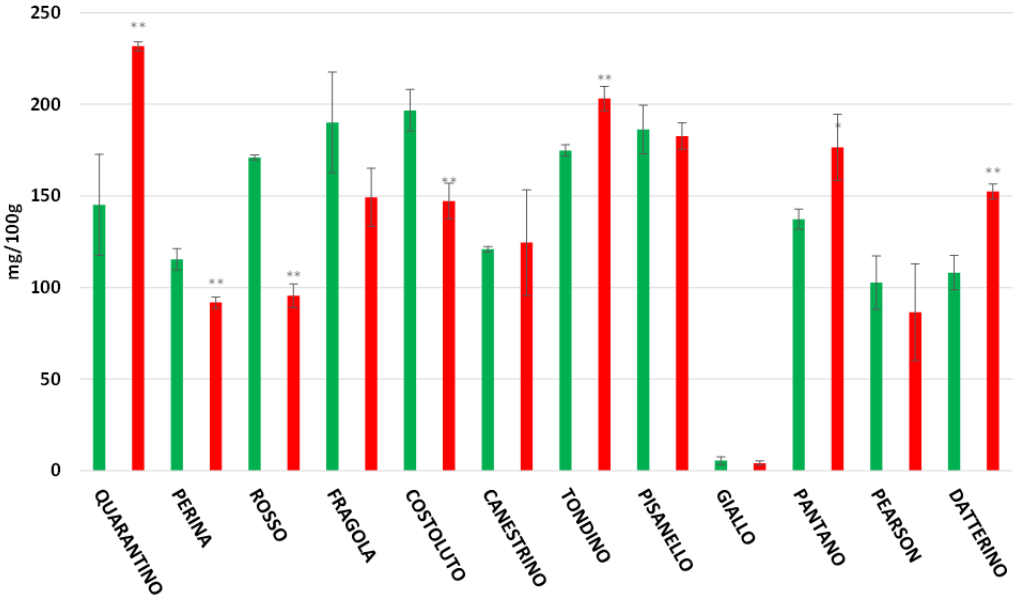


Figure 4.12. Lycopene content in tomato peel of 9 Italian cultivars and three commercial cultivars. Controls (CTRL) in green and drought-stressed group (DS) in red. The bars indicate the standard deviation. A significantly difference is shown between CTRL and DS of each cultivar by * for a $p \leq 0.05$ and ** for $p \leq 0.01$.

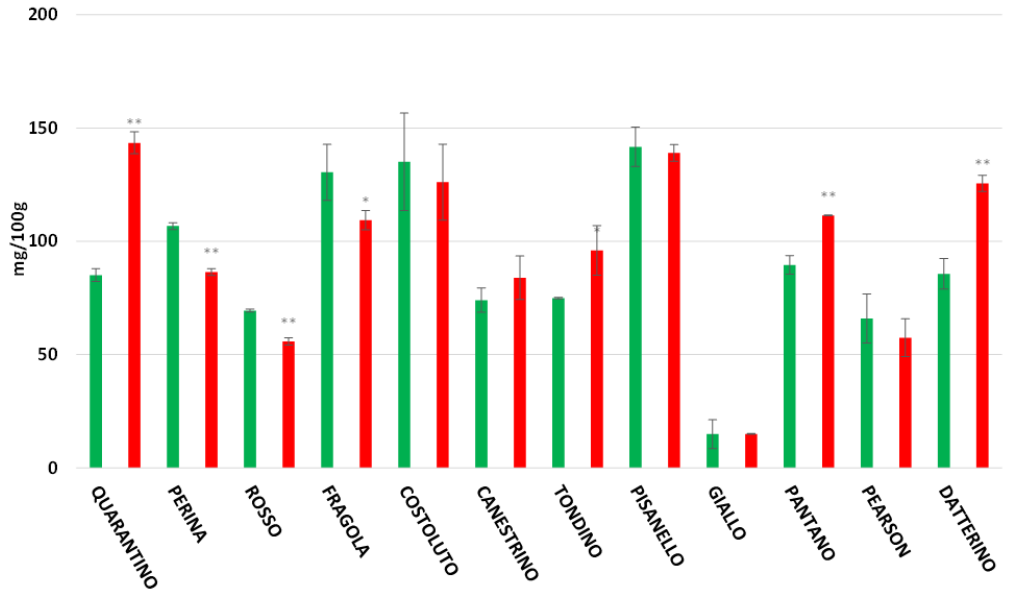


Figure 4.13. Lycopene Content in tomato pulp of 9 Italian cultivars and three commercial cultivars. Controls (CTRL) in green and drought-stressed group (DS) in red. The bars indicate the standard deviation. A significantly difference is shown between CTRL and DS of each cultivar by * for a $p \leq 0.05$ and ** for $p \leq 0.01$.

4.3.8. Rutin, Caffeic Acid and Naringenin

Rutin is the flavonoid most found in tomatoes [164,165]. Chemically, it is a glycoside composed of the flavonol quercetin aglycone and the disaccharide rutinose. In this study, rutin was not identified in the pulp, while it was found in high amounts in the peel of all cultivars (**Figure 4.14**). These showed an increase in the concentration of rutin in the peel of stressed tomatoes, except for the cultivars Perina and Costoluto in which the stressed peel underwent a decrease in concentration. In the cultivars Rosso and Giallo there are no clear differences between CTRL and DS. Quercetin was not found in any of the considered samples.

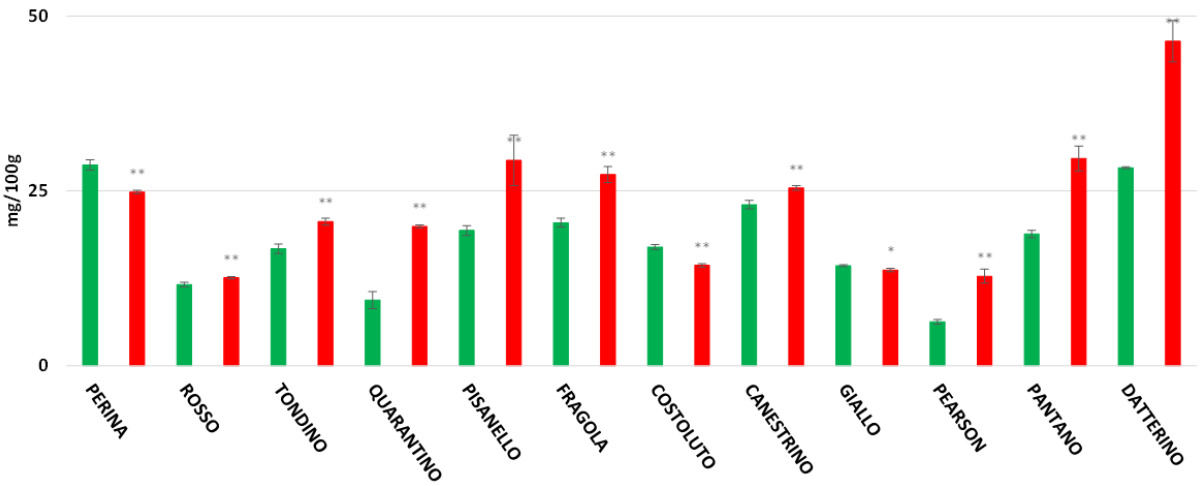


Figure 4.14. Rutin content in tomato peel of 9 Italian cultivars and three commercial cultivars. Controls (CTRL) in green and drought-stressed group (DS) in red. The bars indicate the standard deviation. A significant difference is shown between CTRL and DS of each cultivar by * for a $p \leq 0.05$ and ** for $p \leq 0.01$.

Caffeic acid is part of the hydroxycinnamic acids and belongs to the family of polyphenols. In the present work caffeic acid was found only in four cultivars: Perina, Rosso, Quarantino, and Pisanello (**Figure 4.15**). High concentrations were found in the peel of Perina and Rosso cultivars, with higher values in drought-stressed plants. Small concentrations were instead found in the pulp of the cultivar Perina, both CTRL and DS, while in the cultivar Rosso caffeic acid was found only in the pulp of the CTRL group. In the cultivar Quarantino caffeic acid was found only in the pulp with lower contents in the DS group than in the CTRL. The cultivar Pisanello contains caffeic acid only in the pulp of control fruits while it is absent in stressed fruits.

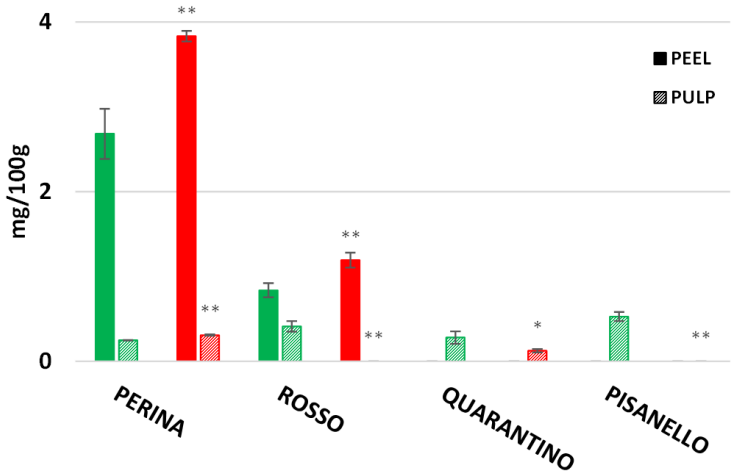


Figure 4.15. Caffeic acid content in 4 Italian tomato cultivars. The concentration of peel in full color and pulp in stripes color. CTRL indicates the control group of tomato and DS the drought-stressed group. The bars indicate the standard deviation. A significant difference is shown between CTRL and DS of each cultivar by * for a $p \leq 0.05$ and ** for $p \leq 0.01$.

Naringenin is a flavanone belonging to the flavonoid family. **Figure 4.16** shows that in most cultivars, naringenin is present only in the fruit peel. The cultivars Fragola, Costoluto,

and the commercial Datterino are the only cultivars that also show naringenin in the pulp. While the Fragola and Datterino cultivars show the presence of this flavonoid only in the pulp of control fruits, Costoluto contains naringenin also in the pulp of stressed fruits. The highest content of naringenin is present in the peel of cultivar Perina, both CTRL and DS, with an increase in the peel of stressed fruits, as well as for cultivar Giallo. In the cultivars Tondino, Pearson, and Pantano, naringenin is only present in the peel of control fruits while it is not present in stressed fruits. In contrast, in the cultivars Costoluto and Datterino, naringenin is present only in the peel of the DS group and not in the CTRL. The cultivars Rosso, Pisanello, and Fragola do not show major differences in the concentration of naringenin between control and stressed peels.

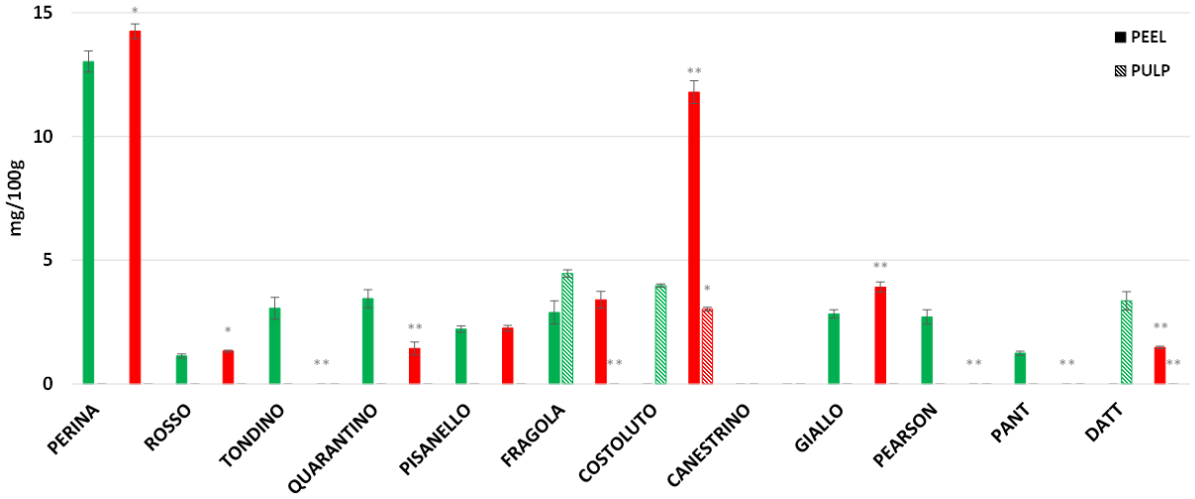


Figure 4.16. Naringenin content in tomatoes of 9 Italian cultivars and three commercial cultivars. The concentration of peel in full color and pulp in stripes color. CTRL indicates the control group of tomato and DS the drought-stressed group. The bars indicate the standard deviation. A significant difference is shown between CTRL and DS of each cultivar by * for a $p \leq 0.05$ and ** for $p \leq 0.01$.

4.4. Discussion

Climate change leads to increasingly sudden adverse events that can damage agriculture and the food livelihood of the population. These critical climatic conditions are also likely to affect southern Europe, including Italy [197]. Several physiological, biochemical, and molecular changes occur in plants because of stressful conditions; for example, the scarce availability of water reduces metabolic processes such as photosynthesis [198].

In Chapter 2. we investigated how local tomato cultivars (the same as in the present study) respond to drought in morpho-physiological terms [105]. The previous study found that drought stress causes a decrease in plant growth and photosynthetic efficiency; however, some local cultivars have proven to be tolerant of stress. The Perina and Quarantino cultivars were the most tolerant, with the first cultivar more tolerant in the vegetative phase while the second cultivar was in the reproductive phase. It should be noted that a higher demand for

water supply is necessary for tomato plants just at the flowering stage [24] and a water shortage during flowering not only reduces flower development, but also increases their fall [199]. This is also confirmed in the present work, which highlights a fall of flowers, both open and fertilized, and a general delay in their development in stressed plants. For all cultivars, a general decrease in the ripening and developmental time of fruits in stressed tomato plants was observed. The cultivars exhibiting this behavior are expected to complete their life cycle. The different behaviors observed between control (CTRL) and stressed (DS) plants are less evident in the Perina and Quarantino cultivars, where development remains similar between CTRL and DS groups.

Earlier germination is generally observed in stressed samples compared to controls, except for Quarantino, where germination is similar, and Perina, where no germination is observed after four days in both CTRL and DS. After eight days, germination remains similar between CTRL and DS for Quarantino and most cultivars, while Perina shows a lower germination rate in stressed samples. When stress affects the final stage of fruit ripening, germination decreases while no effect is noted when stress acts early in fruit development [7]. Under conditions of environmental stress, it is also well-known that germination is delayed or completely inhibited depending on the intensity of stress and the timing of initiation [200].

Oxidative damage (i.e., the production of reactive oxygen species, ROS) is one of the main consequences of water deficit. Plants have an innate antioxidant system that mitigates the effects of stress and involves the synthesis of antioxidant molecules, as already shown in the cultivars under study [25]. Nevertheless, differences have been found between cultivars so that plants of different genotypes do not implement the same mechanisms and consequently the amounts of antioxidant molecules can be different [25]. Drought-induced oxidative stress does not only have downsides: following stressful conditions, plants can increase the content of antioxidant molecules in fruits, resulting in improved quality and thus benefits to human health. In this study, analyses were performed on the tomato fruit by separating the peel from the pulp, which can have very different concentrations of biomolecules. In the peel, which is normally considered a waste, there is a higher concentration of biomolecules; this is not surprising because the peel is in direct contact with the environment and pathogens/parasites [165]. In general, the data obtained in the present study indicate that total antioxidants increase in the stressed group for most local cultivars, while the stressed group of commercial cultivars often exhibit a decrease in antioxidant concentration compared to controls. Among the stressed cultivars with a higher antioxidant power, both in the peel and in the pulp, the cultivars Perina and Canestrino di Lucca exhibit a high value even in the control group.

Data about total polyphenols reveal a situation like the one outlined for the antioxidant power, with an increase in the stressed group compared to the control. From the analysis of peel, the cultivar with the highest concentration of polyphenols is Perina. Compared to all other cultivars, Rosso di Pitigliano increases the concentration of polyphenols in the stressed group. A lower concentration of these compounds in the stressed group is shown in the cultivar Quarantino, both in the peel and in the pulp; it should be remembered that this cultivar tolerated better drought stress during the reproductive phase. A countertrend observed with respect to other compounds is the flavonoid content. The CTRL group of Quarantino is the cultivar with the highest concentration in the peel while Datterino and Perina are those with the highest concentration in the DS group. Thus, from the data of the present study, it is possible to state that the increase in the above molecules varies among cultivars, in agreement with other work in the literature. For example, work on Cucumis melo L. showed that antioxidant power is affected by genotype [201]. In our case, the cultivars with a marked increase in antioxidant molecules are Perina and Rosso di Pitigliano.

The most abundant compounds present in tomato fruits are flavonoids, such as rutin, quercetin, naringenin and caffeic acid, and vitamin C while the most abundant carotenoid is lycopene [163]. These compounds have beneficial effects on human health; in fact, several studies confirm that lycopene plays a role in the prevention of prostate cancer and cardiovascular disease. This is because lycopene may have an inhibitory effect on cholesterol synthesis and may increase the degradation of LDL [202]. In this study, lycopene content in the peel was much higher than in the pulp, but most cultivars decrease lycopene content due to irrigation conditions. There is conflicting data in the literature on this topic. Riggi [203], Atkinson [204], and Klunklin [205] found that drought stress lowers lycopene content compared to well-watered plants. In contrast, Theobald [206] stated that lycopene content increases by more than 27% in drought-stressed fruits. An increase in lycopene content has also been found in tomato fruits grown in southern Italy [207].

Vitamin C is a potent antioxidant that contributes to immune defense by supporting various cellular functions of the innate and adaptive immune systems. Vitamin C also promotes oxidative scavenging activity in the skin, thereby protecting cells from oxidative stress [208]. In our work, there is no increase in vitamin C in stressed plants compared to controls for most cultivars, as with lycopene. This behavior agrees with Seminario [209], where drought stress was shown to cause a reduction in ascorbic acid biosynthesis in soybean plants. The data are also in agreement with Shao [210], in which no increase was reported in tomatoes after drought stress. Other studies have shown that vitamin C increases in relation to water depletion, especially during fruit ripening [207,211], although the magnitude of this

effect may also be cultivar dependent [212]. The vitamin content values found in these cultivars are comparable to those described by Ilahy [213].

Rutin is important for several pharmacological activities, including antioxidant, cytoprotective, vasoprotective, anticarcinogenic, neuroprotective, and cardioprotective activities [214]. The analysis of rutin, naringenin and caffeic acid in this study revealed that their concentration in the pulp is extremely low, if not completely absent. In general, it turned out that the exocarp (peel) is the part where these molecules are most abundant. This was expected since the peel is the part of the fruit most exposed to environmental stresses. The results showed that cultivars behave very differently from each other, with the content of rutin, naringenin, and caffeic acid depending on both genotype and stress conditions. These differences can be attributed to the genetic biodiversity of the cultivars investigated. Perina contains the highest concentration of caffeic acid and naringenin, and large amounts of rutin (highest among controls). For most cultivars, the concentration is higher in stressed fruits than in the control. On the whole, the reported values are higher than those found in the literature [215].

The results of the present work show general agreement with those of Klunklin and Savage [205], i.e., different tomato crops respond differently and therefore generate different concentrations of metabolites when affected by abiotic or biotic stress. Tomato peel, which is much more enriched in bioactives than pulp, is usually considered a waste by processing industries. Actually, the data contained in this work and others indicate that it could be recycled and valorized. In support of this is to mention the recent work of Grassino et al. [216], in which the authors propose the exploitation of the peel for the recovery of bioactives. Approximately 8.5 million tons of peel waste is discarded globally by tomato processing industries; however, valuable bioactive constituents such as lycopene would allow for the revalorization of tomato byproducts that could be incorporated into functional foods [217].

Chapter 5. Conclusions

In this Ph.D. thesis, a detailed analysis of different physiological, morphological, and biochemical parameters was performed, highlighting critical differences of Tuscan tomato cultivars in drought responses. This made it possible to classify tomato cultivars based on their tolerance capacity. Local cultivars show a more pronounced genotype-dependent response to drought than commercial cultivars, both in vegetative and reproductive growth phases, thus emphasizing a different behavior for all nine local and four commercial cultivars. Two groups of plants have been identified: one composed of the cultivars most tolerant to drought, the other of more susceptible plants. In the vegetative phase, the most tolerant cultivar is Perina while in the reproductive phase the cultivar showing more adequate responses is Quarantino. This indicates that the relationship between plants and water deficit also depends on the individual growth phase. Perina and Quarantino are the cultivars that perform at intermediate level (that is, they exhibit an average tolerance) respectively in the reproductive and vegetative phase. In the vegetative phase, four cultivars representative of the tolerant group (Perina and Fragola), of the susceptible group (Pisanello) and of the intermediate group (Quarantino) have been identified. These four Tuscan tomato cultivars were characterized using a biochemical analysis panel. The results revealed critical differences between cultivars in the drought response. Some mechanisms, such as increasing HSP70 and cyclophilin levels, are common and implemented by all cultivars, although with some differences (for example, Perina increases the content of HSP70 more than Pisanello or does not use the same cyclophilins). These data confirm the important protective role of HSP70 and cyclophilins in the correct folding of proteins. Indeed, considering the previous data on the morpho-physiological aspects of the cultivars mentioned above, Perina was the most tolerant cultivar, while Pisanello was the most susceptible.

The content of dehydrin and osmotin is highest when plants are severely affected by drought stress. While dehydrins are substantially expressed by all the cultivars under stress, osmotins are found only in Pisanello. This can be related to the fact that the Pisanello cultivar, identified as the most susceptible, is mostly affected in the photosynthetic system. Therefore, Pisanello is the only cultivar that requires the expression of osmotin, which is known to play a role in the protection of chlorophyll. Analysis of RuBisCO confirmed this hypothesis. Indeed, a drastic decrease in the content of RuBisCO is observed in the Pisanello cultivar under drought stress. In addition, the 9905 isoform of RuBisCO is apparently typical of the most tolerant cultivars (such as Perina and Fragola) but apparently Pisanello does not use it. RuBisCO is an enzyme with several co/post-translational modification sites; therefore,

under stress these modifications can generate isoforms more suitable to counteract a challenging situation such as drought. This concept is further supported by the evidence that Pisanello, compared to other cultivars, shows a very pronounced generic dephosphorylation pattern. Phosphorylation/dephosphorylation contributes to an increase or decrease in sucrose synthase (SuSy) activity. The content of this enzyme increases in all stressed cultivars, especially in Pisanello. SuSy allows the cleavage of sucrose into fructose and UDP-glucose, thus feeding the biosynthesis pathway of osmoprotective sugars. However, the Pisanello cultivar still produces sucrose in large quantities without breaking it down into glucose and fructose, which could be beneficial during stress.

At the biochemical level we have confirmed the results previously obtained from morpho-physiological analyses on the tolerance or susceptibility of tomato cultivars to drought stress. More specifically, we examined the biochemical mechanisms that are activated by drought and that increase tolerance. The Perina cultivar is confirmed as the most tolerant, as it can activate all the mechanisms necessary for tolerance. In particular, it keeps the photosynthetic system active by probably selecting the best RuBisCO isoforms and increasing the content of aquaporins, beneficial for the transport of CO_2 and H_2O.

At the reproductive stage, the experimental evidence of this dissertation showed that, in the absence of drought, Perina is the tomato cultivar with the highest antioxidant power and polyphenol content. On the other hand, the cultivar Quarantino is characterized by a high content of total flavonoids in control and lycopene and vitamin C in stressed plants. It is worth noting that Perina and Quarantino show, although with differences, an improved response to drought. In particular, Quarantino responds more effectively to stress during the reproductive phase. This suggests that specific Tuscan tomato cultivars may be better suited to proper irrigation water management without affecting natural resources and contributing to sustainable agriculture. The second perspective concerns bioactive phytochemicals, such as sterols, carotenes and polyphenols extracted from tomato by-products that could be useful to formulate functional foods and to prevent diseases (such as cardiovascular and Alzheimer's). In fact, the processing waste (peels) of tomato subjected to drought could have an antioxidant action even at low concentrations once integrated into the diet.

From a more general point of view, the data of this PhD thesis confirm that biodiversity is a huge reservoir from which to recover crucial genetic traits, both in terms of productivity and tolerance to abiotic stresses. In the future, the most drought-tolerant tomato cultivars could be selected for breeding programs, also according to their productivity. Another point in favor of using drought-tolerant plants is that sustainable agriculture could benefit more from drought stress-tolerant cultivars because, when used in combination with

appropriate irrigation plans, they can improve agrobiodiversity and save significant amounts of irrigation water.

Bibliography

1. Lionello, P.; Scarascia, L. The relation between climate change in the Mediterranean region and global warming. *Reg. Environ. Chang.* **2018**, *18*, 1481–1493, doi:10.1007/s10113-018-1290-1.

2. Food and Agriculture Organization (FAO) Rome Statement on Water Scarcity in Agriculture. Available online: http://www.fao.org/3/cb2397en/cb2397en.pdf (accessed on Jun 8, 2021).

3. Hanjra, M.A.; Qureshi, M.E. Global water crisis and future food security in an era of climate change. *Food Policy* **2010**, *35*, 365–377, doi:10.1016/j.foodpol.2010.05.006.

4. Chaves, M.M.; Flexas, J.; Pinheiro, C. Photosynthesis under drought and salt stress: Regulation mechanisms from whole plant to cell. *Ann. Bot.* 2009, *103*, 551–560.

5. Singh, M.; Kumar, J.; Singh, S.; Singh, V.P.; Prasad, S.M. Roles of osmoprotectants in improving salinity and drought tolerance in plants: a review. *Rev. Environ. Sci. Biotechnol.* **2015**, *14*, 407–426, doi:10.1007/S11157-015-9372-8/FIGURES/6.

6. Murchie, E.H.; Ruban, A. V. Dynamic non-photochemical quenching in plants: from molecular mechanism to productivity. *Plant J.* **2020**, *101*, 885–896, doi:10.1111/tpj.14601.

7. Pervez, M.A.; Ayub, C.M.; Khan, H.A.; Shahid, M.A.; Ashraf, I. Effect of drought stress on growth, yield and seed quality of tomato (Lycopersicon esculentum L.). *Pak. J. Agri. Sci* **2009**, *46*, 174–178.

8. Gill, S.S.; Tuteja, N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol. Biochem.* **2010**, *48*, 909–930, doi:10.1016/J.PLAPHY.2010.08.016.

9. Cruz De Carvalho, M.H. Drought stress and reactive oxygen species: Production, scavenging and signaling. *Plant Signal. Behav.* 2008, *3*, 156–165.

10. Negri, V. Agro-biodiversity conservation in Europe: Ethical issues. *J. Agric. Environ. Ethics* **2005**, *18*, 3–25, doi:10.1007/s10806-004-3084-3.

11. Pascual, U.; Narloch, U.; Nordhagen, S.; Drucker, A.G. The economics of agrobiodiversity conservation for food security under climate change. *Econ. Agrar. y Recur. Nat.* **2011**, *11*, 191–220, doi:10.22004/AG.ECON.117623.

12. Iglesias, A.; Garrote, L. Adaptation strategies for agricultural water management under climate change in Europe. *Agric. Water Manag.* 2015, *155*, 113–124.

13. Slama, A.; Mallek-Maalej, E.; Mohamed, H. Ben; Rhim, T.; Radhouane, L. A return to the genetic heritage of durum wheat to cope with drought heightened by climate change. *PLoS One* **2018**, *13*, e0196873, doi:10.1371/journal.pone.0196873.

14. Martínez-Cuenca, M.R.; Pereira-Dias, L.; Soler, S.; López-Serrano, L.; Alonso, D.; Calatayud, Á.; Díez, M.J. Adaptation to water and salt stresses of solanum pimpinellifolium and solanum lycopersicum var. cerasiforme. *Agronomy* **2020**, *10*, 1169, doi:10.3390/agronomy10081169.

15. Romano, A.; Sorgonà, A.; Lupini, A.; Araniti, F.; Stevanato, P.; Cacco, G.; Abenavoli, M.R. Morpho-physiological responses of sugar beet (Beta vulgaris L.) genotypes to drought stress. *Acta Physiol. Plant.* **2013**, *35*, 853–865, doi:10.1007/s11738-012-1129-1.

16. Tellah, S.; Badiani, M.; Triflò, P.; Lo Gullo, M.A.; Ounane, G. Morpho-physiological traits contributing to water stress tolerance in a peanut (Arachis hypogaea L.) landraces collection from the Algerian Maghreb - Tellah, S. - Badiani, M. - Triflò, P. - Lo Gullo, M.A. - Ounane, G. - Pisa University Press. *Agrochim. Int. J. Plant Chem. Soil Sci. Plant Nutr. Univ. Pisa* **2014**, *58*, 126–147.

17. Abenavoli, M.R.; Leone, M.; Sunseri, F.; Bacchi, M.; Sorgonà, A. Root Phenotyping For Drought Tolerance in Bean Landraces From Calabria (Italy). *J. Agron. Crop Sci.* **2016**, *202*, 1–12, doi:10.1111/JAC.12124.

18. Zimmerer, K.S.; De Haan, S. Agrobiodiversity and a sustainable food future. *Nat. Plants* 2017, *3*, 1–3.

19. Food and Agriculture Organization (FAO) Agrobiodiversity. A Training Manual for Farmer Groups in East Africa.

20. Ficiciyan, A.M.; Loos, J.; Tscharntke, T. Similar Yield Benefits of Hybrid, Conventional, and Organic

Tomato and Sweet Pepper Varieties Under Well-Watered and Drought-Stressed Conditions. *Front. Sustain. Food Syst.* **2021**, *5*, 628537, doi:10.3389/fsufs.2021.628537.

21. Food and Agriculture Organization (FAO) Tomato | Land & Water | Food and Agriculture Organization of the United Nations | Land & Water | Food and Agriculture Organization of the United Nations Available online: http://www.fao.org/land-water/databases-and-software/crop-information/tomato/en/ (accessed on Jun 9, 2021).

22. Food and Agriculture Organization (FAO) FAOSTAT Available online: https://www.fao.org/faostat/en/#data/QCL/visualize (accessed on Jan 12, 2021).

23. Bartels, D.; Sunkar, R. Drought and Salt Tolerance in Plants. *CRC. Crit. Rev. Plant Sci.* **2005**, *24*, 23–58, doi:10.1080/07352680590910410.

24. Nuruddin, M.M.; Madramootoo, C.A.; Dodds, G.T. Effects of Water Stress at Different Growth Stages on Greenhouse Tomato Yield and Quality. *HortScience* **2003**, *38*, 1389–1393, doi:10.21273/hortsci.38.7.1389.

25. Conti, V.; Mareri, L.; Faleri, C.; Nepi, M.; Romi, M.; Cai, G.; Cantini, C. Drought stress affects the response of italian local tomato (solanum lycopersicum L.) varieties in a genotype-dependent manner. *Plants* **2019**, *8*, 336, doi:10.3390/plants8090336.

26. Landi, S.; Nurcato, R.; De Lillo, A.; Lentini, M.; Grillo, S.; Esposito, S. Glucose-6-phosphate dehydrogenase plays a central role in the response of tomato (Solanum lycopersicum) plants to short and long-term drought. *Plant Physiol. Biochem.* **2016**, *105*, 79–89, doi:10.1016/j.plaphy.2016.04.013.

27. Sánchez-Rodríguez, E.; Rubio-Wilhelmi, Mm.; Cervilla, L.M.; Blasco, B.; Rios, J.J.; Rosales, M.A.; Romero, L.; Ruiz, J.M. Genotypic differences in some physiological parameters symptomatic for oxidative stress under moderate drought in tomato plants. *Plant Sci.* **2010**, *178*, 30–40, doi:10.1016/j.plantsci.2009.10.001.

28. Farooq, M.; Wahid, A.; Kobayashi, N.; Fujita, D.; Basra, S.M.A. Plant drought stress: Effects, mechanisms and management. In *Sustainable Agriculture*; Springer Netherlands, 2009; pp. 153–188 ISBN 9789048126651.

29. Wang, C.; Yang, A.; Yin, H.; Zhang, J. Influence of water stress on endogenous hormone contents and cell damage of maize seedlings. *J. Integr. Plant Biol.* **2008**, *50*, 427–434, doi:10.1111/j.1774-7909.2008.00638.x.

30. Pirasteh-Anosheh, H.; Saed-Moucheshi, A.; Pakniyat, H.; Pessarakli, M. Stomatal responses to drought stress. In *Water Stress and Crop Plants: A Sustainable Approach*; wiley, 2016; Vol. 1–2, pp. 24–40 ISBN 9781119054450.

31. Yoo, C.Y.; Pence, H.E.; Jin, J.B.; Miura, K.; Gosney, M.J.; Hasegawa, P.M.; Mickelbart, M. V. The Arabidopsis GTL1 transcription factor regulates water use efficiency and drought tolerance by modulating stomatal density via transrepression of SDD1. *Plant Cell* **2010**, *22*, 4128–4141, doi:10.1105/tpc.110.078691.

32. Hepworth, C.; Doheny-Adams, T.; Hunt, L.; Cameron, D.D.; Gray, J.E. Manipulating stomatal density enhances drought tolerance without deleterious effect on nutrient uptake. *New Phytol.* **2015**, *208*, 336–341, doi:10.1111/nph.13598.

33. Alves, A.A.C.; Setter, T.L. Response of cassava to water deficit: Leaf area growth and abscisic acid. *Crop Sci.* **2000**, *40*, 131–137, doi:10.2135/cropsci2000.401131x.

34. Baker, N.R.; Rosenqvist, E. Applications of chlorophyll fluorescence can improve crop production strategies: An examination of future possibilities. *J. Exp. Bot.* 2004, *55*, 1607–1621.

35. Anjum, S.A.; Xie, X.-Y.; Wang, L.-C.; Saleem, M.F.; Man, C.; Lei, W. Morphological, physiological and biochemical responses of plants to drought stress. *African J. Agric. Res.* **2011**, *6*, 2026–2032, doi:10.5897/AJAR10.027.

36. Hao, S.; Cao, H.; Wang, H.; Pan, X. The physiological responses of tomato to water stress and re-water in different growth periods. *Sci. Hortic. (Amsterdam).* **2019**, *249*, 143–154, doi:10.1016/j.scienta.2019.01.045.

37. Sade, N.; Gebremedhin, A.; Moshelion, M. Risk-taking plants: anisohydric behavior as a stress-resistance trait. *Plant Signal. Behav.* 2012, *7*, 767–770.

38. Sade, N.; Galkin, E.; Moshelion, M. Measuring Arabidopsis, Tomato and Barley Leaf Relative Water Content (RWC). *BIO-PROTOCOL* **2015**, *5*, doi:10.21769/bioprotoc.1451.

39. Xu, Z.; Zhou, G. Responses of leaf stomatal density to water status and its relationship with photosynthesis in a grass. *J. Exp. Bot.* **2008**, *59*, 3317–3325, doi:10.1093/jxb/ern185.

40. Galmés, J.; Conesa, M.A.; Ochogavía, J.M.; Perdomo, J.A.; Francis, D.M.; Ribas-Carbó, M.; Savé, R.; Flexas, J.; Medrano, H.; Cifre, J. Physiological and morphological adaptations in relation to water use efficiency in Mediterranean accessions of Solanum lycopersicum. *Plant, Cell Environ.* **2011**, *34*, 245–260, doi:10.1111/j.1365-3040.2010.02239.x.

41. Flexas, J.; Ribas-Carbó, M.; Hanson, D.T.; Bota, J.; Otto, B.; Cifre, J.; McDowell, N.; Medrano, H.; Kaldenhoff, R. Tobacco aquaporin NtAQP1 is involved in mesophyll conductance to CO2 in vivo. *Plant J.* **2006**, *48*, 427–439, doi:10.1111/j.1365-313X.2006.02879.x.

42. Muñoz-Perea, C.G.; Allen, R.G.; Westermann, D.T.; Wright, J.L.; Singh, S.P. Water use efficiency among dry bean landraces and cultivars in drought-stressed and non-stressed environments. *Euphytica* **2007**, *155*, 393–402, doi:10.1007/s10681-006-9340-z.

43. Franks, P.J.; W. Doheny-Adams, T.; Britton-Harper, Z.J.; Gray, J.E. Increasing water-use efficiency directly through genetic manipulation of stomatal density. *New Phytol.* **2015**, *207*, 188–195, doi:10.1111/nph.13347.

44. Liu, F.; Stützel, H. Biomass partitioning, specific leaf area, and water use efficiency of vegetable amaranth (Amaranthus spp.) in response to drought stress. *Sci. Hortic. (Amsterdam).* **2004**, *102*, 15–27, doi:10.1016/j.scienta.2003.11.014.

45. Fraser, L.H.; Greenall, A.; Carlyle, C.; Turkington, R.; Friedman, C.R. Adaptive phenotypic plasticity of Pseudoroegneria spicata: Response of stomatal density, leaf area and biomass to changes in water supply and increased temperature. *Ann. Bot.* **2009**, *103*, 769–775, doi:10.1093/aob/mcn252.

46. Toscano, S.; Ferrante, A.; Romano, D. Response of mediterranean ornamental plants to drought stress. *Horticulturae* 2019, *5*, 6.

47. Egilla, J.N.; Davies, F.T.; Boutton, T.W. Drought stress influences leaf water content, photosynthesis, and water-use efficiency of Hibiscus rosa-sinensis at three potassium concentrations. *Photosynthetica* **2005**, *43*, 135–140.

48. Siddiqui, M.H.; Al-Khaishany, M.Y.; Al-Qutami, M.A.; Al-Whaibi, M.H.; Grover, A.; Ali, H.M.; Al-Wahibi, M.S.; Bukhari, N.A. Response of different genotypes of faba bean plant to drought stress. *Int. J. Mol. Sci.* **2015**, *16*, 10214–10227, doi:10.3390/ijms160510214.

49. Zhou, R.; Yu, X.; Ottosen, C.O.; Rosenqvist, E.; Zhao, L.; Wang, Y.; Yu, W.; Zhao, T.; Wu, Z. Drought stress had a predominant effect over heat stress on three tomato cultivars subjected to combined stress. *BMC Plant Biol.* **2017**, *17*, 1–13, doi:10.1186/s12870-017-0974-x.

50. Çelik, Ö.; Ayan, A.; Atak, Ç. Enzymatic and non-enzymatic comparison of two different industrial tomato (Solanum lycopersicum) varieties against drought stress. *Bot. Stud.* **2017**, *58*, 1–13, doi:10.1186/s40529-017-0186-6.

51. Tapia, G.; Méndez, J.; Inostroza, L. Different combinations of morpho-physiological traits are responsible for tolerance to drought in wild tomatoes Solanum chilense and Solanum peruvianum. *Plant Biol.* **2016**, *18*, 406–416, doi:10.1111/plb.12409.

52. Mishra, K.B.; Iannacone, R.; Petrozza, A.; Mishra, A.; Armentano, N.; La Vecchia, G.; Trtílek, M.; Cellini, F.; Nedbal, L. Engineered drought tolerance in tomato plants is reflected in chlorophyll fluorescence emission. *Plant Sci.* **2012**, *182*, 79–86, doi:10.1016/j.plantsci.2011.03.022.

53. Živčák, M.; Brestič, M.; Olšovská, K.; Slámka, P. Performance index as a sensitive indicator of water stress in Triticum aestivum L. *Plant, Soil Environ.* **2008**, *54*, 133–139, doi:10.17221/392-pse.

54. Chaves, M.M.; Zarrouk, O.; Francisco, R.; Costa, J.M.; Santos, T.; Regalado, A.P.; Rodrigues, M.L.; Lopes, C.M. Grapevine under deficit irrigation: hints from physiological and molecular data. *Ann. Bot.* 2010, *105*, 661–676.

55. Liu, F.; Jensen, C.R.; Shahanzari, A.; Andersen, M.N.; Jacobsen, S.E. ABA regulated stomatal control and photosynthetic water use efficiency of potato (Solanum tuberosum L.) during progressive soil drying. *Plant Sci.* **2005**, *168*, 831–836, doi:10.1016/j.plantsci.2004.10.016.

56. Grzesiak, S.; Hordyńska, N.; Szczyrek, P.; Grzesiak, M.T.; Noga, A.; Szechyńska-Hebda, M. Variation among wheat (Triticum easativum L.) genotypes in response to the drought stress: I–selection approaches. *J. Plant Interact.* **2019**, *14*, 30–44, doi:10.1080/17429145.2018.1550817.

57. Talebi, R.; Fayaz, F.; Mohammad Naji, A. Effective selection criteria for assessing drought stress tolerance in durum wheat (Triticum durum Desf.). *Gen. Appl. plant Physiol.* **2009**, *35*, 64–74.

58. Nouraein, M.; Mohammadi, S.A.; Moghaddam, M. Evaluation of drought tolerance indices in wheat recombinant inbred line population. *Ann. Biol. Res.* **2013**, *4*, 113–122.

59. Zhu, J.K. Abiotic Stress Signaling and Responses in Plants. *Cell* **2016**, *167*, 313–324, doi:10.1016/J.CELL.2016.08.029.

60. Liu, F.; Jensen, C.R.; Andersen, M.N.; Liu, F.; Jensen, C.R.; Andersen, M.N. A review of drought adaptation in crop plants: changes in vegetative and reproductive physiology induced by ABA-based chemical signals. *Aust. J. Agric. Res.* **2005**, *56*, 1245–1252, doi:10.1071/AR05062.

61. Singh, R.; Singh, S.; Parihar, P.; Mishra, R.K.; Tripathi, D.K.; Singh, V.P.; Chauhan, D.K.; Prasad, S.M. Reactive Oxygen Species (ROS): Beneficial Companions of Plants' Developmental Processes. *Front. Plant Sci.* **2016**, *0*, 1299, doi:10.3389/FPLS.2016.01299.

62. Cho, E.K.; Hong, C.B. Over-expression of tobacco NtHSP70-1 contributes to drought-stress tolerance in plants. *Plant Cell Rep.* **2006**, *25*, 349–358, doi:10.1007/S00299-005-0093-2.

63. Cho, E.K.; Choi, Y.J. A nuclear-localized HSP70 confers thermoprotective activity and drought-stress tolerance on plants. *Biotechnol. Lett.* **2009**, *31*, 597–606, doi:10.1007/S10529-008-9880-5.

64. Alamillo, J.; Almoguera, C.; Bartels, D.; Jordano, J. Constitutive expression of small heat shock proteins in vegetative tissues of the resurrection plant Craterostigma plantagineum. *Plant Mol. Biol.* **1995**, *29*, 1093–1099, doi:10.1007/BF00014981.

65. Campalans, A., Messeguer, R., Goday, A., & Pagès, M. Plant responses to drought, from ABA signal transduction events to the action of the induced proteins. *Plant Physiol. Biochem.* **1999**, *37*, 327–340.

66. Swindell, W.R.; Huebner, M.; Weber, A.P. Transcriptional profiling of Arabidopsis heat shock proteins and transcription factors reveals extensive overlap between heat and non-heat stress response pathways. *BMC Genomics* **2007**, *8*, 1–15, doi:10.1186/1471-2164-8-125/TABLES/6.

67. Augustine, S.M.; Narayan, J.A.; Syamaladevi, D.P.; Appunu, C.; Chakravarthi, M.; Ravichandran, V.; Subramonian, N. Erianthus arundinaceus HSP70 (EaHSP70) overexpression increases drought and salinity tolerance in sugarcane (Saccharum spp. hybrid). *Plant Sci.* **2015**, *232*, 23–34, doi:10.1016/J.PLANTSCI.2014.12.012.

68. Trivedi, D.K.; Yadav, S.; Vaid, N.; Tuteja, N. Genome wide analysis of Cyclophilin gene family from rice and Arabidopsis and its comparison with yeast. *Plant Signal. Behav.* **2012**, *7*, 1653–1666, doi:10.4161/PSB.22306.

69. Galat, A. Peptidylprolyl cis/trans isomerases (immunophilins): biological diversity--targets--functions. *Curr. Top. Med. Chem.* **2003**, *3*, 1315–1347, doi:10.2174/1568026033451862.

70. Thai, V.; Renesto, P.; Fowler, C.A.; Brown, D.J.; Davis, T.; Gu, W.; Pollock, D.D.; Kern, D.; Raoult, D.; Eisenmesser, E.Z. Structural, biochemical, and in vivo characterization of the first virally encoded cyclophilin from the Mimivirus. *J. Mol. Biol.* **2008**, *378*, 71–86, doi:10.1016/J.JMB.2007.08.051.

71. Berardini, T.Z.; Bollman, K.; Sun, H.; Scott Poethig, R. Regulation of vegetative phase change in Arabidopsis thaliana by cyclophilin 40. *Science* **2001**, *291*, 2405–2407, doi:10.1126/SCIENCE.1057144.

72. Barik, S. Dual-Family Peptidylprolyl Isomerases (Immunophilins) of Select Monocellular Organisms. *Biomol.* 2018, *8*.

73. Marivet, J.; Margis-Pinheiro, M.; Frendo, P.; Burkard, G. Bean cyclophilin gene expression during plant development and stress conditions. *Plant Mol. Biol.* **1994**, *26*, 1181–1189, doi:10.1007/BF00040698.

74. Hong-Bo, S.; Zong-Suo, L.; Ming-An, S. LEA proteins in higher plants: structure, function, gene expression and regulation. *Colloids Surf. B. Biointerfaces* **2005**, *45*, 131–135, doi:10.1016/j.colsurfb.2005.07.017.

75. Close, T.J. Dehydrins: Emergence of a biochemical role of a family of plant dehydration proteins.

Physiol. Plant. **1996**, *97*, 795–803, doi:https://doi.org/10.1111/j.1399-3054.1996.tb00546.x.

76. Riley, A.C.; Ashlock, D.A.; Graether, S.P. Evolution of the modular, disordered stress proteins known as dehydrins. *PLoS One* **2019**, *14*, e0211813, doi:10.1371/JOURNAL.PONE.0211813.

77. Liu, Y.; Song, Q.; Li, D.; Yang, X.; Li, D. Multifunctional Roles of Plant Dehydrins in Response to Environmental Stresses. *Front. Plant Sci.* **2017**, *8*, doi:10.3389/fpls.2017.01018.

78. Katagiri, T.; Ishiyama, K.; Kato, T.; Tabata, S.; Kobayashi, M.; Shinozaki, K. An important role of phosphatidic acid in ABA signaling during germination in Arabidopsis thaliana. *Plant J.* **2005**, *43*, 107–117, doi:https://doi.org/10.1111/j.1365-313X.2005.02431.x.

79. Hara, M.; Shinoda, Y.; Tanaka, Y.; Kuboi, T. DNA binding of citrus dehydrin promoted by zinc ion. *Plant. Cell Environ.* **2009**, *32*, 532–541, doi:10.1111/j.1365-3040.2009.01947.x.

80. Veronese, P.; Ruiz, M.T.; Coca, M.A.; Hernandez-Lopez, A.; Lee, H.; Ibeas, J.I.; Damsz, B.; Pardo, J.M.; Hasegawa, P.M.; Bressan, R.A.; et al. In defense against pathogens. Both plant sentinels and foot soldiers need to know the enemy. *Plant Physiol.* **2003**, *131*, 1580–1590, doi:10.1104/pp.102.013417.

81. Goel, D.; Singh, A.K.; Yadav, V.; Babbar, S.B.; Bansal, K.C. Overexpression of osmotin gene confers tolerance to salt and drought stresses in transgenic tomato (Solanum lycopersicum L.). *Protoplasma* **2010**, *245*, 133–141, doi:10.1007/s00709-010-0158-0.

82. Sokhansanj, A.; Sadat Noori, S.A.; Niknam, V. Comparison of bacterial and plant genes participating in proline biosynthesis with Osmotin gene, with respect to enhancing salinity tolerance of transgenic tobacco plants. *Russ. J. Plant Physiol.* **2006**, *53*, 110–115, doi:10.1134/S1021443706010146.

83. Lutts, S.; Kinet, J.M.; Bouharmont, J. Effects of salt stress on growth, mineral nutrition and proline accumulation in relation to osmotic adjustment in rice (Oryza sativa L.) cultivars differing in salinity resistance. *Plant Growth Regul.* **1996**, *19*, 207–218, doi:10.1007/BF00037793.

84. Hong, Z.; Lakkineni, K.; Zhang, Z.; Verma, D.P. Removal of feedback inhibition of delta(1)-pyrroline-5-carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. *Plant Physiol.* **2000**, *122*, 1129–1136, doi:10.1104/pp.122.4.1129.

85. Vinocur, B.; Altman, A. Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. *Curr. Opin. Biotechnol.* **2005**, *16*, 123–132, doi:10.1016/j.copbio.2005.02.001.

86. Barthakur S, Babu V, B.K. Over-expression of osmotin induces proline accumulation and confers tolerance to osmotic stress in transgenic tobacco. *J Plant Biochem Biotechnol* **2001**, *10*, 31–37.

87. Patel, J.; Mishra, A. Plant aquaporins alleviate drought tolerance in plants by modulating cellular biochemistry, root-architecture, and photosynthesis. *Physiol. Plant.* **2021**, *172*, 1030–1044, doi:https://doi.org/10.1111/ppl.13324.

88. Uehlein, N.; Lovisolo, C.; Siefritz, F.; Kaldenhoff, R. The tobacco aquaporin NtAQP1 is a membrane CO2 pore with physiological functions. *Nature* **2003**, *425*, 734–737, doi:10.1038/nature02027.

89. Hanba, Y.T.; Shibasaka, M.; Hayashi, Y.; Hayakawa, T.; Kasamo, K.; Terashima, I.; Katsuhara, M. Overexpression of the Barley Aquaporin HvPIP2;1 Increases Internal CO2 Conductance and CO2 Assimilation in the Leaves of Transgenic Rice Plants. *Plant Cell Physiol.* **2004**, *45*, 521–529, doi:10.1093/pcp/pch070.

90. Flexas, J.; Ribas-Carbó, M.; Bota, J.; Galmés, J.; Henkle, M.; Martínez-Cañellas, S.; Medrano, H. Decreased Rubisco activity during water stress is not induced by decreased relative water content but related to conditions of low stomatal conductance and chloroplast CO2 concentration. *New Phytol.* **2006**, *172*, 73–82, doi:10.1111/j.1469-8137.2006.01794.x.

91. Singh, R.K.; Deshmukh, R.; Muthamilarasan, M.; Rani, R.; Prasad, M. Versatile roles of aquaporin in physiological processes and stress tolerance in plants. *Plant Physiol. Biochem. PPB* **2020**, *149*, 178–189, doi:10.1016/j.plaphy.2020.02.009.

92. Sreedharan, S.; Shekhawat, U.K.S.; Ganapathi, T.R. Transgenic banana plants overexpressing a native plasma membrane aquaporin MusaPIP1;2 display high tolerance levels to different abiotic stresses. *Plant Biotechnol. J.* **2013**, *11*, 942–952, doi:https://doi.org/10.1111/pbi.12086.

93. Li, R.; Wang, J.; Li, S.; Zhang, L.; Qi, C.; Weeda, S.; Zhao, B.; Ren, S.; Guo, Y.-D. Plasma Membrane Intrinsic Proteins SlPIP2;1, SlPIP2;7 and SlPIP2;5 Conferring Enhanced Drought Stress Tolerance in

Tomato. *Sci. Rep.* **2016**, *6*, 31814, doi:10.1038/srep31814.

94. Wang, L.; Li, Q.-T.; Lei, Q.; Feng, C.; Zheng, X.; Zhou, F.; Li, L.; Liu, X.; Wang, Z.; Kong, J. Ectopically expressing MdPIP1;3, an aquaporin gene, increased fruit size and enhanced drought tolerance of transgenic tomatoes. *BMC Plant Biol.* **2017**, *17*, 246, doi:10.1186/s12870-017-1212-2.

95. Parry, M.A.J.; Andralojc, P.J.; Khan, S.; Lea, P.J.; Keys, A.J. Rubisco Activity: Effects of Drought Stress. *Ann. Bot.* **2002**, *89*, 833–839, doi:10.1093/aob/mcf103.

96. Feller, U.; Anders, I.; Mae, T. Rubiscolytics: fate of Rubisco after its enzymatic function in a cell is terminated. *J. Exp. Bot.* **2008**, *59*, 1615–1624, doi:10.1093/jxb/erm242.

97. Demirevska, K.; Zasheva, D.; Dimitrov, R.; Simova-Stoilova, L.; Stamenova, M.; Feller, U. Drought stress effects on Rubisco in wheat: Changes in the Rubisco large subunit. *Acta Physiol. Plant.* **2009**, *31*, 1129–1138, doi:10.1007/S11738-009-0331-2.

98. Stein, O.; Granot, D. An Overview of Sucrose Synthases in Plants. *Front. Plant Sci.* **2019**, *10*, doi:10.3389/fpls.2019.00095.

99. Baroja-Fernández, E.; Muñoz, F.J.; Li, J.; Bahaji, A.; Almagro, G.; Montero, M.; Etxeberria, E.; Hidalgo, M.; Sesma, M.T.; Pozueta-Romero, J. Sucrose synthase activity in the sus1/sus2/sus3/sus4 Arabidopsis mutant is sufficient to support normal cellulose and starch production. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 321–326, doi:10.1073/pnas.1117099109.

100. Ruan, Y.-L. Sucrose Metabolism: Gateway to Diverse Carbon Use and Sugar Signaling. *Annu. Rev. Plant Biol.* **2014**, *65*, 33–67, doi:10.1146/annurev-arplant-050213-040251.

101. Ruan, Y.-L.; Jin, Y.; Yang, Y.-J.; Li, G.-J.; Boyer, J.S. Sugar input, metabolism, and signaling mediated by invertase: roles in development, yield potential, and response to drought and heat. *Mol. Plant* **2010**, *3*, 942–955, doi:10.1093/mp/ssq044.

102. Akbudak, M.A.; Yildiz, S.; Filiz, E. Pathogenesis related protein-1 (PR-1) genes in tomato (Solanum lycopersicum L.): Bioinformatics analyses and expression profiles in response to drought stress. *Genomics* **2020**, *112*, 4089–4099, doi:10.1016/j.ygeno.2020.07.004.

103. Solankey, S.S.; Singh, R.K.; Baranwal, D.K.; Singh, D.K. Genetic Expression of Tomato for Heat and Drought Stress Tolerance: An Overview. *Int. J. Veg. Sci.* **2015**, *21*, 496–515, doi:10.1080/19315260.2014.902414.

104. Muñoz-Mayor, A.; Pineda, B.; Garcia-Abellán, J.O.; Antón, T.; Garcia-Sogo, B.; Sanchez-Bel, P.; Flores, F.B.; Atarés, A.; Angosto, T.; Pintor-Toro, J.A.; et al. Overexpression of dehydrin tas14 gene improves the osmotic stress imposed by drought and salinity in tomato. *J. Plant Physiol.* **2012**, *169*, 459–468, doi:10.1016/j.jplph.2011.11.018.

105. Conti, V.; Romi, M.; Parri, S.; Aloisi, I.; Marino, G.; Cai, G.; Cantini, C. Morpho-Physiological Classification of Italian Tomato Cultivars (Solanum lycopersicum L.) According to Drought Tolerance during Vegetative and Reproductive Growth. *Plants* **2021**, *10*, 1826, doi:10.3390/plants10091826.

106. Conti, V.; Romi, M.; Guarnieri, M.; Cantini, C.; Cai, G. Italian Tomato Cultivars under Drought Stress Show Different Content of Bioactives in Pulp and Peel of Fruits. *Foods 2022, Vol. 11, Page 270* **2022**, *11*, 270, doi:10.3390/FOODS11030270.

107. Faurobert, M.; Pelpoir, E.; Chaïb, J. Phenol extraction of proteins for proteomic studies of recalcitrant plant tissues. *Methods Mol. Biol.* **2007**, *355*, 9–14, doi:10.1385/1-59745-227-0:9.

108. Hachmann, J.P.; Amshey, J.W. Models of protein modification in Tris-glycine and neutral pH Bis-Tris gels during electrophoresis: effect of gel pH. *Anal. Biochem.* **2005**, *342*, 237–245, doi:10.1016/j.ab.2005.04.015.

109. Parrotta, L.; Aloisi, I.; Suanno, C.; Faleri, C.; Kiełbowicz-Matuk, A.; Bini, L.; Cai, G.; Del Duca, S. A low molecular-weight cyclophilin localizes in different cell compartments of Pyrus communis pollen and is released in vitro under Ca^{2+} depletion. *Plant Physiol. Biochem. PPB* **2019**, *144*, 197–206, doi:10.1016/J.PLAPHY.2019.09.045.

110. Persia, D.; Cai, G.; Del Casino, C.; Faleri, C.; Willemse, M.T.M.; Cresti, M. Sucrose Synthase Is Associated with the Cell Wall of Tobacco Pollen Tubes . *Plant Physiol.* **2008**, *147*, 1603–1618, doi:10.1104/pp.108.115956.

111. Garavaglia, B.S.; Garofalo, C.G.; Orellano, E.G.; Ottado, J. Hsp70 and Hsp90 expression in citrus and pepper plants in response to Xanthomonas axonopodis pv. citri. *Eur. J. Plant Pathol.* **2009**, *123*, 91–97, doi:10.1007/S10658-008-9336-8.

112. Piccini, C.; Cai, G.; Dias, M.C.; Araújo, M.; Parri, S.; Romi, M.; Faleri, C.; Cantini, C. Olive Varieties under UV-B Stress Show Distinct Responses in Terms of Antioxidant Machinery and Isoform/Activity of RubisCO. *Int. J. Mol. Sci. 2021, Vol. 22, Page 11214* **2021**, *22*, 11214, doi:10.3390/IJMS222011214.

113. Polenta, G.; Budde, C.; Sivakumar, D.; Nanni, M.; Guidi, S. Evaluation of biochemical and quality attributes to monitor the application of heat and cold treatments in tomato fruit (Lycopersicon esculentum Mill.). *J. Food Qual.* **2015**, *38*, 153–163, doi:10.1111/JFQ.12139.

114. Kiełbowicz-Matuk, A.; Rey, P.; Rorat, T. The abundance of a single domain cyclophilin in Solanaceae is regulated as a function of organ type and high temperature and not by other environmental constraints. *Physiol. Plant.* **2007**, *131*, 387–398, doi:10.1111/j.1399-3054.2007.00968.x.

115. Liu, H.; Shen, J.; Yuan, C.; Lu, D.; Acharya, B.R.; Wang, M.; Chen, D.; Zhang, W. The Cyclophilin ROC3 Regulates ABA-Induced Stomatal Closure and the Drought Stress Response of Arabidopsis thaliana. *Front. Plant Sci.* **2021**, *12*, 965, doi:10.3389/FPLS.2021.668792/BIBTEX.

116. Sharma, A. D.; Kaur, P. Combined effect of drought stress and heat shock on cyclophilin protein expression in Triticum aestivum. *Gen. Appl. Plant Physiol.* **2009**, *35*, 88–92.

117. Sekhar, K., Priyanka, B., Reddy, V. D., & Rao, K. V. Isolation and characterization of a pigeonpea cyclophilin (CcCYP) gene, and its over-expression in Arabidopsis confers multiple abiotic stress tolerance. *Plant. Cell Environ.* **2010**, *33*, 1324–1338, doi:https://doi.org/10.1111/j.1365-3040.2010.02151.x.

118. Sharma, A.D.; Singh, P. Effect of Water Stress on Expression of a 20 kD Cyclophilin-like Protein in Drought Susceptible and Tolerant Cultivars of Sorghum. *J. Plant Biochem. Biotechnol.* **2003**, *12*, 77–80, doi:10.1007/BF03263165.

119. Lee, S.S.; Park, H.J.; Yoon, D.H.; Kim, B.-G.; Ahn, J.C.; Luan, S.; Cho, H.S. Rice cyclophilin OsCYP18-2 is translocated to the nucleus by an interaction with SKIP and enhances drought tolerance in rice and Arabidopsis. *Plant. Cell Environ.* **2015**, *38*, 2071–2087, doi:10.1111/pce.12531.

120. Goñi, O.; Quille, P.; O'Connell, S. Ascophyllum nodosum extract biostimulants and their role in enhancing tolerance to drought stress in tomato plants. *Plant Physiol. Biochem. PPB* **2018**, *126*, 63–73, doi:10.1016/j.plaphy.2018.02.024.

121. ZamaniBahramabadi, E.; Jonoubi, P.; Rezanejad, F. Dehydrin Content in Fresh and Desiccated Pistachio (Pistacia vera L.) Seeds. *Iran. J. Sci. Technol. Trans. A Sci.* **2019**, *43*, 2099–2105, doi:10.1007/s40995-019-00712-6.

122. Szegő, A.; Badics, E.; Gubala, D.; Oszlányi, R.; Oyuntogtokh, B.E.; Kappel, N.; Papp, I.; Kiss-Bába, E. Diverse responsiveness of dehydrin genes to abscisic acid and water stress treatments in cucumber F1 cultivar hybrids. *https://doi.org/10.1080/14620316.2019.1628665* **2019**, *94*, 726–734, doi:10.1080/14620316.2019.1628665.

123. Garay-Arroyo, A.; Colmenero-Flores, J.M.; Garciarrubio, A.; Covarrubias, A.A. Highly Hydrophilic Proteins in Prokaryotes and Eukaryotes Are Common during Conditions of Water Deficit *. *J. Biol. Chem.* **2000**, *275*, 5668–5674, doi:10.1074/JBC.275.8.5668.

124. Borovskii, G.B.; Stupnikova, I. V; Antipina, A.I.; Vladimirova, S. V; Voinikov, V.K. Accumulation of dehydrin-like proteins in the mitochondria of cereals in response to cold, freezing, drought and ABA treatment. *BMC Plant Biol.* **2002**, *2*, 5, doi:10.1186/1471-2229-2-5.

125. Velasco-Conde, T.; Yakovlev, I.; Majada, J.P.; Aranda, I.; Johnsen, Ø. Dehydrins in maritime pine (Pinus pinaster) and their expression related to drought stress response. *Tree Genet. Genomes* **2012**, *8*, 957–973, doi:10.1007/s11295-012-0476-9.

126. Riyazuddin, R.; Nisha, N.; Singh, K.; Verma, R.; Gupta, R. Involvement of dehydrin proteins in mitigating the negative effects of drought stress in plants. *Plant Cell Rep.* **2021**, doi:10.1007/s00299-021-02720-6.

127. Samarah, N.H.; Mullen, R.E.; Cianzio, S.R.; Scott, P. Dehydrin-Like Proteins in Soybean Seeds in Response to Drought Stress during Seed Filling. *Crop Sci.* **2006**, *46*, 2141–2150,

doi:https://doi.org/10.2135/cropsci2006.02.0066.

128. Lopez, C.G.; Banowetz, G.M.; Peterson, C.J.; Kronstad, W.E. Dehydrin Expression and Drought Tolerance in Seven Wheat Cultivars. *Crop Sci.* **2003**, *43*, 577–582, doi:https://doi.org/10.2135/cropsci2003.5770.

129. Arumingtyas, E.L.; Savitri, E.S.; Purwoningrahayu, R.D. Protein Profiles and Dehydrin Accumulation in Some Soybean Varieties (*Glycine max* L. Merr) in Drought Stress Conditions. *Am. J. Plant Sci.* **2013**, *2013*, 134–141, doi:10.4236/AJPS.2013.41018.

130. Hakim; Ullah, A.; Hussain, A.; Shaban, M.; Khan, A.H.; Alariqi, M.; Gul, S.; Jun, Z.; Lin, S.; Li, J.; et al. Osmotin: A plant defense tool against biotic and abiotic stresses. *Plant Physiol. Biochem. PPB* **2018**, *123*, 149–159, doi:10.1016/j.plaphy.2017.12.012.

131. Grillo, S.; Leone, A.; Xu, Y.; Tucci, M.; Francione, R.; Hasegawa, P.M.; Monti, L.; Bressan, R.A. Control of osmotin gene expression by ABA and osmotic stress in vegetative tissues of wild-type and ABA-deficient mutants of tomato. *Physiol. Plant.* **1995**, *93*, 498–504, doi:https://doi.org/10.1111/j.1399-3054.1995.tb06849.x.

132. Abdin, M.Z.; Kiran, U.; Alam, A. Analysis of osmotin, a PR protein as metabolic modulator in plants. *Bioinformation* **2011**, *5*, 336–340, doi:10.6026/97320630005336.

133. Patade, V.Y.; Khatri, D.; Kumari, M.; Grover, A.; Mohan Gupta, S.; Ahmed, Z. Cold tolerance in Osmotin transgenic tomato (Solanum lycopersicum L.) is associated with modulation in transcript abundance of stress responsive genes. *Springerplus* **2013**, *2*, 117, doi:10.1186/2193-1801-2-117.

134. Jia, J.; Liang, Y.; Gou, T.; Hu, Y.; Zhu, Y.; Huo, H.; Guo, J.; Gong, H. The expression response of plasma membrane aquaporins to salt stress in tomato plants. *Environ. Exp. Bot.* **2020**, *178*, 104190, doi:https://doi.org/10.1016/j.envexpbot.2020.104190.

135. Ouziad, F.; Wilde, P.; Schmelzer, E.; Hildebrandt, U.; Bothe, H. Analysis of expression of aquaporins and Na + /H + transporters in tomato colonized by arbuscular mycorrhizal fungi and affected by salt stress. *Environ. Exp. Bot.* **2006**, *57*, 177–186, doi:10.1016/j.envexpbot.2005.05.011.

136. Balarynova, J.; Fellner, M. Expression of genes encoding PIP aquaporins in tomato seeds exposed to blue radiation and mercury. *Biol. Plant.* **2019**, *63*, 1–10, doi:10.32615/bp.2019.001.

137. Fang, L.; Abdelhakim, L.O.A.; Hegelund, J.N.; Li, S.; Liu, J.; Peng, X.; Li, X.; Wei, Z.; Liu, F. ABA-mediated regulation of leaf and root hydraulic conductance in tomato grown at elevated CO2 is associated with altered gene expression of aquaporins. *Hortic. Res.* **2019**, *6*, 104, doi:10.1038/s41438-019-0187-6.

138. Aroca, R.; Del Mar Alguacil, M.; Vernieri, P.; Ruiz-Lozano, J.M. Plant responses to drought stress and exogenous ABA application are modulated differently by mycorrhization in tomato and an ABA-deficient mutant (sitiens). *Microb. Ecol.* **2008**, *56*, 704–719, doi:10.1007/s00248-008-9390-y.

139. Ricardi, M.M.; González, R.M.; Zhong, S.; Domínguez, P.G.; Duffy, T.; Turjanski, P.G.; Salgado Salter, J.D.; Alleva, K.; Carrari, F.; Giovannoni, J.J.; et al. Genome-wide data (ChIP-seq) enabled identification of cell wall-related and aquaporin genes as targets of tomato ASR1, a drought stress-responsive transcription factor. *BMC Plant Biol.* **2014**, *14*, 29, doi:10.1186/1471-2229-14-29.

140. Reuscher, S.; Akiyama, M.; Mori, C.; Aoki, K.; Shibata, D.; Shiratake, K. Genome-Wide Identification and Expression Analysis of Aquaporins in Tomato. *PLoS One* **2013**, *8*, e79052.

141. Parrotta, L.; Aloisi, I.; Faleri, C.; Romi, M.; Del Duca, S.; Cai, G. Chronic heat stress affects the photosynthetic apparatus of Solanum lycopersicum L. cv Micro-Tom. *Plant Physiol. Biochem. PPB* **2020**, *154*, 463–475, doi:10.1016/j.plaphy.2020.06.047.

142. Hasanagić, D.; Koleška, I.; Kojić, D.; Vlaisavljević, S.; Janjić, N.; Kukavica, B. Long term drought effects on tomato leaves: anatomical, gas exchange and antioxidant modifications. *Acta Physiol. Plant.* **2020**, *42*, 121, doi:10.1007/s11738-020-03114-z.

143. Castillejo, M.A.; Maldonado, A.M.; Ogueta, S.; Jorrin, J. V. Proteomic Analysis of Responses to Drought Stress in Sunflower (Helianthus annuus) Leaves by 2DE Gel Electrophoresis and Mass Spectrometry . *Open Proteomics J.* **2008**, *1*, 59–71, doi:10.2174/1875039700801010059.

144. Houtz, R.L.; Magnani, R.; Nayak, N.R.; Dirk, L.M.A. Co- and post-translational modifications in Rubisco: unanswered questions. *J. Exp. Bot.* **2008**, *59*, 1635–1645, doi:10.1093/jxb/erm360.

145. Heinlein, M.; Starlinger, P. Tissue- and cell-specific expression of the two sucrose synthase isoenzymes in developing maize kernels. *Mol. Gen. Genet. MGG* **1989**, *215*, 441–446, doi:10.1007/BF00427041.

146. Kleczkowski, L.A.; Kunz, S.; Wilczynska, M. Mechanisms of UDP-Glucose Synthesis in Plants. *CRC. Crit. Rev. Plant Sci.* **2010**, *29*, 191–203, doi:10.1080/07352689.2010.483578.

147. Pelah, D.; Wang, W.; Altman, A.; Shoseyov, O.; Bartels, D. Differential accumulation of water stress-related proteins, sucrose synthase and soluble sugars in Populus species that differ in their water stress response. *Physiol. Plant.* **1997**, *99*, 153–159, doi:https://doi.org/10.1111/j.1399-3054.1997.tb03443.x.

148. Xue, G.-P.; McIntyre, C.L.; Glassop, D.; Shorter, R. Use of expression analysis to dissect alterations in carbohydrate metabolism in wheat leaves during drought stress. *Plant Mol. Biol.* **2008**, *67*, 197–214, doi:10.1007/s11103-008-9311-y.

149. Nemati, F.; Ghanati, F.; Ahmadi Gavlighi, H.; Sharifi, M. Comparison of sucrose metabolism in wheat seedlings during drought stress and subsequent recovery. *Biol. Plant.* **2018**, *62*, 595–599, doi:10.1007/s10535-018-0792-5.

150. Du, Y.; Zhao, Q.; Chen, L.; Yao, X.; Zhang, H.; Wu, J.; Xie, F. Effect of Drought Stress during Soybean R2–R6 Growth Stages on Sucrose Metabolism in Leaf and Seed. *Int. J. Mol. Sci.* 2020, *21*.

151. Shi, K.; Fu, L.-J.; Dong, D.-K.; Zhou, Y.-H.; Yu, J.-Q. Decreased energy synthesis is partially compensated by a switch to sucrose synthase pathway of sucrose degradation in restricted root of tomato plants. *Plant Physiol. Biochem. PPB* **2008**, *46*, 1040–1044, doi:10.1016/j.plaphy.2008.07.008.

152. Ponnu, J.; Wahl, V.; Schmid, M. Trehalose-6-Phosphate: Connecting Plant Metabolism and Development. *Front. Plant Sci.* **2011**, *2*, doi:10.3389/fpls.2011.00070.

153. Ramon, M.; Rolland, F. Plant development: introducing trehalose metabolism. *Trends Plant Sci.* **2007**, *12*, 185–188, doi:10.1016/j.tplants.2007.03.007.

154. Duncan, K.A.; Hardin, S.C.; Huber, S.C. The Three Maize Sucrose Synthase Isoforms Differ in Distribution, Localization, and Phosphorylation. *Plant Cell Physiol.* **2006**, *47*, 959–971, doi:10.1093/pcp/pcj068.

155. Raghavendra, A.S.; Gonugunta, V.K.; Christmann, A.; Grill, E. ABA perception and signalling. *Trends Plant Sci.* **2010**, *15*, 395–401, doi:10.1016/j.tplants.2010.04.006.

156. Yang, J.; Zhang, J.; Li, C.; Zhang, Z.; Ma, F.; Li, M. Response of sugar metabolism in apple leaves subjected to short-term drought stress. *Plant Physiol. Biochem. PPB* **2019**, *141*, 164–171, doi:10.1016/j.plaphy.2019.05.025.

157. Parvaiz, A.; Satyawati, S. Salt stress and phyto-biochemical responses of plants - A review. *Plant, Soil Environ.* **2008**, *54*, 89–99, doi:10.17221/2774-PSE.

158. Le Gall, H.; Philippe, F.; Domon, J.-M.; Gillet, F.; Pelloux, J.; Rayon, C. Cell Wall Metabolism in Response to Abiotic Stress. *Plants (Basel, Switzerland)* **2015**, *4*, 112–166, doi:10.3390/plants4010112.

159. Kubo, Y.; Xue, Y.; Nakatsuka, A.; Mathooko, F.M.; Inaba, A.; Nakamura, R. Expression of a Water Stress-induced Polygalacturonase Gene in Harvested Cucumber Fruit. *J. Japanese Soc. Hortic. Sci.* **2000**, *69*, 273–279, doi:10.2503/jjshs.69.273.

160. Tenhaken, R. Cell wall remodeling under abiotic stress. *Front. Plant Sci.* **2015**, *5*, doi:10.3389/fpls.2014.00771.

161. Novaković, L.; Guo, T.; Bacic, A.; Sampathkumar, A.; Johnson, K.L. Hitting the Wall-Sensing and Signaling Pathways Involved in Plant Cell Wall Remodeling in Response to Abiotic Stress. *Plants (Basel, Switzerland)* **2018**, *7*, doi:10.3390/plants7040089.

162. Perveen, R.; Suleria, H.A.R.; Anjum, F.M.; Butt, M.S.; Pasha, I.; Ahmad, S. Tomato (*Solanum lycopersicum*) Carotenoids and Lycopenes Chemistry; Metabolism, Absorption, Nutrition, and Allied Health Claims—A Comprehensive Review. *Crit. Rev. Food Sci. Nutr.* **2015**, *55*, 919–929, doi:10.1080/10408398.2012.657809.

163. Canene-Adams, K.; Campbell, J.K.; Zaripheh, S.; Jeffery, E.H.; Erdman, J.W. The Tomato As a Functional Food. *J. Nutr.* **2005**, *135*, 1226–1230, doi:10.1093/jn/135.5.1226.

164. Martí, R.; Roselló, S.; Cebolla-Cornejo, J. Tomato as a Source of Carotenoids and Polyphenols Targeted

to Cancer Prevention. *Cancers (Basel).* **2016**, *8*, 58, doi:10.3390/cancers8060058.

165. Tamasi, G.; Pardini, A.; Bonechi, C.; Donati, A.; Pessina, F.; Marcolongo, P.; Gamberucci, A.; Leone, G.; Consumi, M.; Magnani, A.; et al. Characterization of nutraceutical components in tomato pulp, skin and locular gel. *Eur. Food Res. Technol.* **2019**, *245*, 907–918, doi:10.1007/s00217-019-03235-x.

166. Chandra, H.M.; Ramalingam, S. Antioxidant potentials of skin, pulp, and seed fractions of commercially important tomato cultivars. *Food Sci. Biotechnol.* **2011**, *20*, 15–21, doi:10.1007/s10068-011-0003-z.

167. Giovannetti, M.; Avio, L.; Barale, R.; Ceccarelli, N.; Cristofani, R.; Iezzi, A.; Mignolli, F.; Picciarelli, P.; Pinto, B.; Reali, D.; et al. Nutraceutical value and safety of tomato fruits produced by mycorrhizal plants. *Br. J. Nutr.* **2012**, *107*, 242–251, doi:10.1017/S000711451100290X.

168. Jesús Periago, M.; Rincón, F.; Dolores Agüera, M.; Ros, G. Mixture Approach for Optimizing Lycopene Extraction from Tomato and Tomato Products. *J. Agric. Food Chem.* **2004**, *52*, 5796–5802, doi:10.1021/jf049345h.

169. Toor, R.K.; Savage, G.P. Antioxidant activity in different fractions of tomatoes. *Food Res. Int.* **2005**, *38*, 487–494, doi:10.1016/j.foodres.2004.10.016.

170. Hernández, I.; Cela, J.; Alegre, L.; Munné-Bosch, S. Antioxidant Defenses Against Drought Stress BT - Plant Responses to Drought Stress: From Morphological to Molecular Features. In; Aroca, R., Ed.; Springer Berlin Heidelberg: Berlin, Heidelberg, 2012; pp. 231–258 ISBN 978-3-642-32653-0.

171. De Storme, N.; Geelen, D. The impact of environmental stress on male reproductive development in plants: Biological processes and molecular mechanisms. *Plant, Cell Environ.* 2014, *37*, 1–18.

172. Hussain, M.; Farooq, S.; Hasan, W.; Ul-Allah, S.; Tanveer, M.; Farooq, M.; Nawaz, A. Drought stress in sunflower: Physiological effects and its management through breeding and agronomic alternatives. *Agric. Water Manag.* 2018, *201*, 152–166.

173. Razzaq, H.; Nadeem Tahir, M.H.; Ahmad Sadaqat, H.; Sadia, B. Screening of sunflower (Helianthus annus L.) accessions under drought stress conditions, an experimental assay. *J. soil Sci. plant Nutr.* **2017**, *17*, 662–671, doi:10.4067/S0718-95162017000300009.

174. Dickin, E.; Wright, D. The effects of winter waterlogging and summer drought on the growth and yield of winter wheat (Triticum aestivum L.). *Eur. J. Agron.* **2008**, *28*, 234–244, doi:10.1016/j.eja.2007.07.010.

175. Wahb-Allah, M.A.; Alsadon, A.A.; Ibrahim, A.A. Drought Tolerance of Several Tomato Genotypes Under Greenhouse Conditions. *World Appl. Sci. J.* **2011**, *15*, 933–940.

176. Harmanto; Salokhe, V.M.; Babel, M.S.; Tantau, H.J. Water requirement of drip irrigated tomatoes grown in greenhouse in tropical environment. *Agric. Water Manag.* **2005**, *71*, 225–242, doi:10.1016/j.agwat.2004.09.003.

177. Sivakumar, R.; Srividhya, S. Impact of drought on flowering, yield and quality parameters in diverse genotypes of tomato (Solanum lycopersicum L.). *Adv. Hortic. Sci.* **2016**, *30*, 3–11, doi:10.13128/ahs-18696.

178. Lamin-Samu, A.T.; Farghal, M.; Ali, M.; Lu, G. Morpho-Physiological and Transcriptome Changes in Tomato Anthers of Different Developmental Stages under Drought Stress. *Cells* **2021**, *10*, 1809, doi:10.3390/cells10071809.

179. Morresi, C.; Cianfruglia, L.; Armeni, T.; Mancini, F.; Tenore, G.C.; D'Urso, E.; Micheletti, A.; Ferretti, G.; Bacchetti, T. Polyphenolic compounds and nutraceutical properties of old and new apple cultivars. *J. Food Biochem.* **2018**, *42*, doi:10.1111/jfbc.12641.

180. Sica, P.; Galvao, A.; Scariolo, F.; Maucieri, C.; Nicoletto, C.; Pilon, C.; Sambo, P.; Barcaccia, G.; Borin, M.; Cabrera, M.; et al. Effects of Drought on Yield and Nutraceutical Properties of Beans (Phaseolus spp.) Traditionally Cultivated in Veneto, Italy. *Horticulturae* **2021**, *7*, 17, doi:10.3390/horticulturae7020017.

181. Sumalan, R.M.; Ciulca, S.I.; Poiana, M.A.; Moigradean, D.; Radulov, I.; Negrea, M.; Crisan, M.E.; Copolovici, L.; Sumalan, R.L. The Antioxidant Profile Evaluation of Some Tomato Landraces with Soil Salinity Tolerance Correlated with High Nutraceuticaland Functional Value. *Agronomy* **2020**, *10*, 500, doi:10.3390/agronomy10040500.

182. Ruiz, J.J.; García-Martínez, S.; Picó, B.; Gao, M.; Quiros, C.F. Genetic Variability and Relationship of Closely Related Spanish Traditional Cultivars of Tomato as Detected by SRAP and SSR Markers. *J. Am. Soc. Hortic. Sci.* **2005**, *130*, 88–94, doi:10.21273/JASHS.130.1.88.

183. Cesare, M.M.; Felice, F.; Conti, V.; Cerri, L.; Zambito, Y.; Romi, M.; Cai, G.; Cantini, C.; Di Stefano, R. Impact of Peels Extracts from an Italian Ancient Tomato Variety Grown under Drought Stress Conditions on Vascular Related Dysfunction. *Molecules* **2021**, *26*, 4289, doi:10.3390/molecules26144289.

184. Wei, Z.; Fang, L.; Li, X.; Liu, J.; Liu, F. Endogenous ABA level modulates the effects of CO2 elevation and soil water deficit on growth, water and nitrogen use efficiencies in barley and tomato plants. *Agric. Water Manag.* **2021**, *249*, 106808, doi:https://doi.org/10.1016/j.agwat.2021.106808.

185. Azzi, L.; Deluche, C.; Gévaudant, F.; Frangne, N.; Delmas, F.; Hernould, M.; Chevalier, C. Fruit growth-related genes in tomato. *J. Exp. Bot.* **2015**, *66*, 1075–7086, doi:10.1093/jxb/eru527.

186. Mazzucato, A.; Taddei, A.R.; Soressi, G.P. The parthenocarpic fruit (pat) mutant of tomato sets seedless fruits and has aberrant anther and ovule development. *Development* **1998**, *125*, 107–114.

187. Benzie, I.F.F.; Strain, J.J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Anal. Biochem.* **1996**, *239*, 70–76, doi:10.1006/abio.1996.0292.

188. Singleton, V.L.; Rossi, J.A. Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144–158.

189. Smirnova, L.P.; Pervykh, L.N. Quantitative determination of the total content of flavonoids in a cholagogic species. *Pharm. Chem. J.* **1999**, *33*, 151–154, doi:10.1007/BF02508454.

190. Olives Barba, A.I.; Cámara Hurtado, M.; Sánchez Mata, M.C.; Fernández Ruiz, V.; López Sáenz De Tejada, M. Application of a UV-vis detection-HPLC method for a rapid determination of lycopene and β-carotene in vegetables. *Food Chem.* **2006**, *95*, 328–336, doi:10.1016/j.foodchem.2005.02.028.

191. Scherer, R.; Rybka, A.C.P.; Ballus, C.A.; Meinhart, A.D.; Filho, J.T.; Godoy, H.T. Validation of a HPLC method for simultaneous determination of main organic acids in fruits and juices. *Food Chem.* **2012**, *135*, 150–154, doi:10.1016/j.foodchem.2012.03.111.

192. Tokuşoğlu, Ö.; Ünal, M.K.; Yildirim, Z. HPLC-UV and GC-MS characterization of the flavonol aglycons quercetin, kaempferol, and myricetin in tomato pastes and other tomato-based products. *Acta Chromatogr.* **2003**, 196–207.

193. Kumar, N.; Bhandari, P.; Singh, B.; Gupta, A.P.; Kaul, V.K. Reversed phase-HPLC for rapid determination of polyphenols in flowers of rose species. *J. Sep. Sci.* **2008**, *31*, 262–267, doi:10.1002/jssc.200700372.

194. Srivastava, K.; Kumar, S.; Kumar, S.; Prakash, P.; Vaishampayan, A. Screening of tomato genotypes for reproductive characters under high temperature stress conditions. *Sabrao J. Breed. Genet.* **2012**, *44*, 263–276.

195. Ruíz, R.; Guardiola, J.L. Carbohydrate and mineral nutrition of orange fruitlets in relation to growth and abscission. *Physiol. Plant.* **1994**, *90*, 27–36, doi:10.1111/J.1399-3054.1994.TB02188.X.

196. ALONI, B.; KARNI, L.; ZAIDMAN, Z.; SCHAFFER, A.A. Changes of Carbohydrates in Pepper (Capsicum annuumL.) Flowers in Relation to Their Abscission Under Different Shading Regimes. *Ann. Bot.* **1996**, *78*, 163–168, doi:10.1006/anbo.1996.0109.

197. Brunetti, M.; Maugeri, M.; Monti, F.; Nanni, T. Temperature and precipitation variability in Italy in the last two centuries from homogenised instrumental time series. *Int. J. Climatol.* **2006**, *26*, 345–381, doi:10.1002/joc.1251.

198. LAWLOR, D.W. Limitation to Photosynthesis in Water-stressed Leaves: Stomata vs. Metabolism and the Role of ATP. *Ann. Bot.* **2002**, *89*, 871–885, doi:10.1093/aob/mcf110.

199. Mahendran, S.; Bandara', D.C. Effects of Soil Moisture Stress at Different Growth Stages on Vitamin C, Capsaicin and P-Carotene Contents of Chilli (Capsicum annuum L.) Fruits and their Impact on Yield. *Trop. Agric. Res.* **2000**, *12*, 95–106.

200. Foolad, M.R.; Subbiah, P.; Kramer, C.; Hargrave, G.; Lin, G.Y. *Genetic relationships among cold, salt and drought tolerance during seed germination in an interspecific cross of tomato*; 2003; Vol. 130;.

201. Henan, I.; Tlili, I.; Him, T.R.; Ali, A.B.E.N.; Jebari, H. Carotenoid content and antioxidant activity of local varieties of muskmelon (Cucumis melo L .) grown in Tunisia. *J. New Sci.* **2016**, *29*, 1672–1675.

202. Arab, L.; Steck, S. Lycopene and cardiovascular disease. *Am. J. Clin. Nutr.* **2000**, *71*, 1691S-1695S, doi:10.1093/ajcn/71.6.1691S.

203. Riggi, E.; Patané, C.; Ruberto, G. Content of carotenoids at different ripening stages in processing tomato in relation to soil water availability. *Aust. J. Agric. Res.* **2008**, *59*, 348–353, doi:10.1071/AR07215.

204. J. Atkinson, N.; P. Dew, T.; Orfila, C.; E. Urwin, P. Influence of Combined Biotic and Abiotic Stress on Nutritional Quality Parameters in Tomato (Solanum lycopersicum). *J. Agric. Food Chem.* **2011**, *59*, 9673–9682, doi:10.1021/jf202081t.

205. Klunklin, W.; Savage, G. Effect on Quality Characteristics of Tomatoes Grown Under Well-Watered and Drought Stress Conditions. *Foods* **2017**, *6*, 56, doi:10.3390/foods6080056.

206. Theobald, J.; Bacon, M.; Davies, W. Delivering enhanced fruit quality to the UK tomato industry through implementation of partial root-zone drying. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **2007**, *146*, S241, doi:10.1016/J.CBPA.2007.01.557.

207. Favati, F.; Lovelli, S.; Galgano, F.; Miccolis, V.; Di Tommaso, T.; Candido, V. Processing tomato quality as affected by irrigation scheduling. *Sci. Hortic. (Amsterdam).* **2009**, *122*, 562–571, doi:10.1016/j.scienta.2009.06.026.

208. Carr, A.; Maggini, S. Vitamin C and Immune Function. *Nutrients* **2017**, *9*, 1211, doi:10.3390/nu9111211.

209. Seminario, A.; Song, L.; Zulet, A.; Nguyen, H.T.; González, E.M.; Larrainzar, E. Drought stress causes a reduction in the biosynthesis of ascorbic acid in soybean plants. *Front. Plant Sci.* **2017**, *8*, doi:10.3389/fpls.2017.01042.

210. Shao, G.C.; Wang, M.H.; Liu, N.; Yuan, M.; Kumar, P.; She, D.L. Growth and comprehensive quality index of tomato under rain shelters in response to different irrigation and drainage treatments. *Sci. World J.* **2014**, *2014*, doi:10.1155/2014/457937.

211. Barbagallo, R.N.; Di Silvestro, I.; Patanè, C. Yield, physicochemical traits, antioxidant pattern, polyphenol oxidase activity and total visual quality of field-grown processing tomato cv. Brigade as affected by water stress in Mediterranean climate. *J. Sci. Food Agric.* **2013**, *93*, 1449–1457, doi:10.1002/jsfa.5913.

212. Blokhina, O.; Virolainen, E.; Fagerstedt, K. V Antioxidants, oxidative damage and oxygen deprivation stress: a review. *Ann. Bot.* **2003**, *91 Spec No*, 179–194, doi:10.1093/aob/mcf118.

213. Ilahy, R.; Hdider, C.; Lenucci, M.S.; Tlili, I.; Dalessandro, G. Phytochemical composition and antioxidant activity of high-lycopene tomato (Solanum lycopersicum L.) cultivars grown in Southern Italy. *Sci. Hortic. (Amsterdam).* **2011**, *127*, 255–261, doi:10.1016/j.scienta.2010.10.001.

214. Ganeshpurkar, A.; Saluja, A.K. The Pharmacological Potential of Rutin. *Saudi Pharm. J.* 2017, *25*, 149–164.

215. Raffo, A.; La Malfa, G.; Fogliano, V.; Maiani, G.; Quaglia, G. Seasonal variations in antioxidant components of cherry tomatoes (Lycopersicon esculentum cv. Naomi F1). *J. Food Compos. Anal.* **2006**, *19*, 11–19, doi:10.1016/j.jfca.2005.02.003.

216. Ninčević Grassino, A.; Djaković, S.; Bosiljkov, T.; Halambek, J.; Zorić, Z.; Dragović-Uzelac, V.; Petrović, M.; Rimac Brnčić, S. Valorisation of Tomato Peel Waste as a Sustainable Source for Pectin, Polyphenols and Fatty Acids Recovery Using Sequential Extraction. *Waste and Biomass Valorization* **2020**, *11*, 4593–4611, doi:10.1007/s12649-019-00814-7.

217. Szabo, K.; Cătoi, A.F.; Vodnar, D.C. Bioactive Compounds Extracted from Tomato Processing by-Products as a Source of Valuable Nutrients. *Plant Foods Hum. Nutr.* 2018, *73*, 268–277.

Ringraziamenti

LIBERTÀ: questa è la parola con la quale voglio descrivere il mio percorso di dottorato. Sono stati tre anni difficili, faticosi, pieni di imprevisti e di ostacoli. Sono ricaduti per gran parte del tempo nel periodo della grande pandemia che ha messo a dura prova il mondo intero, ma, nonostante tutto, mi sono sentita LIBERA! Seppure sia rimasta in casa senza poter uscire e portare avanti la ricerca che stavo seguendo, ero LIBERA di esprimermi sempre, e questo grazie alle persone che ho avuto intorno a me.

Il Professore *Giampiero Cai* è riuscito fin dall'inizio a trasmettermi tranquillità, ma anche sicurezza in ciò che stavo realizzando. Con la sua empatia e la sua esperienza è riuscito comunque a rendermi indipendente, responsabile della mia ricerca e LIBERA di poterla realizzare come meglio credevo. *Claudio Cantini* si è da sempre occupato con grande interesse di tutte le ricerche che ho condotto. Con la sua razionalità e decisione è stato in grado di trasmettermi gli aspetti più matematici e organizzativi, ma anche di rendermi LIBERA e responsabile nell'affrontare e contribuire a tanti altri progetti che lo vedevano coinvolto. *Marco Romi* è stato un collega ed è un grande amico. Mi ha aiutato in ogni momento, sia a livello pratico (posizionare 200 piante, organizzarle ed effettuare i vari campionamenti non era certamente banale), che come supporto morale, offrendomi sempre ottimi consigli. Anche lui è stato capace di farmi sentire LIBERA di credere in me stessa, rendendomi sempre partecipe di tutte le realizzazioni di nuovi progetti.

Chiara è una grande amica. È stata anche una collega, certo, una grande collega dalla quale ho imparato tanto, ma è, e rimane, soprattutto una vera amica. Con lei ho condiviso l'intero percorso di dottorato e abbiamo passato tanti momenti, sia difficili che felici, insieme. Ci sono stati pianti e grasse risate condivise, ma l'insegnamento più bello che ho ricevuto da lei è stato quello di rimanere sempre se stessi e LIBERI di esprimersi per ciò che si è, dando sfogo se necessario anche alle proprie debolezze, che rendono più veri e spesso più forti. *Claudia*, è stata la mia compagna di stanza, una collega, ma anche un'amica divertente ed empatica. Lei ha reso familiari le mie giornate in laboratorio e in ufficio, con i consigli e commenti di una mamma. *Sara* è stata dapprima una tesista a cui trasmettere il mio poco sapere, ma poi è diventata una dottoranda anche lei, una collega con la quale ho condiviso tante esperienze lavorative e di ricerca. Da lei ho imparato la LIBERTÀ intesa come energia, giovinezza e mente aperta. *Agata*, *Letizia*, *Lavina* e *Cecilia* sono state quattro colleghe vicine e premurose con le quali ho scambiato LIBERAmente tante chiacchiere, risate e consigli.

Che dire poi di "*Babbo e Mamma*" … mi hanno insegnato a sognare LIBERAmente, a credere sempre in me anche quando credere in sé sessi diventava difficile. Mi hanno insegnato che le difficoltà vanno guardate con aria di sfida e che gli ostacoli sono spesso grandi insegnanti di vita. Mi hanno insegnato che posso essere chi voglio, anche se alla fine per loro sarò sempre una piccola fanciulla che corre LIBERA, ma che poi torna sempre da loro. Per mio fratello *Alberto* spero di essere un faro per non perdersi mai, dal quale prenda spunto e il volo per sentirsi sempre LIBERO di esprimersi.

Ed infine… ma non per importanza, c'è *Alessandro*. Lui è stato per me un ancóra forte e robusta alla quale aggrapparmi in ogni momento. In grado di infondermi sicurezza e spensieratezza, è riuscito negli anni a farmi raggiungere tutti gli obiettivi prefissati con passione e determinazione. Mi ha reso LIBERA e felice di pensare e sognare insieme a lui.

Con queste poche parole ci tengo a ringraziare tutti non solo per la realizzazione di questo traguardo, ma per avermi resa ciò che sono.

Veronica Conti

Firmato il 06/06/2022