Unravelling the mechanisms involved in tomato responses to combined nitrogen and water deficit

Joana Filipa Félix Machado
Doctor's Degree in Agrarian Sciences
Department of Geosciences, Environment and Spatial Plannings
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Supervisor
Susana M. P. Carvalho, Assistant Professor, Faculty of Sciences, University of Porto
Co-supervisor
Marta W. Vasconcelos, Assistant Professor, School of Biotechnology, Catholic University of Portugal
Co-supervisor
Ep Heuvelink, Associate Professor, Wageningen University & Research, The Netherlands


U.PORTO
FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

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Joana Filipa Félix Machado

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Abstract

The European Green Deal (2020) strives to make Europe the first climate-neutral continent. To that end, it is of utmost importance to design sustainable strategies that will contribute to increased nitrogen and water use efficiency in economically important crops. Tomato (*Solanum lycopersicum*) is among the most cultivated vegetable crops worldwide and commercial cultivars are sensitive to water and N deficit representing dramatic yield losses. Despite N and water deficit being two of the most important factors limiting plant growth, only a few studies have addressed the interactions between both abiotic stresses, and new insights are required to improve their use efficiency. Considering this, the major aims of this Ph.D. thesis are to: i) explore mechanisms involved in tomato responses to combined N and water deficit and compare these with the response to single N or water deficit, and ii) explore sustainable mitigation strategies that help tomato plants to cope with these combined abiotic stresses. As a first step to assess plant responses to combined N and water deficit (N+W) tomato seedlings cv. Micro-Tom were exposed to a gradual mild N and/or water deficit levels for 16 days. A large number of parameters were analyzed, at different levels of integration, including: (i) morphological traits; (ii) physiological responses; (iii) plant metabolites as well as N metabolism-related enzymes; (iv) transcript accumulation of carbon and N metabolism-related genes; (v) oxidative stress biomarkers; (vi) non-enzymatic and (vii) enzymatic components of the antioxidant system, both at the transcript and enzyme activity level. This holistic approach allowed us to show, for the first time, that although similarities in morphological responses between plants grown under N and N+W deficits can occur, different molecular and biochemical plant responses were employed by plants subjected to the combined deficit. These responses could not be deduced from those observed under single deficit conditions, and they were organ dependent (with roots and shoots responding differently). For instance, at the root level plants grown under combined N+W deficit were comparable to those of plants exposed to single W deficit alone, sharing similar responses mainly related to osmoregulation (e.g. higher proline, total soluble protein, and NO_3^- concentration, along with a higher NR activity and upregulation of *NR* and *GS* genes) and the antioxidant system (higher APX and CAT activity and *Cu,ZnSOD, APX,* and *CAT2* expression profiles). On the other hand, at the shoot level plants grown under combined deficit were more alike to those exposed to single N deficit, sharing similar responses mainly related to N remobilization (e.g. lower proline concentration, higher C/N ratio, GS and NR activity as well as increased levels of *rcbS* and *rcbL* transcripts). Interestingly, shoot characteristics of plants subjected to combined N+W

deficit presented a general enhancement of several non-enzymatic and enzymatic components (higher total antioxidant capacity and anthocyanins accumulation as well as higher SOD, APX, and CAT activity along with higher upregulation of *APX* and *CAT2* genes) that was not observed under the single deficits' exposure. This suggests in the presence of both deficits tomato seedlings can activate N remobilization and osmoregulation mechanisms and increase their antioxidant responses. Since plant responses differ according to the duration of the imposed stresses, in the second part of this thesis, tomato seedlings were exposed to a more prolonged (27 days) N and/or water deficit to further explore and better understand the underlying mechanism. To that end, attention has also been paid to the morphological and physiological parameters, but additionally, a special focus was given to: i) anatomical traits; ii) macronutrient accumulation and partitioning; and iii) amino acids profile. For the studied deficit levels, the impact on plant growth traits under combined deficit was more pronounced than the one found under the single deficits. However, the anatomical traits were affected to the same extent. Here, we showed that plants grown under N+W deficit used different N remobilization and osmoregulatory mechanism that contributed to different physiological responses. For instance, under N+W deficit root synthesis of asparagine and arginine specifically increased. This led to lower N remobilization from the photosynthetic machinery, as demonstrated by the lower decreases in chlorophyll content and photosynthetic rate compared to plants grown under single N deficit. In contrast, plants subjected to single N deficit responded mainly through N remobilization from the photosynthetic machinery, as demonstrated by the decreased chlorophyll content and photosynthetic rate. Furthermore, under combined N+W deficit plants increased reallocation of K^+ (from roots to shoots) and synthesis of proline (at the root level), which contributed to a higher stomatal closure and lower transpiration rate than in plants grown under single W deficit. On the other hand, under single water deficit, plants invested more in the root system as a strategy to increase uptake of water and nutrients. Concerning the second main aim of the thesis, we analyzed the potential of silicon (Si) application and of exploring the genotypic variation (particularly of the old accessions) as tools to mitigate some of the adverse effects caused by combined N and water deficit. Sodium silicate addition (2 mM) to the nutrient solution of tomato seedlings partly alleviated the stress caused by the co-exposure to N and water deficit, increasing the antioxidant system of these plants mainly at the root level (i.e. SOD, APX and CAT activity) thus reducing the negative impact of the stress in several growth traits (e.g. root and total dry weight as well as root length). We also showed in this thesis, for the first time, that tomato has a considerably high variability in its response to the combined

deficit, and that old accessions might represent a valuable gene pool in terms of tolerance to these combined abiotic stresses. Taken together, these findings highlight that tomato plants grown under combined stresses employ specific response mechanisms that cannot be deduced from the responses obtained when both stressors are individually applied. However, understanding the full mechanisms involved in the complex interactions between combined stress factors is still in its infancy. With the large dataset collected in the present study, we expect to motivate future studies addressing tomato responses to combined N and W deficits to deeply understand the interactive consequences of N starvation and water shortage on this crop throughout its life cycle. Moreover, this thesis highlights the potential of Si and genotypic variation as valuable tools to increase tomato tolerance to combined N and water deficit, which is highly relevant under the current population growth and climate change context.

Keywords: antioxidant system, combined abiotic stress, gene expression, genotypic variation, nitrogen remobilization, cv Micro-Tom, mitigation strategies, plant metabolism, photosynthesis, osmoregulation, silicon, sustainable agriculture

Resumo

O Green Deal Europeu (2020) pretende tornar a Europa o primeiro continente com impacto neutro no clima. Para isso, é de elevada importância desenhar estratégias sustentáveis que possam contribuir para aumenta a eficiência do uso de azoto e água em culturas economicamente importantes. O tomate (*Solanum lycopersicum*) está entre as culturas hortícolas mais cultivadas mundialmente, mas cultivares comerciais são sensíveis à deficiência de água e N, representando perdas dramáticas de produtividade. Apesar dos déficits de azoto e de água serem dois dos fatores mais importantes a limitar o crescimento das plantas, apenas alguns estudos abordaram as interações entre ambos os stresses abióticos, e novos conhecimentos são necessários para melhorar a eficiência do seu uso. Tendo isto em consideração, os principais objetivos desta tese de doutoramento são: i) explorar os mecanismos envolvidos nas respostas do tomateiro ao déficit combinado de azoto e água e compará-los com a resposta ao déficit individual de azoto ou de água e ii) explorar estratégias de mitigação sustentáveis que ajudem as plantas de tomate a lidar com a combinação destes stresses abióticos. Como primeiro passo para avaliar as respostas das plantas ao déficit combinado de azoto e água, plântulas de tomate foram expostas a um nível moderado de déficit de azoto e/ou água por 16 dias. Um grande número de parâmetros foi analisado, a diferentes níveis de integração, incluindo: (i) características morfológicas; (ii) respostas fisiológicas; (iii) metabolitos secundários, bem como enzimas relacionadas com o metabolismo de azoto; (iv) acumulação de fatores de transcrição de genes relacionados com o metabolismo de carbono e azoto; (v) biomarcadores de stresse oxidativo; (vi) componentes não enzimáticas e (vii) enzimáticas do sistema antioxidante, tanto ao nível da transcrição como da atividade enzimática. Esta abordagem holística permitiu mostrar pela primeira vez que, apesar semelhanças nas respostas morfológicas entre plantas cultivadas sob déficit individual de azoto e déficit combinado de azoto e água poderem ocorrer, diferentes respostas moleculares e bioquímicas foram utilizadas pelas plantas submetidas ao déficit combinado. Estas respostas não puderam ser deduzidas das observadas nas plantas sob stress individual, e foram dependentes do órgão (raízes e parte aérea a responderem diferenciadamente). Por exemplo, ao nível radicular plantas crescidas em déficit combinado de azoto e água foram comparáveis às das plantas expostas apenas ao déficit individual de água, compartilhando respostas semelhantes principalmente relacionadas com a osmorregulação (por exemplo, prolina, proteína solúvel total e concentração de NO_3^- mais elevadas, juntamente com uma maior atividade da NR e um aumento da expressão dos genes relacionados com a NR e GS)

e com o sistema antioxidante (maior atividade da APX e CAT e da expressão dos genes *Cu,ZnSOD*, *APX* e *CAT2*). Por outro lado, ao nível da parte aérea as plantas crescidas em déficit combinado foram mais idênticas às expostas ao déficit individual de azoto, compartilhando respostas semelhantes principalmente relacionadas com a remobilização de azoto (por exemplo, menor concentração de prolina, maior relação C/N, atividade da GS e NR, bem como como níveis aumentados dos transcritos rcbS e rcbL). Curiosamente, as características da parte aérea das plantas submetidas ao déficit combinado de azoto e água apresentaram um aumento de vários componentes não enzimáticos e enzimáticos (maior capacidade antioxidante total e acumulação de antocianinas, bem como maior atividade da SOD, APX e CAT, juntamente com uma regulação positiva dos genes *APX* e CAT*2*) que não foi observada sob a exposição aos déficits individuais. Isto sugere que na presença de ambos os déficits as plântulas de tomate podem ativar mecanismos de remobilização e osmorregulação de azoto e aumentam a sua resposta antioxidante. Uma vez que a resposta das plantas difere de acordo com a duração dos déficits impostos, na segunda parte desta tese, plântulas de tomate foram expostas a um déficit mais prolongado (27 dias) de azoto e/ou água (stresse prolongado) para melhor explorar e compreender os mecanismos subjacentes. Para isso, atenção foi novamente dada aos parâmetros morfológicos e fisiológicos, mas adicionalmente um foco especial foi dado a: i) características anatómicas; ii) acumulação e partição de macronutrientes; e iii) perfil de aminoácidos. Para os níveis de déficit estudados, o impacto nas características de crescimento foi mais pronunciado nas plantas crescidas em déficit combinado do que o observado nas plantas crescidas em déficit individual. No entanto, as características anatómicas foram afetadas na mesma medida. Aqui, mostramos que as plantas crescidas sob déficit combinado de azoto e água usaram diferentes mecanismos de remobilização de azoto e de osmorregulação que contribuíram para diferentes respostas fisiológicas. Por exemplo, sob deficiência combinada de azoto e água, a síntese radicular de asparagina e arginina aumentou especificamente. Isso levou a uma menor remobilização de azoto da maquinaria fotossintética, conforme demonstrado pelas menores reduções no teor de clorofila e da taxa fotossintética destas plantas em comparação às das crescidas em déficit individual de azoto. Por outro lado, as plantas submetidas a déficit individual de azoto responderam ao stresse principalmente através da remobilização de azoto da maquinaria fotossintética, conforme demonstrado pela diminuição do teor de clorofila e da taxa fotossintética. Além disso, em déficit combinado de azoto e água, as plantas aumentaram a realocação de K^+ (da raíz para a folha), e a síntese de prolina (a nível radicular), o que contribuiu para um maior fecho estomático e menor taxa de

transpiração do que observado nas plantas crescidas em déficit individual de água. Por outro lado, sob déficit individual de água, as plantas investiram mais no sistema radicular como estratégia para aumentar a absorção de água e nutrientes. Quanto ao segundo objetivo principal da tese, analisámos o potencial da aplicação de silício (Si) e de explorar a variação fenotípica (particularmente de acessos tradicionais) como ferramentas para mitigar alguns dos efeitos adversos causados pelo déficit combinado de azoto e água. A adição de silicato de sódio (2 mM) à solução nutritiva das plântulas de tomate aliviou parcialmente o stresse causado pela co-exposição ao déficit de azoto e água, aumentando o sistema antioxidante dessas plantas principalmente ao nível da raiz (i.e. atividade SOD, APX e CAT) o que reduziu o impacto negativo do stresse em vários parâmetros de crescimento (por exemplo, peso seco da raiz e total, bem como comprimento da raiz). Também mostramos nesta tese, pela primeira vez, que o tomateiro apresenta uma variação fenotípica considerável na sua resposta ao déficit combinado, e que acessos tradicionais, podem representar um pool genético valioso em termos de tolerância a estes stresses abióticos combinados. No seu conjunto, estas descobertas destacam que as plantas de tomate crescidas em stresses combinados empregam mecanismos de resposta específicos que não podem ser deduzidos das respostas obtidas quando ambos os stresses são aplicados individualmente. No entanto, a compreensão de todos os mecanismos envolvidos na complexa interação entre os fatores do stress combinado ainda está numa fase inicial. Com o elevado conjunto de dados recolhidos neste trabalho, esperamos motivar estudos futuros que abordem as respostas do tomate aos déficits combinados de azoto e água de modo que seja possível entender detalhadamente as consequências interativas da carência de azoto e da escassez de água nesta cultura, ao longo de todo o seu ciclo de vida. Além disso, esta tese destaca o potencial do Si e da variação fenotípica como ferramentas valiosas de aumentar a tolerância do tomateiro ao déficit combinado de azoto e água, o que é altamente relevante no contexto atual de crescimento populacional e mudanças climáticas.

Palavras-chave: agricultura sustentável, cv. Micro-Tom, estratégias de mitigação, expressão génica, fotossíntese, metabolismo da planta, remobilização de azoto, osmorregulação, silício, sistema antioxidante, stresse abiótico combinado, variabilidade fenotípica.

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Abbreviations and Acronyms

$^1O_2$·	SINGLET OXYGEN
A	PHOTOSYNTHETIC RATE
ABA	ABSCISIC ACID
ABTS$^{•+}$	2,2-AZINOBIS-(3-ETHYLBENZOTHIAZOLINE-6-SULPHONIC ACID)
C/N	CARBON TO NITROGEN RATIO
ACC	1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID
AMT	AMMONIUM TRANSPORTERS
ANOVA	ANALYSIS OF VARIANCE
AOX	ANTIOXIDANT
APX	ASCORBATE PEROXIDASE
AS	ASPARAGINE SYNTHETASE
AsA	ASCORBIC ACID
ATGs	AUTOPHAGY-RELATED GENES
B	BORON
BR	BRASSINOSTEROID
C	CARBON
Ca	CALCIUM
CAT	CATALASE
cDNA	COMPLEMENTARY DEOXYRIBONUCLEIC ACID
Cl	CHLORINE
CO_2	CARBON DIOXIDE
CTR	CONTROL
Cu	COPPER
cv.	CULTIVAR
DHA	DEHYDROASCORBATE

DNA	DEOXYRIBONUCLEIC ACID
DW	DRY WEIGHT
E	TRANSPIRATION RATE
ETc	EVAPOTRANSPIRATION
ETR	ELECTRON TRANSPORT RATE
FC	FIELD CAPACITY
Fe	IRON
F_v/F_m	MAXIMUM PHOTOCHEMICAL FLUORESCENCE
F_v/F_0	POTENTIAL ACTIVITY OF PSII
F_v'/F_m'	PHOTOCHEMICAL EFFICIENCY OF PSII IN THE LIGHT
FW	FRESH WEIGHT
GABBA	GAMMA-AMINOBUTYRIC ACID
GAE	GALLIC ACID EQUIVALENTS
GOGAT	GLUTAMATE SYNTHASE
GR	GLUTATHIONE REDUCTASE
GS	GLUTAMINE SYNTHASE
g_s	STOMATAL CONDUCTANCE
H_2O_2	HYDROGEN PEROXIDE
HATS	HIGH-AFFINITY TRANSPORT SYSTEMS
$HO^{\cdot}$	HYDROXYL RADICAL
HPLC	HIGH PERFORMANCE LIQUID CHROMATOGRAPHY
HPS	HIGH PRESSURE SODIUM
ICP-OES	INDUCTIVELY COUPLED PLASMA-ATOMIC EMISSION SPECTROMETRY
K	POTASSIUM
LA	LEAF AREA
LAR	LEAF AREA RATIO

LATS	LOW-AFFINITY TRANSPORT SYSTEMS
LP	LIPID PEROXIDATION
LWC	LEAF WEIGHT CONTENT
LWR	LEAF WEIGHT RATIO
MDA	MALONDIALDEHYDE
Mg	MAGNESIUM
Mn	MANGANESE
mRNA	MESSENGER RNA
N	NITROGEN
N+W	NITROGEN AND WATER
Na_2SiO_3	SODIUM SILICATE
NH_4^+	AMMONIUM
NiR	NITRITE REDUCTASE
NO_2	NITROGEN DIOXIDE
NO_3^-	NITRATE
NPQ	NONPHOTOCHEMICAL QUENCHING
NR	NITRATE REDUCTASE
NRTs	NITRATE TRANSPORTERS
NUE	NITROGEN USE EFFICIENCY
NUtE	NITROGEN UTILIZATION EFFICIENCY
O_2^-	SUPEROXIDE ANION
P	PHOSPHORUS
PAL	PHENYLALANINE AMMONIA-LYASE
PCA	PRINCIPAL COMPONENT ANALYSIS
pH	POTENTIAL OF HYDROGEN
PO_4^{3-}	PHOSPHATE

POD	PEROXIDASE
PPFD	PHOTOSYNTHETIC PHOTON FLUX DENSITY
PPM	PARTS PER MILLION
PSII	PHOTOSYSTEM II
PFTE	POLYTETRAFLUOROETHYLENE
QE	QUERCETIN EQUIVALENTS
qP	QUENCHING COEFFICIENT
QTL	QUANTITATIVE TRAIT LOCI
RCB	RuBisCO-CONTAINING BODIES
RNA	RIBONUCLEIC ACID
ROS	REACTIVE OXYGEN SPECIES
RT-qPCR	REVERSE TRANSCRIPTION QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION
RuBisCO	RIBULOSE BISPHOSPHATE CARBOXYLASE-OXYGENASE
S	SULPHUR
S/R	SHOOT-ROOT RATIO
SDGs	SUSTAINABLE DEVELOPMENT GOALS
SEM	STANDARD ERROR OF THE MEAN
Si	SILICON
SLA	SPECIFIC LEAF AREA
SO_2^{-4}	SULPHATE
SOD	SUPEROXIDE DISMUTASE
SPAD	SOIL PLANT ANALYSIS DEVELOPMENT
T/S	TRICHOMES TO STOMATA RATIO
TMA	TOTAL MONOMERIC ANTHOCYANINS
TPC	TOTAL PHENOLIC CONTENT

TSS	TOTAL SOLUBLE SOLIDS
UV	ULTRAVIOLET
W	WATER
WUE	WATER USE EFFICIENCY
Zn	ZINC
ΦPSII	QUANTUM EFFICIENCY OF PSII PHOTOCHEMISTRY

CHAPTER 1

General Introduction

Overview

The world's population is expected to grow to almost 9.5 billion by 2050 meaning that the world food production needs to increase by 70% to feed the world population by that time (Kyalo Willy *et al.*, 2019; van Dijk *et al.*, 2021). However, global climatic change and reduced resource availability threaten our efforts to increase crop yields (Fernandes *et al.*, 2022). Agriculture uses large amounts of inputs, such as water and nitrogen (N) fertilizers, following unsustainable practices that lead to resource depletion and pollution (FAO, 2020a). This sector is by far the largest water user, accounting for about 80% of global freshwater withdrawals (Elbehri, 2015). Additionally, N fertilizer application is projected to increase even further (from 110 M t in 2014 to 236 M t by 2050) (Liang *et al.*, 2019), as this nutrient plays a key role as a crop yield determinant. Nonetheless, the excessive use of N fertilizers seriously impacts the environment (eutrophication of freshwater ecosystems, acidification of agricultural soils, greenhouse gas emissions) and human health (Wang *et al.*, 2019a; Coskun *et al.*, 2017b; Liu *et al.*, 2016). Therefore, a significant challenge in agricultural production systems is to increase crop yield under suboptimal conditions.

Tomato (*Solanum lycopersicum*) is among the most cultivated vegetable crops worldwide, but most commercial cultivars are sensitive to water and N deficit leading to dramatic yield losses (Heuvelink *et al.*, 2020). Consequently, research has been focused on the development of tomato cultivars that are either N- or water-use efficient (NUE, WUE). The focus has been on elucidating the complex biological processes (spanning from gene expression to metabolite accumulation and phenotype translation) involved in the response to either N or W deficit recently reviewed by Machado *et al.*, 2022). However, many essential plant traits depend simultaneously on N and water input (Gonzalez-Dugo *et al.*, 2010). Hence crosstalk likely exists between the biochemical and molecular mechanisms that tomato plants use to perceive and respond to the combination of N and water deficit. Recent studies revealed that the acclimation of several plant species to a combination of different environmental stresses is unique and, therefore, cannot be directly deduced from studying the response of plants obtained when these stresses are applied individually (Pandey *et al.*, 2015; Wu *et al.*, 2022; Zhou *et al.*, 2019a; Zhou *et al.*, 2017). Furthermore, under the current context of climate change and environmental instability, crops often do not face single abiotic stresses but rather a combination of several adverse growth conditions. Assessing opportunities for more sustainable agricultural practices requires an understanding of the mechanisms involved in plant responses to combined stresses. Still, most studies have focused on tomato responses to single N or water deficit rather than on a combination of both.

Besides understanding the basis of tomato response to combined N and water deficit, finding sustainable strategies that enable tomato plants to cope with limited resources and improve NUE and WUE is of utmost urgency. These strategies include both biotechnological and agronomic approaches (recently reviewed by Fernandes *et al.*, 2022). Elicitors are hazard-free compounds capable of inducing defense responses in plants against various abiotic stresses, playing a vital role in helping the plant to mitigate stress-induced changes by modulating an array of processes at the cellular and molecular levels. Among these elicitors, several studies have reported that silicon (Si application to crops, including tomato, enhances their tolerance to drought (recently reviewed by Thorne *et al.*, 2020; Bokor *et al.*, 2021; Malik *et al.*, 2021; Wang *et al.*, 2021; Verma *et al.*, 2022). However, only a few studies investigated the effect of Si in mitigating N deficiency. Studies on Si application might be of importance also for plants grown under the combined N and water deficit since Si can improve the uptake and translocation of different nutrients in some crops (Ali *et al.*, 2020; Minden *et al.*, 2020; Pavlovic *et al.*, 2021). Additionally, plant breeding is a powerful tool to enhance crop resilience to these abiotic stresses, as plants often exhibit genotypic plasticity to minimize the adverse effects of harsh environments (Monforte, 2020). Moreover, so far, the old tomato accessions represent an unexploited genetic resource, but it has been highlighted that they may represent a valuable gene pool for tomato improvement (Conesa *et al.*, 2019; Roohanitaziani *et al.*, 2020). Finally, research focused on agronomical strategies for stress mitigation relies predominantly on studies on single deficits. Thus, the benefits of applying these strategies to plants subjected to combined N and water deficit remain poorly explored.

Aim and outline of the thesis

The knowledge gaps mentioned above led to the main goals of this thesis: 1) explore the mechanisms involved in tomato responses to combined N and water (N+W) deficit and compare these with the response to single N or W deficit and 2) explore sustainable mitigation strategies that help tomato plants to better cope with these combined abiotic deficits. Among the many existing tomato cultivars, the dwarf ‘Micro-Tom’ has become a popular model system for tomato research (Shikata and Ezura, 2016), being the main reason why we choose it for the current study. The specific goals of this thesis are to:

i) evaluate the morpho-physiological responses and primary metabolism of young tomato plants subjected to combined N+W deficit

ii) unravel the antioxidant responses of young tomato plants grown under combined N+W deficit

iii) explore the effect of combined N+W deficit on young tomato plants regarding anatomical traits, as well as on macronutrient accumulation and partitioning and amino acids profile

iv) evaluate if silicon supplementation can be used as a strategy to help young tomato plants cope with N deficit alone and/or when combined with W deficit

v) perform a phenotypic evaluation of 40 tomato genotypes grown under combined N+W deficit.

The thesis aims are in line with the European Green Deal that fosters innovation targeting a healthy and environmentally friendly food system and with the United Nations Sustainable Development Goals (SDGs): SDG2 (Zero Hunger; particularly it's 2.4 milestone that aims to 'ensure sustainable food production systems and implement resilient agricultural practices that increase productivity and production, that help maintain ecosystems, that strengthen capacity for adaptation to climate change) and SDG12 (Ensure sustainable consumption and production patterns).

The present thesis is organized into four Chapters, including a total of seven sub-chapters (Figure 1), each formatted as a scientific paper comprising an Abstract, Introduction, Materials and methods, Results, Discussion, and Conclusions. Briefly, **Chapter 1** holds a General Introduction, followed by two in-depth literature reviews focused on: drought and N stress effects and tolerance mechanisms in tomato (**Chapter 1.1**); and the role of silicon in mitigating drought and nutrient deficiency (**Chapter 1.2**). In **Chapter 2**, the mechanisms involved in tomato responses to combined N+W deficit and the differences with the application of these deficits individually on the model crop 'Micro-Tom' are assessed through: i) the evaluation of the morpho-physiological responses and primary metabolism (**Chapter 2.1**), ii) the responses of the plant antioxidant system (**Chapter 2.2**) and iii) plant anatomical traits, macronutrients accumulation and partitioning, and amino acids profile (**Chapter 2.3**). **Chapter 3** explores two sustainable mitigation strategies with the potential of helping tomato plants to better cope with the combined N+W deficit, namely: i) silicon application (**Chapter 3.1**); and ii) genotypic variability as a first step for breeding towards combined stress tolerance (**Chapter 3.2**). The main achievements, limitations, and prospects of this Ph.D. study are discussed in **Chapter 4**, which is followed by the bibliography used throughout the entire document (Figure 1).

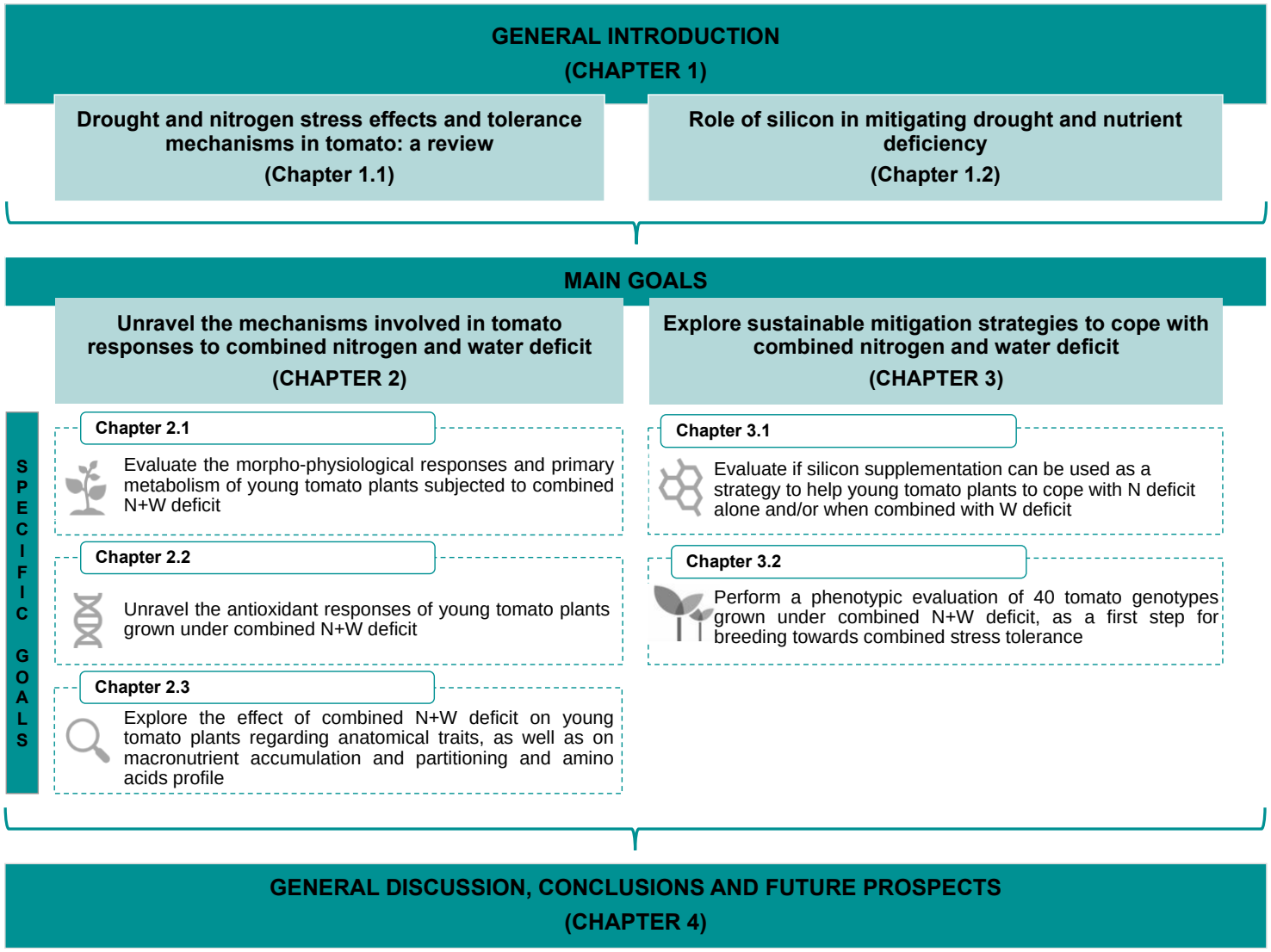


Figure 1 - Schematic presentation of the thesis structure highlighting the goals of each chapter

Sub-chapter 1.1

Drought and nitrogen stress effects and tolerance mechanisms in tomato: a review

The content of this sub-chapter is published as:

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Drought and nitrogen stress effects and tolerance mechanisms in tomato: a review

Abstract

Being two of the most impactful crop-yield determinants, water, and nitrogen (N) greatly affect plant performance, spanning from genes through the whole-plant level. Therefore, adequate water and N management is of utmost importance to face the global societal challenge of sustainably feeding the increasing world population while facing the constraints of climate change. Tomato is an emerging model system because of its economic importance, large genotypic variation, and its recently completely sequenced genome. Moreover, given that tomato plants have high water and N demands, this crop is very attractive for studying plant responses and tolerance mechanisms to deficits in these resources. This explains the huge amount of studies carried out on this topic in recent decades, but so far that valuable information has not yet been reviewed. In this chapter, we critically analyze the impact of drought and N deficiency on tomato at the morphological, physiological, biochemical, and molecular levels. Further, the reported tolerance mechanisms to drought and N deficit are outlined and the current knowledge gaps are summarized. More studies exploring the key genes controlling tolerance in tomato will be crucial to identify new important targets for breeding interventions, making agriculture more productive and sustainable.

1. Introduction

In less than 30 years, humanity will face the challenge of having to feed 9.8 billion inhabitants, which requires a dramatic increase in global agriculture production. The excessive use of water and nitrogen (N) fertilizers is unquestionably the most negatively impactful practice in modern agriculture. Irrigated agriculture accounts for 80% of the total available freshwater, which is making this natural resource increasingly scarce and of poor quality (Elbehri, 2015). N fertilization is a major limiting factor determining crop yield (Liang *et al.*, 2019). However, increased N fertilization has serious impacts on human health and the environment due to the eutrophication of freshwater ecosystems, acidification of agricultural soils, and emission of greenhouse gases (Liang *et al.*, 2019). Therefore, new efforts allowing sustainable management of water and N fertilizers usage will be imperative for feeding the world's growing population.

Despite the need for using fewer resources, it is important to take into account that water and N deficits will significantly affect the crop physiology and yield. Therefore, it is highly important to understand how plants perceive, are affected, and respond to both stresses. Drought stress induces a wide range of physiological and biochemical processes in plants such as stomatal regulation, photosynthetic rapid reaction, and activation of the antioxidant defense system (Laxa *et al*., 2019; Osakabe *et al.*, 2014) and can reduce crop yields up to 70% (Siracusa *et al.*, 2018). N is one of the major components of proteins, nucleic acids, lipids, and some hormones in plants thus having a direct effect on cell division and growth (Tian *et al.*, 2011). Hence, N stress symptoms result from impaired metabolism causing premature senescence and decreased growth, and consequently reduced yields (Fageria and Baligar, 2005).

Tomato (*Solanum lycopersicum*) is the most important vegetable crop in the world, both for fresh and processing markets, with approximately 182.2 million tons produced on more than 5.7 million hectares (FAO, 2020b; Heuvelink *et al.*, 2020). In Europe, the total value of annual tomato production exceeds EUR 7.3 billion, accounting for 21.1% of total fresh vegetables produced with the highest planted area (10.8% of the European Union's total fresh vegetables area) (Cicco, 2019). Moreover, tomato is emerging as a model system because of its (1) economic importance, (2) large genotypic variation, and (3) genome that has been recently completely sequenced (Heuvelink *et al.*, 2020). Tomato production requires high amounts of water and N fertilizers, due to its long growing seasons and high greenhouse temperatures, tomato is quite sensitive to water and N deficits (Du *et al.*, 2018). An adequate water supply is essential for vegetative and reproductive growth being particularly important in flowering and fruit enlargement stages (Srinivasa Rao *et al.*, 2001). Similarly, a proper amount of N fertilizer during plant growth allows maximum yield and economic benefits, because N is an essential macronutrient that can affect several aspects of plant growth and metabolic pathways, including water relations and photosynthesis (Du *et al.*, 2018; Fageria and Baligar, 2005). To halt the excessive use of water and N fertilizers worldwide for economic gains, it is imperative to improve drought and N stress tolerance in tomato cultivars for sustainable and environmental-friendly production. Knowledge of the morphophysiological, biochemical, and molecular responses to drought and N stress is essential for a holistic perception of plant tolerance mechanisms to water and N-limited conditions in higher plants.

In this chapter, we will review the effect of both drought and N stresses on the morphological, physiological, biochemical, and molecular mechanisms in tomato plants and identify potential knowledge gaps. The identification of the machinery

related to water use efficiency and N use efficiency (WUE/NUE) will be crucial to identify new key targets for breeding interventions to make agriculture more productive and sustainable.

2. Tomato plant responses to drought stress

The mechanisms involved in response to drought stress and its effects on plants have been reviewed often in the last decades (e.g., Chaves *et al.*, 2003; Kapoor *et al.*, 2020; Nadeem *et al.*, 2019; Sun *et al.*, 2020b) and are synthesized in Figure 1. Drought is one of the most relevant environmental stresses affecting plant metabolism at different levels. Approximately one-third of agricultural land experiences an inadequate water supply (Klunklin and Savage, 2017). Plants respond and adapt to drought through important biochemical and physiological changes at the cellular and molecular levels (Osakabe *et al.*, 2014). Drought alters water relations that lead to the formation of reactive oxygen species (ROS), reduced seed germination, impaired plant growth and photosynthesis, and biomass and yield losses (Shao *et al.*, 2008).

In the section below we review the impact of drought on the tomato crop at several integration levels and we thoroughly explore the reported tolerance mechanisms to drought.

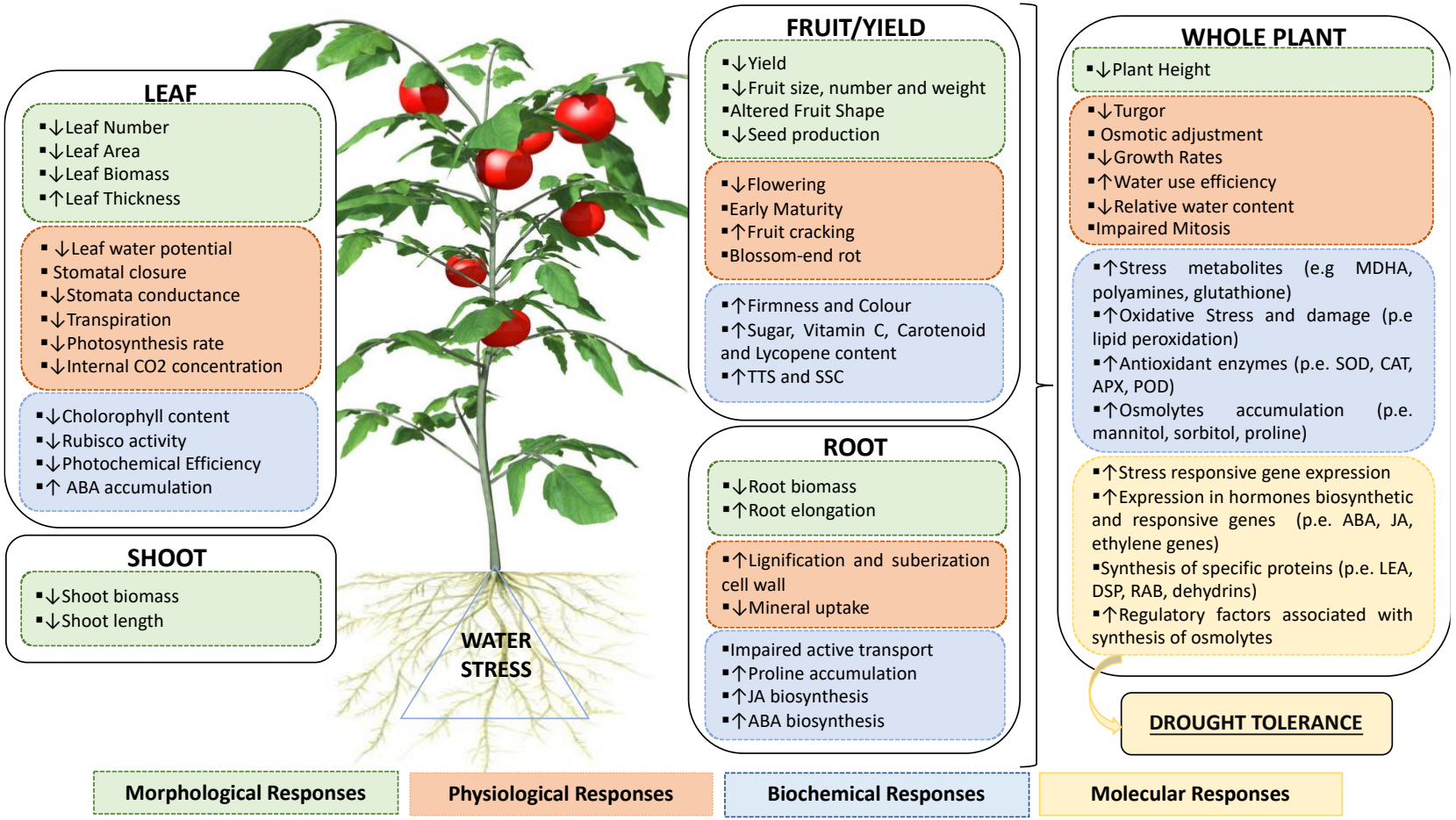


Figure 1 - Schematic overview of the morphological, physiological, biochemical, and molecular processes occurring at specific tomato plant organs or the whole-plant level in response to drought stress.

2.1. Impact on plant growth, yield, and fruit quality

2.1.1. Plant growth and development

Drought has effects on plant growth from the onset of seed germination. Successful seed germination is crucial for seedling establishment and crop yield. However, one of the initial effects of drought stress is poor or slow germination and reduced seedling vigor and establishment (Bhatt and Srinivasa Rao, 1987; Esan *et al.*, 2018; Foolad *et al.*, 2003; Pervez *et al.*, 2009). Studies have demonstrated that the extension growth of the radicles is affected by drought stress suggesting that the effect on germination occurs prior to this process (Bhatt and Srinivasa Rao; 1987). Accordingly, it was found that total recovery of germination (100%) can occur if non-germinated seeds are displaced from this stressful condition (Esan *et al.*, 2018). Drought stress can have major impacts on subsequent plant growth and development due to the reduction of water potential, relative water content, and turgor, which consequently increases the concentration of solutes in the cytosol (Nonami, 1998). These changes impair both cell elongation and expansion, leading to growth inhibition (Jaleel *et al.*, 2009; Salehi-Lisar *et al.*, 2012). When plants are exposed to drought, the water pressure inside the leaves decreases, leading to reduced carbon assimilation, imbalance of mineral nutrition, and accumulation of abscisic acid (ABA), causing wilt and reduction of plant growth (Jaleel *et al.*, 2009; Salehi-Lisar *et al.*, 2012).

The development of an optimal leaf area (LA) is of extreme importance for photosynthesis and, consequently, for plant growth. Leaf expansion depends on the turgor pressure and supply of assimilates. Drought stress limits leaf expansion by disrupting water flow from the xylem to the elongating cells, reducing turgor pressure, and impairing photosynthesis (Hsiao and Xu, 2000; Nonami, 1998). Accordingly, a reduction of LA is one of the main traits affected by drought stress which limits crop productivity (Sinclair and Muchow, 2001). Moreover, data suggest that the reduction of specific leaf area (SLA) is likely a strategy to cope with drought stress. Thicker leaves commonly have a higher content of chlorophyll and proteins per LA unit, thus having a greater photosynthetic capability, and therefore a higher biomass production (Chatterjee and Solankey, 2015). Pokluda *et al.* (2010) have shown that tomato plants respond to drought stress by significantly decreasing SLA and leaf weight content (LWC). Other parameters related to leaf growth, namely the number of leaves per plant, number of branches per plant, total LA, SLA, LA ratio, leaf weight ratio, and LA index are significantly affected by drought stress in tomato (Selim and El-Nady, 2011). In particular, studies in three different tomato cultivars (‘Arvento’, ‘LA1994’, and ‘LA2093’) have

demonstrated that drought stress significantly decreases the LA, leaf number, and leaf angle, and increases the leaf temperature, leading to plant wilting (Zhou *et al.*, 2017). Al Hassan *et al.* (2015) also showed that for cherry tomato, drought stress impairs stem growth and leaf number. Results from Kamanga also demonstrated that drought stress resulted in significant reductions in shoot dry weight, LA, and shoot height of 14 different commercial tomato cultivars (Kamanga, 2020).

In many agronomically important crops, drought stress is initially perceived by the root system, which continues to grow underneath the soil even though shoot growth is inhibited under these conditions (Spollen and Sharp, 1991). Although the growth of the primary root is usually not severely affected by drought stress, the lateral roots growth is significantly reduced mainly by suppression of the activation of the lateral root meristems (Deak and Malamy, 2005; Du and Scheres, 2018; Franco *et al.*, 2011; Koevoets *et al.*, 2016). Experiments in tomato plants have demonstrated that drought stress triggers a decrease in lateral root number and density, and that elongation of the primary root axis is favored over that of the lateral ones (Hooker and Thorpe, 1997). Further, Nuruddin (2001) showed that tomato plants tend to develop a deeper root system at lower soil water potentials. Interestingly, data on sandy soils have also demonstrated that drought stress strongly induces the development of deeper roots (Alaoui *et al.*, 2014). Besides root architecture, root weight, length, and volume are also affected and are important indicators of WUE (Senthilkumar *et al.*, 2017). Water deficit leads to decreased root hydraulic conductivity and long-lasting root anatomical changes, such as xylem elements with smaller diameters and lower total root xylem area (Hernandez-Espinoza and Barrios-Masias, 2020).

Although drought stress may decrease root elongation and LA expansion, these two processes are not equally affected, as roots are less sensitive than shoots to growth inhibition by low water potentials (Wu and Cosgrove, 2000). Thus increased root/shoot ratio is a frequent trait observed in some tolerant tomato genotypes in response to different drought conditions (Du *et al.*, 2018; Manoj and Uday, 2007; Mingo *et al.*, 2004; Moles *et al.*, 2018) as also shown by Nahar and Gretzmache (2011) in seven tomato cultivars. However, an opposite pattern was observed on the economically relevant cultivar ‘Moneymaker’ (Moles *et al.*, 2018), suggesting that a reduced root/shoot ratio can be part of an alternative strategy activated by certain cultivars to cope with drought in which losses in biomass are compensated by the maintenance of the water status in the tissues (Liu and Li, 2005).

As a consequence of the effect on root and shoot development, the plant growth rate has been shown to be significantly impacted by drought stress. Different studies

have reported a negative effect on the fresh and dry weight of leaves, stems, and roots, and consequently in the total plant fresh and dry weight when tomato plants were exposed to drought conditions (Al Hassan *et al.*, 2015; Khan *et al.*, 2015; Selim and El-Nady, 2011; Sibomana *et al.*, 2013; Zhou *et al.*, 2019a). Changes in plant height, stem diameter, and internode length were also reported for tomato plants under similar conditions (Al Hassan *et al.*, 2015; Sibomana *et al.*, 2013; Zhou *et al.*, 2017). In conclusion, drought stress strongly impacts overall tomato plant growth and developmental traits, beginning with germination and impacting traits related to LA, root morphology, and biomass partitioning.

2.1.2. Yield and fruit quality

As a consequence of the impact on plant dry weight, drought affects crop productivity, reducing 50% to 70% of crop yield worldwide (Siracusa *et al.*, 2018). The flowering and fruit development stages are the most sensitive to water deficits in tomato (Chen *et al.*, 2013; Khapte *et al.*, 2019). A deficitary irrigation impacts flower number, which consequently results in fewer fruits produced and/or in fruits with decreased size, and ultimately to less marketable yield (Giardini *et al.*, 1988). Further, drought during seed development decreases seed yield by shortening the final seed-filling periods, thus impacting yield (Pervez *et al.*, 2009). Chen *et al.* (2013) showed that a reduction in irrigation supply (one-third or two-thirds of full irrigation) at the seedling stage has no negative significant influence on the yield and quality of tomato, which suggests that a certain water saving can be achieved at the seedling stage. Similarly, Sivakumar and Srividhya (2016) observed that moderate drought stress can be seen as a positive factor since it speeds up flower initiation. Nevertheless, Hao *et al.* (2019) concluded that compared with the seedling and flowering stages, the application of mild drought stress while the fruit is expanding was considered to be the optimal period to provide satisfactory fruit quality and yield while conserving water resources. On the opposite side, fruit ripening showed to be a highly sensitive stage, and any heterogeneous distribution of irrigation, even if temporary, may induce physiological disorders such as fruit cracking (Srinivasa Rao *et al.*, 2001) and blossom end rot (BER). The latter is a frequent physiological disorder caused by the limited capacity of tomato plants to internally translocate calcium toward fruits under drought conditions (Djangsou *et al.*, 2019; Millones-Chanamé *et al.*, 2019; Tabatabaie *et al*., 2004).

Fresh fruit weight is an important quality feature of tomato that is cultivar-dependent and also highly affected by drought (Wang *et al.*, 2011). Madrid *et al.* (2009) showed that the requirements of tomato fruit for photosynthesis assimilates and water

are drastically increased during the fruit maturation stage, which greatly increases the total water consumption. If the irrigation is reduced, the rate of water absorption will be lower than that of crop transpiration, which induces an internal water deficit affecting photosynthesis and resulting in reduced LA, cell size, and intercellular volume. Thus, fruit water accumulation, and consequently fruit weight, are decreased (Madrid *et al.*, 2009). Sivakumar and Srividhya (2016) also observed that drought stress led to a reduction of soluble protein content, sucrose phosphate synthase (involved in sucrose biosynthesis) activity, fruit volume, diameter, yield, and an increased flower abscission percentage. Nevertheless, water management methods, such as irrigation deficit, in which plants are irrigated with smaller amounts of water during the cultivation period, have been proposed as efficient tools to reduce stomatal conductance without signs of drought stress (Ćosić *et al.*, 2015), while potentially improving fruit quality.

The use of irrigation deficit was recently reviewed by Khapte *et al.* (2019) and it can apparently benefit tomato fruit quality by increasing the level of total soluble solids (sugars, amino acids, and organic acids) while avoiding the negative impact on yield. In the case of tomato, higher levels of lycopene, carotenoids, and total soluble solids content (TSS) are desired traits. A rise in TSS increases the value of fresh fruits and improves their quality by affecting flavor, taste, and water content (Klunklin and Savage, 2017). Irrigation deficit also promotes the ripeness rate in tomato and increases fruit color, as demonstrated by Wang *et al.* (2011). The authors related this phenomenon to an increase in ethylene content, which in turn increases carotenoid concentration. Qi *et al.* (2003) demonstrated that water deficit during fruit maturation increases the activity of sucrose synthase and sucrose phosphate synthase, thus enlarging the gradient of sucrose concentration between leaves and fruits. Consequently, this potentiates the transport of more assimilates into the fruits, increasing the rate and amount of fructose and glucose transformation from sucrose, thus improving fruit TSS (Kan, 2008; Klunklin and Savage, 2017). Nangare *et al.* (2016) also observed improved fruit quality parameters like firmness, TSS, lycopene, ascorbic acid, acidity, and color with only a marginal decrease in fruit shape and size, under 60% ETc (evapotranspiration). Patanè *et al.* (2011) also reported an improvement in the content of TSS, vitamin C, and acidity in tomato fruits under 80% of ETc without significant losses on marketable yield. Significant increases in TSS and lycopene were also observed in drought-stressed tomatoes (Sivakumar and Srividhya, 2016). Additionally, improvement in soluble sugars, vitamin C content, firmness (Hao *et al.*, 2019), and lycopene content seem to be positively affected by deficit irrigation (Nangare *et al.*, 2016; Patanè *et al.*, 2011; Sivakumar and Srividhya, 2016; Takács *et al.*, 2020). Takács *et al.* (2020) compared

three different water supply levels (100%, 75%, and 50% of ETc) and concluded that, although 100% of ETc produced the best marketable yield, 75% ETc increased lycopene content, which could compensate for the lower marketable yield. However, results are sometimes inconsistent (Khapte *et al.*, 2019), and as such this strategy must be further explored to better understand its potential interactions with other growing conditions.

Barbagallo *et al.* (2012) investigated the yield, chemical composition, and sensorial quality of processing tomato cultivar 'Brigade' grown in the field without irrigation, after plant establishment, in comparison with those fully irrigated (100% ETc), and concluded that although regular irrigation is important throughout the growing season to achieve satisfactory yields, under drought stress conditions there is an increase in antioxidant activity due to a rise in ascorbic acid and total phenolic contents and a decline in polyphenol oxidase (PPO) activity, thus improving the nutraceutical quality of the product. Djurović *et al.* (2016) also observed that 50% of ETc increased the organic acid and antioxidant content of tomatoes.

In both the fresh and processing tomato markets, the improvement of organoleptic and functional quality together with the reduction of the impact of agriculture on the environment are of major relevance. The abovementioned studies suggest that the management of drought stress by applying water-saving irrigation strategies at the right moments might improve the quality and nutritional properties of tomato. Thus, an optimization of deficit-irrigation techniques, possibly combined with other sustainable agronomical practices (e.g., grafting, elicitors, biostimulants, and other growth-promoting substances) that have demonstrated an improvement of plant responses to drought stress conditions, is of utmost importance.

2.2. Physiological and biochemical mechanisms underlying drought stress and tolerance

2.2.1. Photosynthesis and stomatal behavior

Photosynthesis is a key metabolic pathway responsible for growth and development, thus playing a central role in plant performance (Lawlor and Tezara, 2009; Pinheiro and Chaves, 2011). The effects of drought stress on photosynthesis have already been extensively reviewed (Chaves *et al.*, 2009; Flexas *et al.*, 2012; Pinheiro and Chaves, 2011; Zargar *et al.*, 2017). Decreased transpiration rate, CO_2 assimilation rate, stomatal conductance, and net photosynthesis were all reported under drought stress (Raja *et al.*, 2020). The reduced photosynthetic rate seems to be mainly caused by stomatal closure (as a short-term response), metabolic impairments of the photosynthetic machinery (as a long-term response), or both (Tezara *et al.*, 1999).

Recently, Liang *et al.* (2020) showed that the response of photosynthesis to drought stress transits from stomatal limitation to nonstomatal limitation, as the stress severity increases.

Adaptation of plants to long-term drought stress leads to adjustments in some leaf anatomical characteristics (Franks and Beerling, 2009; Galmés *et al.*, 2013). For instance, stomatal size often decreases to allow a faster response to excessive water losses and to enhance WUE (Franks and Beerling, 2009). Results have demonstrated that the higher tolerance of 'Ramellet', a tomato landrace adapted to summer drought conditions in the Mediterranean region, is a consequence of a lower leaf mesophyll conductance to CO_2, and stomata conductance (Galmés *et al.*, 2011). Galmés *et al.* (2013) have demonstrated that approximately 90% of the impairment in photosynthesis was caused by diffusion limitations to CO_2 transport from the atmosphere to the sites of carboxylation. By contrast, the biochemical limitations to CO_2 assimilation (RuBisCO activity) seem to be a minor component of photosynthesis limitation (Flexas *et al.*, 2006; Galmés *et al.*, 2011; Galmés *et al.*, 2013; Parry *et al.*, 2002). Nevertheless, accessions with higher concentrations of RuBisCO-activated sites have higher rates of CO_2 assimilation (Galmés *et al.*, 2011; Galmés *et al.*, 2013). Drought-adapted tomato plants present optimal distribution, density, size, and area index of chloroplasts, which could influence leaf gas exchange and be partially responsible for the improved WUE (Galmés *et al.*, 2011; Galmés *et al.*, 2013).

Research has shown that there is considerable variation among tomato accessions regarding another key leaf structural and anatomical traits associated with mesophyll conductance (Galmés *et al.*, 2013). Thick leaves with denser cuticles and cell walls also reduce leaf water loss and improve tolerance to drought stress (Galmés *et al.*, 2011; Kamanga, 2020). A major role for trichomes in gas exchange and the determination of WUE was also established (Kamanga, 2020). The ratio of trichomes to stomata (T/S) seems to have a positive relationship with WUE, suggesting that this trait could be used to develop and screen drought-tolerant varieties (Galdon-Armero *et al.*, 2018).

Drought stress also impairs photosynthesis by damaging the chloroplasts and by inactivating the electron transfer reaction, which can potentially reduce the amount of available oxygen, resulting in the production of ROS that can lead to serious photo-oxidative damages (Lawlor and Cornic, 2002). Chlorophyll fluorescence parameters (F_v/F_o and F_v/F_m), the efficiency of excitation transfer ($F_{v'}/F_{m'}$), the instantaneous quantum yield of photosystem II (ΦPSII), electron transport rate (ETR) of ΦPSII, and photochemical quenching coefficient (qP), as well as light harvesting efficiency,

photochemical conversion, and photosynthetic electron transport (Pervez *et al.*, 2009; Zhang *et al.*, 2018) are all negatively affected by drought.

Oppositely, nonphotochemical quenching (NPQ) is increased in tomato exposed to drought (Raja *et al.*, 2020; Yuan *et al.*, 2016). Interestingly, Liang *et al.* (2020) found that the NPQ increase is transient suggesting that tomato leaves dissipate the excess of excitation energy by heat dissipation under mild drought stress. The authors suggested that as drought stress increases, heat dissipation capacity is reduced and PSII is damaged (Liang *et al.*, 2020).

Besides the photosynthesis reduction, drought also leads to a deterioration of the thylakoid membranes, which causes substantial damage to photosynthetic pigments that control the absorption of energy (chlorophyll) and dissipate excess energy (carotenoids) (Baquedano and Castillo, 2006; Raja *et al.*, 2020; Sivakumar *et al.*, 2018; Tamburino *et al.*, 2017). However, in the majority of cases, carotenoids are known to be less sensitive to drought stress than chlorophyll, which has been demonstrated for several crops (Salehi- Lisar *et al.*, 2012).

2.2.2. Water relations and osmotic adjustment

Osmotic adjustment and cellular-compatible solute accumulation are widely recognized as key mechanisms to tolerate drought, as the increased solute concentration helps to maintain water potential gradients required to ensure continued uptake of water under these conditions (Chen and Jiang, 2010; Wani *et al.*, 2013). In tomato, several studies have reported a significant increase in the accumulation of organic solutes such as glucose, fructose, sucrose, and proline, during drought stress (Hayat *et al.*, 2008; Nahar and Ullah, 2017; Nahar and Ullah, 2018; Nahar *et al.*, 2011; Raja *et al.*, 2020; Tapia *et al.*, 2016), which are cultivar-dependent (Moles *et al.*, 2018; Montesinos-Pereira *et al.*, 2014; Tapia *et al.*, 2016). Although the increase in particular solutes is not directly related to tolerance/sensitivity to drought stress, some studies appear to demonstrate the relevance of this process (Montesinos-Pereira *et al.*, 2014; Patanè *et al.*, 2016; Sánchez-Rodríguez *et al.*, 2010a,b). Polyamine synthesis and accumulation, for example, as a result of proline degradation, was higher in moderate drought-tolerant cherry tomato cultivar ‘Zarina’, than in ‘Josefina’, which is a more sensitive cultivar (Sánchez-Rodríguez *et al.*, 2010a,b).

Although previous results on solute accumulation point to interesting mechanisms that might contribute to a higher WUE, more studies are required to better clarify these processes.

2.2.3. Oxidative stress and scavenging system

Drought induces oxidative stress and increases the production of ROS, including antioxidant substrates and antioxidant enzymes (Sánchez-Rodríguez *et al.*, 2010a,b, Zhou *et al.*, 2019b), being the ability of plants to control oxidant levels highly correlated with stress tolerance (Laxa *et al.*, 2019).

Importantly, ROS play a dual role in plant metabolism during abiotic stress. Under high concentrations, they function as a toxic byproduct while under low concentrations they function as a signal transduction molecule triggering stress response. The major source of ROS in plants is the chloroplast, where stress-induced changes in the redox status of electron transport lead to the formation of singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and hydroxyl radical (HO^-). They can directly act on membrane lipids, inactivate metabolic enzymes, and damage nucleic acids, leading to cell death (Laxa *et al.*, 2019; Mittler *et al.*, 2011). The effects of drought stress on increased malondialdehyde (MDA, related to lipid peroxidation) content in tomato plants have been reported, indicating that the balance of the antioxidant enzymatic system is disrupted, resulting in ROS accumulation and increased lipid peroxidation, causing membrane damage (Liang *et al.*, 2020; Raja *et al.*, 2020; Zhou *et al.*, 2019b). Yuan *et al.* (2016) have shown that this increase occurs at all developmental stages in tomato.

Among the ROS, H_2O_2 works as an oxidative burst signal due to its high permeability across the membranes and relatively long half-life (Mittler *et al.*, 2011). In tomato, the activity of superoxide dismutase (SOD), a ubiquitous antioxidant enzyme that catalytically converts O_2^- to H_2O_2, gradually increased in 14 different cultivars exposed to drought stress (Aghaie *et al.*, 2018; Hayat *et al.*, 2008; Liang *et al.*, 2020; Raja *et al.*, 2020; Yuan *et al.*, 2016; Zhou *et al.*, 2019b). Besides SOD, the activity of peroxidase (POD) and catalase (CAT) also increases in tomato under prolonged drought. However, the activity of these enzymes sharply drops at the maturity stage (Yuan *et al.*, 2016). As drought stress becomes more severe, the activities of SOD, POD, and CAT start decreasing (Liang *et al.*, 2020).

The activities of glutathione reductase (GR) and ascorbate peroxidase (APX) increase in drought-stressed tomatoes. Oppositely, the activity of the dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), PPO, and GPX (glutathione peroxidase) decline under drought stress (Raja *et al.*, 2020; Sánchez-Rodríguez *et al.*, 2011a). Thipyapong *et al.* (2004) proposed that a decrease in PPO lowers H_2O_2 concentration, which could improve tomato tolerance against drought. Sánchez-Rodríguez et al. (2011b) demonstrated that tomato cultivar ‘Zarina’ increased

the synthesis of phenylpropanoids and flavonoids, suggesting that polyphenols play a significant physiological role in drought-stress tolerance in tomato.

Little is known about these responses in fruits subjected to drought. Murshed *et al.* (2013), while analyzing the fruits, found a relationship between drought and oxidative stress, with changes in ROS-scavenging enzymes (increase in SOD, CAT, and APX activity) and oxidative stress biomarkers (increase in H_2O_2 and MDA). Regarding the enzymes from the ascorbate- glutathione cycle, the degree to which the AsA and DHA concentrations changed under drought stress was extremely variable among different drought stress treatments and different fruit development stages (Murshed *et al.*, 2013).

In sum, drought stress leads to an increased production of ROS in tomato affecting proteins, lipids, and DNA (leaves and fruits), which ultimately compromises drought responses. Increased content of polyphenols in drought-tolerant cultivars appears to indicate that the antioxidant system can be a useful tool for breeding programs.

2.2.4. Nutrient uptake

Drought stress affects plant mineral nutrition, not only by decreasing nutrient uptake at the root level but also the mineral translocation to the shoots due to lower transpiration rate and membrane permeability (Rouphael *et al.*, 2012).

Under drought conditions, N uptake diminishes (reviewed by Gonzalez- Dugo *et al.*, 2010). Interestingly, tolerant cultivars seem to present increased amounts of N as compared to sensitive ones, which intensifies the synthesis of amino acids and proteins (Sánchez-Rodríguez *et al.*, 2011a). Sánchez- Rodríguez *et al.* (2010b) found lower N uptake and accumulation under water deficit in four sensitive cherry tomato cultivars. However, in the tolerant cultivar ‘Zarina’, an increase was found, suggesting a cultivar dependency for this trait. The authors suggested that these mechanisms in the cultivar ‘Zarina’ are essential for drought-stress tolerance, by acting on N metabolism as well as by helping to maintain or augment biomass. Additionally, Sánchez-Rodríguez *et al.* (2011a) reported that moderate water deficit also decreased the NO_2 content in sensitive cultivars, as well as nitrate reductase (NR) activity. The decrease in tomato NR activity was previously reported by Hayat *et al.* (2008) and may be considered a biochemical adaptation to conserve energy by halting nitrate assimilation. Similar results were found for other macronutrients (P, K, Ca, Mg, S) and micronutrients (Fe, Cu, Mn, Zn, Cl, B), where lower concentrations were found in sensitive cultivars. Oppositely, the cultivar ‘Zarina’ showed increased concentrations and uptake of N, P, Mg, K, Fe, and Cl.

Genotypic variability in nutrient uptake suggests that these different responses against drought might play an important role in drought-stress tolerance.

In short, although it is clear that drought stress hampers mineral uptake in tomato, more research should be done regarding the mechanisms involved, as the imbalances in nutrients ultimately have serious effects on various growth and developmental processes.

2.3. Molecular mechanisms underlying drought tolerance

Water deficit imposes selective pressure on the evolution of plant morphology and physiology. Breeding for drought-tolerant crops is a pressing issue due to the increasing frequency and duration of droughts caused by climate change. The activation of molecular pathways modulates the expression level of specific genes responsible for the accumulation of various osmolytes, improvement of the antioxidant system, reduction of transpiration, inhibition of shoot growth, and decrease of tillering, including hormonal regulation (Foolad, 2007; Joshi *et al.*, 2016). Although ABA is reported to be abundant under drought conditions, inducing the expression of various stress-related genes, it has been shown that drought-inducible gene expression is also headed by ABA-independent regulatory systems (Joshi *et al*., 2016).

The genetic determinants of tomato response to drought are still poorly understood. Many quantitative trait loci (QTL) have been identified (Fooland *et al.*, 2003; Gur and Zamir, 2004). However, the genomic regions covered by the QTL usually cover many genes, limiting the understanding of those involved in drought tolerance. Foolad et al. (2003) identified four drought-tolerant QTLs highly relevant for seed germination under drought stress, while Gur and Zamir (2004) reported three QTLs regulating increased yield under drought conditions. More recently, high-throughput analysis has identified thousands of genes whose expression is altered by drought stress (Candar-Cakir *et al.*, 2016; Iovieno *et al.*, 2016; Liu *et al.*, 2017; Liu *et al.*, 2018), but few studies have deeply analyzed the relevance of downregulated or upregulated genes on tomato drought-stress tolerance improvement (summarized in Table 1).

After a long and high-pressure selection process, mostly based on yield traits, modern tomato cultivars have a very narrow genetic basis, and the vast majority are now drought-stress sensitive (Shirasawa *et al.*, 2010). However, elite genes for stress tolerance appear to be well conserved in some wild tomato species, as *Solanum pennellii* and accessions from this species show high levels of drought tolerance (Bolger *et al.*, 2014). A model of the different processes and genes involved in the *S. pennellii* drought tolerance response was proposed by Egea *et al.* (2018). Several genes linked to different

physiological and metabolic processes have been identified, especially those involved in N assimilation, GOGAT/GS cycle, and GABA shunt. Upregulation of genes linked to jasmonic acid/ethylene (JA/ET) biosynthesis was also observed under drought stress in drought-tolerant species. Gong *et al.* (2010) studied the gene expression of two drought-tolerant introgression lines of *S. pennellii*, and one drought-sensitive cultivar (*S. lycopersicum* cultivar 'M82') showing that the drought-tolerant lines induced around 400 drought-responsive genes encoding for transcription factors and signaling proteins involved in the cell-wall structure, wax biosynthesis, and plant height. Key signaling molecular pathways regulating gluconeogenesis, purine and pyrimidine nucleotide biosynthesis, tryptophan degradation, starch degradation, methionine biosynthesis, and the removal of superoxide radicals were also specifically affected by drought stress, showing that tomato plants can adapt to drought conditions through decreasing energy dissipation, increasing ATP energy provision, and reducing oxidative damage (Gong *et al.*, 2010). Also, genome-wide studies have identified genetic variation in fruit quality components upon drought stress (Albert *et al.*, 2016). The authors proposed 41 putative candidate genes under three constitutive QTLs and 15 interactive or specific QTLs as being implicated in drought stress response in tomato. Regarding the interactive and specific QTLs, genes related to protein protection (chaperone and heat/cold shock proteins), water and solute transport (aquaporins and other transporters), sugar metabolism (sucrose phosphate synthase and invertases), and hormonal signaling (auxin, gibberellin, and ethylene) were identified (Albert *et al.*, 2016). However, improved genetic tools for the construction of knockout mutants on tomato are needed to validate the relevance of such genes on overall drought stress response. Therefore, further studies to establish genetic determinants of drought stress tolerance in tomato are of utmost importance to globally understand the complex regulatory network(s) controlling this process.

Table 1 - Summary of genes in which overexpression has been associated with improved tolerance to drought stress in tomato.

Gene name	Encoded protein	Protein function on drought tolerance	Reference
AREB1	Abscisic acid-insensitive 5 like protein	⇒ *AREB1*-overexpressing transgenic tomato plants showed increased tolerance to drought stress as assessed by physiological responses such as higher relative water content and chlorophyll fluorescence and lower damage by lipoperoxidation. ⇒ Involved in ABA signals and in the up-regulation of genes encoding oxidative stress-related proteins, lipid transfer proteins (LTPs), transcription regulators and late embryogenesis abundant proteins.	Orellana *et al.*, 2010
CML44	Probable calcium-binding protein CML44	⇒ Transgenic plants overexpressing *CML44* gene showed increased germination and better seedling growth, as well as higher antioxidant enzymes activity, gas exchange and water retention capacity and lower MDA accumulation and membrane damage. ⇒ Greater stress tolerance of transgenic plants was further reflected by the up-/down-regulation of stress-related genes including SOD, GST, CAT, POD, LOX, PR and ERD.	Munir *et al.*, 2016
ERF5	Ethylene-responsive transcription factor 5	⇒ Increased tolerance to drought as well as the levels of relative water content.	Pan *et al.*, 2012
HsfA1a	Heat stress transcription factor A-1a	⇒ Silencing *HsfA1a* leads to hypersensitive response to drought stress with increased reduction of the stomatal aperture, increased level of ubiquitinated proteins, and compromised authophagosome formation. ⇒ Overexpression of *HsfA1a* increases plant drought tolerance, in a mechanism independent of the phytohormone ABA. ⇒ HsfA1a acts as a positive regulator of the expression of the autophagy genes *Atg10* and *Atg18f*.	Wang *et al.*, 2015
JUB1	Transcription factor JUNGBRUNNEN 1	⇒ *SlJUB1* knockout plants showed increased ion leakage and H_2O_2 levels and decreased expression of various drought-responsive genes. ⇒ Overexpression of *SlJUB1* leads to activation of *DELLA* and of the stress-related genes *DREB2* and *DREB1*, responsible for decreasing ROS levels, increasing thereby drought tolerance.	Thirumalaikumar *et al.*, 2018

MAPK1-3	Mitogen Activated Protein Kinases	⇒ Mapk1, Mapk2, and Mapk3 play a crucial role in enhancing tomato plants' drought tolerance by influencing stomatal activity and H_2O_2 production via the ABA-H_2O_2 pathway.	Li *et al.*, 2013
		⇒ The *mapk3* knockout mutants exhibit severe wilting symptoms, higher H_2O_2 content, lower antioxidant enzymes activity, and severe membrane damages under drought stress. The *mapk3* knockout was shown to lead to up- or down-regulation of drought stress-responsive genes including *SlLOX*, *SlGST*, and *SlDREB*, suggesting that *mapk3* is involved in drought response in tomato plants by protecting cell membranes from oxidative damage and modulating transcription of stress-related genes.	Wang *et al.*, 2017
		⇒ Transgenic plants overexpressing *mapk3* showed an increased photosynthetic activity as well as content of chlorophyll, proline, and sugars under drought stress.	Muhammad *et al.*, 2019
MX1	MIXTA-like MYB transcription factor	⇒ Substantially increases drought tolerance, by inducing trichome formation and cuticle deposition. ⇒ Improved fruit quality due to higher accumulation of carotenoids (including lycopene, b-carotene, phytoene and lutein), and upregulation of *PSY*, *PDS*, *ZDS*, *PTOX* and *LCY* that encode key carotenoid biosynthesis genes.	Ewas *et al.*, 2016
NPR1	Regulatory protein NPR1	⇒ Increased tolerance to drought stress. ⇒ *SlNPR1* knockout plants showed severe oxidative damage and cell membrane damage and down-regulation activity of antioxidant enzymes (APX, CAT, POD, and SOD). Down-regulated expression of drought related key genes, including *GST*, *DHN*, and *DREB*.	Li *et al.*, 2019a
PIP	Aquaporins (Plasma membrane intrinsic proteins)	⇒ Overexpression of *PIP2;1*, *PIP2;7* and *PIP2;5* resulted in better water status and survival rates than wild type plants	Li *et al.*, 2016
PKE1		⇒ Significantly improved the accumulation of chlorophyll and proline and limited the MDA content. ⇒ Upregulation of several ion transporter genes, abiotic-related transcriptional factors, and reactive oxygen species-scavenging genes in *PKE1* overexpression lines.	Li *et al.*, 2018 Li *et al.*, 2019b
PKE1		⇒ Significantly improved the accumulation of chlorophyll and proline and limited the MDA content.	Li *et al.*, 2018 Li *et al.*, 2019a

		⇒ Upregulation of several ion transporter genes, abiotic-related transcriptional factors, and reactive oxygen species-scavenging genes in *PKE1* overexpression lines.	
SHN1	Ethylene-responsive transcription factor WIN1	⇒ Overexpression of *SHN1* showed mild growth retardation phenotype with shiny and dark green leaves. SHN1 was shown to modulate wax accumulation, as demonstrated by higher cuticular wax deposition on leaf epidermal tissue and the induction of several wax-related synthesis genes.	Al-Abdallat, *et al.*, 2014
WRKY8	WRKY transcription factor 8	⇒ Alleviated wilting or chlorosis phenotype, with higher levels of stress-induced osmotic substances like proline and higher transcript levels of the stress-responsive genes *AREB*, *DREB2A* and *RD29*. ⇒ Smaller stomatal aperture, maintaining higher water content in leaves and reduced H_2O_2 and MDA which was then followed by higher levels of antioxidant enzyme activities under stress.	Gao *et al.*, 2020
ZIP1	Basic leucine zipper 1	⇒ ZIP1 exerts an essential role in salt and drought stress tolerance by modulating an ABA-mediated pathway. ⇒ Silencing of *ZIP1* resulted in reduced expression of multiple ABA biosynthesis- and signal transduction-related genes in transgenic plants, as demonstrated by decreased ABA, chlorophyll contents and CAT activity, and increased MDA content.	Zhu *et al.*, 2018

3. Tomato plant responses to nitrogen stress

Tomato plants have high demands for fertilizers and are quite sensitive to N deficits (Du *et al.*, 2018; Scholberg *et al.*, 2000). Several reports have indicated that open-field grown tomato plants require high amounts of N, ranging from 100 to 574 kg/ha (with levels oftentimes higher than when grown in greenhouse conditions), which can be affected by different environmental and management factors, one of them being the N form supplied (Du *et al.*, 2018; Siddiqi *et al.*, 2002). Nitrate (NO_3^-) and ammonium (NH_4^+) are the most common forms of N supply in agriculture which have different uptake mechanisms and impacts on plant physiological and biochemical processes (Guo *et al.*, 2007). It is commonly accepted that tomato plants have a better performance on NO_3^- or on a mix of NO_3^- and NH_4^+, than with NH_4^+ supply (Siddiqi *et al.*, 2002).

The precise N fertilization that should be supplied in soil production systems is difficult to quantify particularly in open-field because part of these fertilizers can be lost through leaching (depending on soil type and rainfall) and denitrification. Consequently, it is difficult to adjust the exact N fertilizer supply to the plant demand, often making N availability vary from the N- deficient state to the N-excess state, depending on the agricultural practices (Jangam and Raghuram, 2015). While an excess of N can lead to damaging effects on the environment and human health, plants under N-deficit conditions can also have their growth, development, and yield compromised (Jangam and Raghuram, 2015; Okunlola *et al.*, 2015). Therefore, new sustainable agricultural practices and strategies to improve NUE are needed (Anjana and Iqbal, 2007). Understanding the molecular, biochemical, and physiological changes occurring in tomato upon N deficiency will be a step forward for NUE improvement. In the following section, we analyze how N stress affects tomato plants, and we summarize the existing morphological and molecular traits conferring a higher NUE (Figure 2).

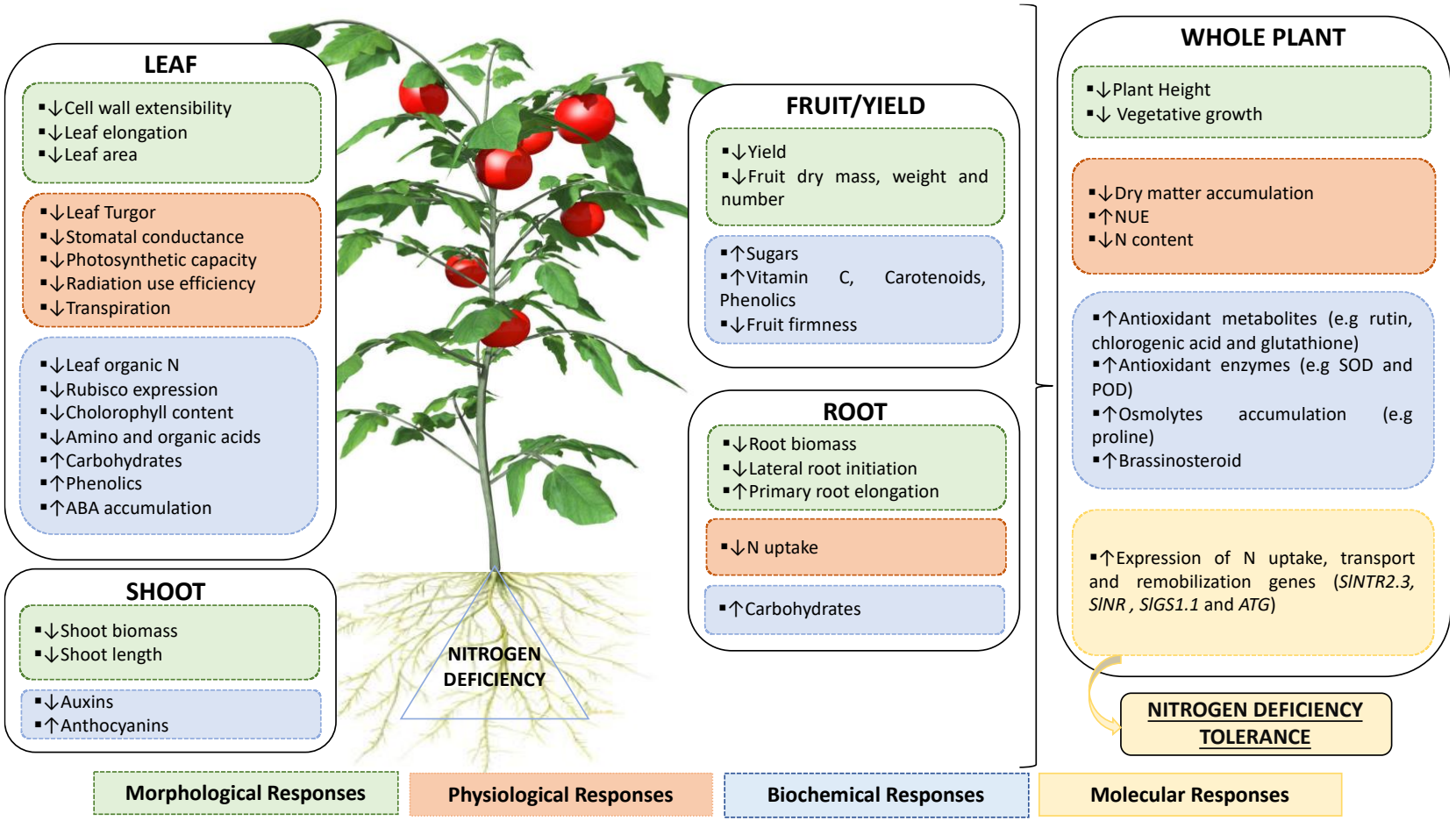


Figure 2 - Schematic overview of the morphological, physiological, biochemical, and molecular processes occurring at specific tomato plant organs or the whole-plant level in response to nitrogen deficiency.

3.1. Impact of nitrogen on plant growth, yield, and fruit quality

3.1.1. Plant growth

N encourages vegetative growth and it is a key element for plant growth, constituting the majority of plant dry matter, along with carbon (C) (Cohen *et al.*, 2019; Nawaz *et al.*, 2012). Several studies have demonstrated that while N availability favors tomato growth, N deficiency strongly disturbs plant development (Luna *et al.*, 2014; Nawaz *et al.*, 2012; Okunlola *et al.*, 2015; Scholberg *et al.*, 2000; Tei *et al.*, 2002). The effect of N deficiency on tomato plants is described by different mechanisms. First, N and carbohydrates are required for protein synthesis; therefore the availability of N promotes plant growth by increasing protein synthesis. On the other hand, when the N supply is insufficient, an excess of carbohydrate deposits in vegetative cells affects apical growth (Okunlola *et al.*, 2015). Secondly, N availability influences auxin levels at the shoot apical meristem, affecting all aerial plant parts (Vernoux *et al.*, 2010). As changes in root, architecture help plants to cope with N deficiency by improving NUE, there is often a relocation of auxin from shoots to roots in plants under N stress for lateral root growth, decreasing the amount of auxin found on the apical meristem, and consequently affecting the shoot development (Kiba *et al.*, 2011; Rajagopal and Rao, 1974). Chapin *et al.* (1988) reported that in tomato plants growing in hydroponic conditions, the shoot water content does not decline with N stress and authors have pointed out that reduced growth may involve a “root signal” that is able to induce the synthesis of ABA in leaves leading to reduced cell-wall extensibility. Finally, N supply influences photosynthetic activity (De Groot *et al.*, 2003; Guidi *et al.*, 1997; Tei *et al.*, 2002). This effect is mainly a consequence of the high number of N-rich substances used during this process, which can lead to even greater deposition of carbohydrates on vegetative cell walls (De Groot *et al.*, 2003; Okunlola *et al.*, 2015). An in-depth analysis of the effect of N stress on photosynthesis is given in Section 3.2.1 of this sub-chapter.

The duration and intensity of the N stress influence the severity of growth impairment. For instance, tomato seedlings under N deficiency (one-eighth of optimum N) or N starvation conditions for 20, 30, and 40 days after sowing, showed that at 20 days only minor growth changes were detected among treatments. However, at 40 days control plants had almost four times the height of plants subjected to N starvation and more than double the height of plants subjected to N deficiency (Rajagopal and Rao, 1974).

The impact of N deficiency is also dependent on the plant’s development stage. In tomato, N deficiency does not seem to impact the reproductive system as optimum N or 1/3 N supply did not directly influence truss development and number (Suniaga

Quijada *et al.*, 1992). Authors found that at the reproductive stage, under N deficiency the priority destination of assimilates is the reproductive organs, followed by roots, and finally the shoot vegetative system. In agreement with these findings, Guidi *et al.* (1997) reported a reduction of root dry mass when N deficiency was employed at the reproductive stage but not at the vegetative stage.

As mentioned above, the impact of N stress on plant growth is highly dependent on the form of N supply. Tomato plants are very prone to NH_4^+ toxicity when it is the sole source of N (Gerendás and Sattelmacher, 1990; Siddiqi *et al.*, 2002). NH_4^+ supply can cause acidification of the root zone, constricting root growth, leading to cation imbalances (especially Ca, K, and Mg), uncoupling of chloroplastic electron transport from photophosphorylation, and reducing LA (Guo *et al.*, 2007; ten Hoopen *et al.*, 2010; Siddiqi *et al.*, 2002). A 67% vegetative growth reduction was reported on tomato supplied exclusively with NH_4^+ when compared to a mixture of NO_3^- and NH_4^+ (Siddiqi *et al.*, 2002). In fact, supplying NH_4^+ at levels above 0.10 of the total N restricts stem and leaf fresh and dry weight, as well as yield, being progressively more severe as the NH_4^+ supply fraction is increased (Akl *et al.*, 2003).

NUE depends on two main steps, that is, how efficiently plants uptake N from the soil (uptake efficiency) and how plants can use the N more efficiently (utilization efficiency) (Garnett *et al.*, 2009). Root morphology is highly affected by soil N availability, particularly with the NO_3^- form (Gruber *et al.*, 2013; Kant *et al.*, 2011). Generally, low N availability decreases tomato root growth and suppresses lateral root initiation (Guidi *et al.*, 1997; Kant *et al.*, 2011). Root architecture has been pointed out as a crucial breeding trait to improve NUE and reduce N fertilizer losses (Garnett *et al.*, 2009). Accordingly, some tolerant cultivars display more vigorous roots than sensitive ones, implying an association between root development and NUE (Fan *et al.*, 2010; Liu *et al.*, 2009). On tomato, two cultivars with contrasting NUE, ‘Regina Ostuni’ (‘RO’) (higher) and ‘UC82’ (lower), were identified and characterized (Abenavoli *et al.*, 2016). Root length and fineness were significantly higher in ‘RO’ compared to ‘UC82’, regardless of NO_3^- supply dose. Thinner roots are related to higher root-soil surface, increasing uptake efficiency (Goss *et al.*, 1993).

3.1.2. Yield and fruit quality

Crop yield and fruit quality are closely related to N availability. Several studies have reported that fruit yield is favored by a wide range of N fertilization rates, ranging from around 100 kg N/ha (Fátima *et al.*, 2012) up to 600 kg N/ha (Montagu and Goh, 1990). These differences are dependent on the developmental stage, cultivar, N source,

and environmental conditions (Beńard *et al.*, 2009; Elia and Conversa, 2012; Warner *et al.*, 2004). Excessive N fertilization can also lead to the remobilization of N from reproductive organs to the leaves and favors vegetative growth to the detriment of fruit growth, resulting in lower yields (Hernández *et al.*, 2020; Scholberg *et al.*, 2000). Elia and Conversa (2012) showed that increasing N fertilization from 0 to 200 kg N/ha increased fruit yield by 28.1% whereas from 0 to 300 kg N/ha only increased 23.3%. Similarly, Du *et al.* (2017) concluded that 250 kg N/ha increased yields by 4.8% compared to 350 kg N/ha N fertilization, reinforcing that excessive N fertilization can have detrimental effects on tomato yield. Recently, a study by Du *et al.* (2019) showed that aerated fertigation, an innovative method, promoted N uptake and improved the yield of tomato plants grown in soil under protected cultivation even when N application was low.

It is important to mention that higher yield does not necessarily lead to higher fruit quality, because it often leads to a decline of the organoleptic quality traits, as a result of a decrease in sugars or organic acids content (Beńard *et al.*, 2009; Hernández *et al.*, 2020). For instance, Wang et al. (2007) reported enhanced titratable acidity and soluble sugars in cherry tomato (*Lycopersicon esculentum* var. cerasiforme) with higher N supplies, while maximum yields were found at lower N levels. Conversely, Beńard *et al.* (2009) concluded that increasing N from 4 to 12 mM produced higher yields but reduced soluble sugars.

The impact of N on the synthesis of bioactive compounds with antioxidant capacity (vitamin C, phenolics, and carotenoids) is an emerging area (Beńard *et al.*, 2009; Hernández *et al.*, 2020), due to the high content of these antioxidants, tomato exhibits antimicrobial, anti-inflammatory, anti-allergic, and antimutagenic properties (Hernández *et al.*, 2020; Tavallali *et al.*, 2018). Interestingly, data have pointed out that N availability directly affects vitamin C and phenolics compounds in fruits (Beńard *et al.*,2009; Hernández *et al.*, 2020). However, the effect of N on carotenoid accumulation appears to be more ambiguous. While high N dosages appear to favor carotenoid accumulation in the fruit, nonsignificant changes in lycopene and β-carotene content were observed when the N supply was reduced (Beńard *et al.*, 2009; Poiroux- Gonord *et al.*, 2010). The differences found in the literature could be largely due to different doses of mineral N applied and the phenological time at which the reduction was implemented (Hernández *et al.*, 2020).

3.2. Physiological and biochemical mechanisms underlying N stress

3.2.1. Photosynthesis

N is involved in the synthesis of several components of the photosynthetic machinery, including pigments and RuBisCO enzyme, which alone encompasses about 50% of leaf N content and plays a major role in C assimilation (Bassi *et al.*, 2018; Cohen *et al.*, 2019; Shangguan *et al.*, 2000). Therefore, N deficiency is often associated with declines in RuBisCO concentration and leaf gas exchange, with a significant decrease in the leaf photosynthetic CO_2 assimilation capacity (Cohen *et al.*, 2019; Jin *et al.*, 2015). N deficiency also affects photosynthesis by triggering leaf senescence, a reduction in the new LA expansion, and a lack of sink to the photoassimilates produced in such processes, as lowering N supply decreases transport and/or utilization of assimilates (De Groot *et al.*, 2003; Wei *et al.*, 2016).

The impact of N stress on the photosynthetic activity of tomato has been extensively studied (Chapin *et al.*, 1988; Cohen *et al.*, 2019; De Groot *et al.*, 2003; Halpern *et al.*, 2018; Suniaga Quijada *et al.*, 1992; Tei *et al.*, 2002). Suniaga Quijada *et al.* (1992) noted that low N supply, during the early developmental stages of tomato, resulted in lower leaf elongation and lower leaf chlorophyll content, as well as abnormal starch accumulation in the chloroplasts. Further, Tei *et al.* (2002) observed that N availability is positively correlated with the growth rate of tomato plants, mainly due to an increase in light interception as a consequence of a higher LA index. In addition, De Groot *et al.* (2003) showed that the photosynthetic capacity of tomato plants grown hydroponically following 2 days of N starvation was reduced due to a decrease in light absorption and assimilates use. Recently, some studies have been addressing the impacts of photosynthetic acclimation to elevated CO_2 (PAC) on N uptake and availability (Cohen *et al.*, 2019; Halpern *et al.*, 2018). It is well known that short-term high CO_2 enhances leaf photosynthetic rate. On the other hand, PAC arises under long-term exposure to elevated CO_2, where the positive effect on the photosynthetic activity becomes weaker as a consequence of (1) lower stomatal conductance and, therefore, reduced leaf gas exchange, and (2) downregulation of the concentration and expression of internal photosynthetic intermediates, particularly RuBisCO (Figure 3). Moreover, long-term elevated CO_2 reduces NO_3^- uptake due to stomatal closure and reduced transpiration rate (Halpern *et al.*, 2018). These authors also demonstrated that low NO_3^- aggravates photosynthetic performance regardless of the CO_2 concentration, but the negative effect was greater when it was combined with elevated CO_2. Furthermore, they demonstrated that higher NO_3^- fertilization can alleviate the acclimation effect of elevated CO_2 on the photosynthetic machinery of tomato plants, N status, and sink-to-source

imbalance. Finally, it has been shown that despite the negative effect that elevated CO_2 can have on photosynthetic parameters and NO_3^- uptake, it can also generally improve the plant-soil microbiome through a lower pH, resulting in higher N bacterial fixation, and hence, a greater N soil availability (Halpern *et al.*, 2018; He *et al.*, 2019; Wang *et al.*, 2020) (Figure 3).

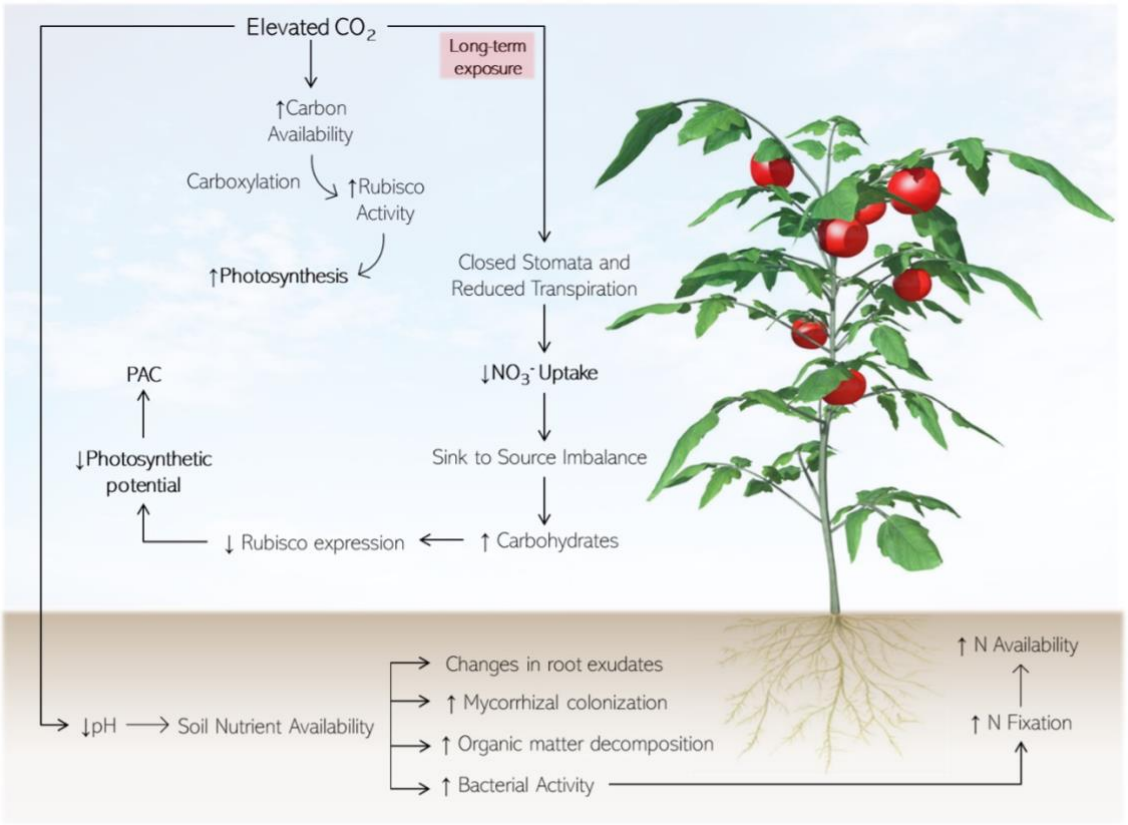


Figure 3 - Interactive effects of elevated CO_2 on N uptake and availability, photosynthesis, photosynthetic acclimation (PAC) and soil microbiome.

3.2.2. Oxidative stress and scavenging system

An effect of N availability on oxidative stress response has been described for several plants (Hsieh *et al.*, 2018; Rubio-Wilhelmi *et al.*, 2011; Tewari *et al.*, 2007). N deficiency diminishes the rate of the Calvin cycle and consequently prevents the oxidation of NADPH and restoration of $NADP^+$ (Rubio-Wilhelmi *et al.*, 2011). N deficiency promotes the generation of ROS, particularly of H_2O_2 and O_2^-, through excitation of the photosystem II that intensifies the Mehler reaction, consequently enhancing the activity of the enzymes POD and SOD (Koleška *et al.*, 2017). In turn, ROS accumulation triggers leaf senescence and inhibition of the photosynthetic capacity by increasing lipid peroxidation, pigment loss, and protein degradation (Koleška *et al.*, 2017; Rubio-Wilhelmi *et al.*, 2011; Tewari *et al.*, 2007).

Several studies on tomato have looked at the effect of N on the oxidative burst and on the scavenging system, particularly on phenolic compound content (Groher *et al.*, 2018; Koleška *et al.*, 2017; Larbat *et al.*, 2012, 2014; Singh *et al.*, 2019). Koleška *et al.* (2017) tested the effect of biostimulants on the prevention of oxidative damage produced by nitrogen-phosphate-potassium (NPK) deficiency and reported an increase in enzymatic antioxidant activity. Singh *et al.* (2019) demonstrated that a high N supply could partly alleviate salt toxicity through the production of osmolytes, including proline.

N deficiency also triggers the accumulation of flavonoids via increases in phenylalanine ammonia-lyase activity (Koleška *et al.*, 2017). Several studies revealed that N deficiency or starvation induces an increase in rutin and chlorogenic acid (two antioxidant flavonoids and the main phenolic compounds found on tomato leaves) (Beńard *et al.*, 2011; Groher *et al.*, 2018; Larbat *et al.*, 2012, 2014).

3.2.3. Nutrient uptake

N is a major macronutrient and its ionic form controls cation and anion uptake to maintain a charge balance and electroneutrality within the plant (Borgognone *et al.*, 2013). In tomato, many studies have evaluated the effect of different N supplies on the uptake of other nutrients (Errebhi Wilcox, 1990; Gunes *et al.*, 1998; Huett and Dettmann, 1988; Kirkby and Knight, 1977; Smart and Bloom, 1993). The understanding of these nutrient interactions is an important step toward achieving an efficient and sustainable strategy for integrated nutrient management (Aulakh and Malhi, 2005). The source of N is also a determining factor in the uptake of other nutrients. Under NO_3^- deficiency and/or higher NH_4^+ supplementation, cations like potassium (K), magnesium (Mg), and calcium (Ca) presented lower uptake levels, and anions like phosphorous (P), sulfate (S), and most micronutrients had higher uptake rates, while with significantly higher NO_3^-/NH_4^+ ratio the inverse response was reported (Borgognone *et al.*, 2013; Smart and Bloom, 1993; Yoneyama *et al.*, 2012). NH_4^+ can induce nutrient deficiency of cations like Ca, K, and Mg by competing with these cations for assimilation and transport sites and by acidifying the rooting medium (as NH_4^+ transport to the roots implies the release of protons), altering the mineral nutrient bioavailability (Gerendás *et al.*, 1997; Haynes and Goh, 1978; ten Hoopen *et al.*, 2010; Rayar and van Hai, 1977). On the contrary, rhizospheric acidification can lead to increased uptake of some anions, while NO_3^-, associated with the proton consumption via $2H^+/NO_3^-$ symport, and consequently increase in the pH of the outer solution, can result in a decrease of anions uptake (Borgognone *et al.*, 2013; Fageria, 2001). P uptake specifically is associated with a lower pH as it induces higher solubility of Ca-phosphates in the rooting medium and increases

the phosphate ion that is preferentially absorbed by roots (Ashraf and Sultana, 2000; Hoffmann *et al.*, 1994).

3.3. Molecular mechanisms underlying nitrogen stress and tolerance

Improving NUE in crop plants is now, more than ever, an urgent environmental and agricultural goal that, despite extensive research over the past decade, remains one of the scientific "Grand Challenges" of the 21st century (Hsieh *et al.*, 2018). To face this challenge and to be able to identify important sites of intervention for higher NUE, we need to have a broad understanding of the genetic machinery behind N uptake, transport, metabolism, and remobilization in crop plants (Hsieh *et al.*, 2018; Jangam and Raghuram, 2015).

Depending on internal and external NO_3^- concentrations, plants have different uptake systems including two high-affinity transport systems (HATS), mainly activated under low NO_3^- availability (levels below 0.1 mM) and one low-affinity transport system (LATS) for cases of higher availability of NO_3^- (Hildebrandt *et al.*, 2002; Lea and Azevedo, 2006; Wang, 2001). This root transport system allows the plant to maximize NO_3^- uptake depending on soil N availability. Once inside the plant roots, NO_3^- may be transported to the shoot for assimilation (storage in the vacuole), released again into the rhizosphere, or reduced to nitrite or NH_4^+ via NR or nitrite reductases (NiR), respectively (Wang, 2001).

NH_4^+ is taken up by the plant directly from the rhizosphere by NH_4^+ transporters (AMT) and is further assimilated into amino acids via the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle and/or the asparagine synthetase (AS) or the glutamate dehydrogenase (GDH) (Lam *et al.*, 1994; Liu *et al.*, 2016; Kant *et al.*, 2011).

Several groups of genes related to N uptake, transport, and assimilation were identified including genes encoding NO_3^- transporters (*NRT*s), genes encoding *AMT*s, genes involved in the transport of NO_3^- to the vacuole (*CLC*), and genes related to the enzymes NR, NiR, of GS/GOGAT, GDH, and AS pathway for NH_4^+ assimilation (Lam *et al.*, 1994; Liu *et al.*, 2016; Wang, 2001). NRTs are further divided into two major groups, one mainly associated with LATS (NRT1) and the other associated with HATS (NRT2) (Wang, 2001).

3.3.1. N uptake and transport

Low N availability strongly induces the expression of genes associated with N uptake, particularly those associated with HATS such as the NRT2 and the high-affinity *AMT* gene family (Hsieh *et al.*, 2018; Peng *et al.*, 2007). A study using an *Arabidopsis*

thaliana Atnrt2.1-nrt2.2 mutant evidenced that this NRT inhibits iHATS expression by up to 80% (Li *et al.*, 2007). N starvation also induced an upregulation of genes belonging to the *NRT2* gene family in tomato (*LeNRT2*-1 and *LeNRT2-2*) (Ono *et al.*, 2000).

Tomato plants grew without N, with 200 μM NO_3^- or 100 μM NH_4^+ as their sole N source, showed that expression of *LeAMT1* and *LeNRT1-1* was independent of the N availability/source, while *LeNRT1-2* expression was only induced in the presence of NO_3^- (Lauter *et al.*, 1996). This can be explained by the fact that (1) LATS can have two or even more differentially regulated transport systems in tomato, cLATS, or NO_3^- iLATS (Huang, 1999); and that (2) *LeNRT1-1* can encode a dual-affinity transporter (Lauter *et al.*, 1996; Ono *et al.*, 2000). Indeed, it was already reported that if *NRT1-1* is posttranscriptionally phosphorylated, it functions as a HATS, whereas if dephosphorylated it functions as a LATS (Masclaux-Daubresse *et al.*, 2010). This last hypothesis was further explored in *A. thaliana*, but it was concluded that *NRT1-1* alone had a minor contribution to NO_3^- uptake at low external NO_3^- concentration. Curiously, the role of *AMT* genes appears to be more controversial. Von Wiren *et al.* (2000) evaluated the expression of three *AMT* genes in hydroponically grown tomato (*LeAMT1-1*, *LeAMT1-2*, *LeAMT1-3*) and observed that *LeAMT1-1* expression was induced by N absence, whereas *LeAMT-2* expression was strongly induced by either NH_4^+ or NO_3^- sources suggesting that these genes encode an inducible and constitutive transport system, respectively. On the other hand, the expression of *LeAMT1-3* demonstrated little similarity with the other two genes and was identified only in leaves and when NO_3^- was present, having hypothetically a different mechanism of regulation in tomato plants.

Further studies in tomato plants aiming for functional characterization of LATS and HATS gene families are needed to fully understand the mechanism of regulation of these transporters to design new strategies to improve N uptake according to N availability.

3.3.2. N assimilation

N assimilation is initiated with the reduction of NO_3^- to NH_4^+, followed by NH_4^+ assimilation. As mentioned above, two enzymes, NR and NiR, have a crucial role in this process by reducing NO_3^- to nitrite and nitrite to NH_4^+, respectively (Masclaux-Daubresse *et al.*, 2010). Studies concerning the effect of N deficiency on the genetic regulation of NR and NiR in tomato plants are scarce and suggest that the expression of NR can be affected by different temporal harvest time points and light conditions, or even by the plant organ analyzed because they differ in the basal amount of NR enzyme (Galangau *et al.*, 1988; Krapp *et al.*, 2011). Accordingly, Galangau *et al.* (1988) stated no significant

changes in the expression level of *NR* in tomato leaves after N starvation, in contrast to a decrease observed in the protein level of this transporter, suggesting that N starvation controls NR activity in tomato at least in part, through a posttranscriptional regulation.

The GS/GOGAT cycle is responsible for the assimilation of N into amino acids but it also plays a significant role in the reassimilation of NH_4^+ produced during N cycling (Liu *et al.*, 2016). Besides the GS/GOGAT cycle, GDH is another alternative pathway responsible for the production of glutamate from NH_4^+. A study by Liu *et al.* (2016) allowed the identification of six GS (*SlGSI, SlGS2, and SlGS1.11.4), two GOGAT (SlNADH-GOGAT and SlFd-GOGAT*), and five GDH (*SlNAD-GDHA1-A3, SlNAD-GDHB1*, and *SlNADP-GDH*) genes in tomato plants. Importantly, *SlGS1.2, SlGS1.3, SlNADHGOGAT*, and *SlGDHB* had decreased transcriptional levels after N starvation, oppositely to *SlGS1.1* whose expression was increased by this condition. *SlGS1.1* may display an important function in N remobilization when plants go through severe N starvation, playing an important role in plant survival to N stress (Liu *et al.*, 2016). Interestingly, the expression of *GDH* was also induced by an excess of NH_4^+ (Vega-Mas *et al.*, 2019).

Asparagine synthetase (AS) is also involved in NH_4^+ assimilation as it enables an ATP-dependent transfer of the amino group of glutamine to aspartate to generate glutamate and asparagine and it is considered an ideal amino acid for N transport and storage because of its high N:C ratio and its stability (Lam *et al.*, 1994; Masclaux-Daubresse *et al.*, 2010). A study by Wang (2001) demonstrated that the expression of an AS gene was induced by NO_3^- while no evidence of the effect of N starvation on AS gene regulation was collected so far.

3.3.3. N remobilization

Under severe N starvation, and depending on the developmental stage, plants may reuse stored N by degrading nitrogenous compounds and remobilizing the newly available N (Bassham, 2007; Lemaitre *et al.*, 2008). Therefore N remobilization is considered time- and environmental-dependent as it is more frequent during the vegetative and reproductive developmental stages and favored by limiting NO_3^- conditions (Lemaitre *et al.*, 2008; Masclaux-Daubresse *et al.*, 2010). During leaf senescence, N remobilization is induced as several proteins are degraded, namely, those involved in photosynthesis, providing great amounts of reutilizable N (Masclaux-Daubresse *et al.*, 2010). GS1 isoforms are well-known markers of N remobilization and, particularly upon N deficiency, they have been described as senescence-related markers ((Lemaitre *et al.*, 2008). In tomato, the expression of the *GS1* gene was upregulated by

N starvation, suggesting that GS might have a role in the utilization of recycled N (Liu *et al.*, 2016).

Autophagy is a highly regulated and conserved process that is crucial for N remobilization inside the cell. Under starvation, activation of autophagy induces the transport of cellular contents to the vacuole for subsequent digestion to recycle nutrients, thus optimizing plant growth, development, and stress responses (Avin-Wittenberg *et al.*, 2018; Bassham, 2007; Wang *et al.*, 2019a). A downregulation of autophagy-related genes (*ATG*s) is associated with increased susceptibility of plants to N and C starvation and inhibits the breakdown of mitochondria-resident proteins during senescence (Wang *et al.*, 2019a). The expression of *ATG* genes was reportedly increased upon N starvation in several plants (Bedu *et al.*, 2020; Wang *et al.*, 2016; Zhou *et al.*, 2015). Brassinosteroid (BR) is a phytohormone that plays a critical role in autophagy (Wang *et al.*, 2019a). Data on tomato plants have revealed that in the early stages of N starvation, the accumulation of *BZR1*, a BR-activated transcription factor, was enhanced to promote N recycling by inducing the expression of *ATG*s. Overall, these results appear to indicate that tomato plants are also dependent on *ATG*s for N remobilization upon N starvation in a process likely regulated by BR.

Despite the importance of understanding genetic regulation upon N deficiency to improve NUE, these mechanisms need further characterization in tomato plants especially regarding N remobilization. Given that tolerance to low N has become a highly relevant breeding goal, it is surprising that only a few studies have compared the genetic variation in N- utilization traits among the accessions of tomato or compared measures of NUE for the same germplasm growing in different environments (Abenavoli *et al.*, 2016). However, this slow breeding progress can be, at least in part, explained by the complexity of abiotic-stress tolerance associated with polygenes (Parajuli e*t al.*, 2018). Nevertheless, Abenavoli *et al.* (2016), reported that in the N stress-tolerant 'RO' tomato genotype grown at low NO_3^- supply (0.1 mM NO_3^-), a higher shoot NO_3^- concentration and higher activity and gene expression of *NR*, as well as higher root *SlNTR2.3* expression, are likely associated with the increased NUE in this cultivar. Furthermore, the authors observed a highly significant difference in the expression of *SlNTR2.3* between contrasting cultivars (at low NO_3^- supply) suggesting that this gene may function as a marker for the identification of improved NUE tomato genotypes, especially because when grown at high NO_3^- supply, the expression was similar for both genotypes. When compared with 'UC82' (a sensitive cultivar), 'RO' showed higher *SlNRT2.3* expression and shoot NO_3^- content at high NO_3^- supply but similar NR activity, suggesting an improved capacity to store NO_3^- on the leaves that may also be a potential phenotypic

marker for improved NUE. Some of the NRT2 transporter activity is dependent on the high-affinity nitrate transporter-activating protein 2.1 (*NAR2.1*). The expression of the two-component high-affinity uptake system *SlNRT2.1/NAR2.1* was significantly higher in UC82 when compared with RO at low NO_3^- supply, but not at 5 mM NO_3^- supply. Further, while *SlNRT2.1* expression did not show any significant difference between genotypes, the *SlNAR2.1* expression was significantly higher in RO, suggesting that it might also have a role in NUE. The authors also concluded that their results may indicate that NUE components may play a greater role than N uptake efficiency in conferring higher NUE in tomato (Abenavoli *et al.*, 2016). Recently, an interesting study by Domínguez-Figueroa et al. (2020) reported that the *A. thaliana* transcription factor CDF3 is involved in N responses and improves NUE in tomato, with *CDF3* expression being transiently induced after 1 day of N starvation. Further analyses of gain-of-function and loss-of-function lines allowed the authors to confirm the role of CDF3 as an important factor in N-regulated processes, specifically that CDF3 regulates (1) the expression of an important set of NO_3^- responsive genes, including *GS-1, GS-2, NR-1*, and NO_3^- transporters *NRT2.1, NRT2.4,* and *NRT2.5*; (2) the expression of carbon assimilation genes like *PK1* and *PEPC1*; (3) a rise in the content of major amino acids (glutamate, glutamine, proline, and asparagine) and total protein; and (4) an increase in enzyme activities of N metabolism (GS, NR, and GDH) in response to N availability (Domínguez-Figueroa *et al.*, 2020). The authors also found that the overexpression of *CDF3* increased N accumulation, improved primary and lateral root elongation, enhanced the amounts of photoassimilates partitioned to the fruits (resulting from increased C and N metabolism), as well as the photosynthetic rate, biomass accumulation, and fruit yield. The recent results from Domínguez-Figueroa *et al.* (2020) suggest that *CDF3* is an important regulatory factor for the NO_3^- response and its potential role in improving NUE in tomato plants.

4. Conclusions and future prospects

Drought stress negatively affects tomato growth at several stages of development (from seed germination until fruit development) reducing fruit yield and quality. This is mostly due to a lower photosynthetic rate (leading to lower yield) and reduced transpiration rate (that affects Ca mobility to the fruits resulting in physiological disorders). N deficiency leads to plants with shorter vegetative growth periods and reduced yields, mainly caused by lower photosynthetic capacity, carbohydrate accumulation, and hormonal readjustments. This literature review also presents an overview of the genes that are differently regulated to aid plants to adapt to both stresses, which might be of interest for future tomato drought and/or N-deficit-tolerant breeding

programs. However, it is concluded that so far only a few studies have been conducted to confirm the molecular regulatory networks and the key genes involved in the tolerance responses to water and N deficit, with the latter ones being almost uncharacterized. Still regarding N stress tolerance, promising new studies have pointed out that N remobilization might be crucial for increased tolerance to N stress on tomato but more studies are needed to confirm that hypothesis.

In the environment, plants are often subjected to several abiotic stresses, including water and N deficiencies, which are closely related. However, only a few studies have addressed the complex interaction between them, and new insights are required in the future to improve WUE and NUE in tomato plants, especially in light of global climate change. Moreover, some studies here reviewed suggest that it is possible to manage drought stress by applying water-saving irrigation strategies to promote the quality and nutritional properties without compromising tomato yield, while also contributing to water conservation. Thus, continuing to explore and improve the use of deficit-irrigation techniques, especially when integrated with other novel sustainable agronomical tools (e.g. grafting, elicitors, biostimulants, and other growth-promoting substances) that have been demonstrated to improve plant response to drought conditions, is of utmost importance.

Sub-chapter 1.2

Role of silicon in mitigating drought and nutrient deficiency

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Role of silicon in mitigating drought and nutrient deficiency

Abstract

In the last decades, climate change are causing environmental issues which are threatening agriculture. Drought, due to climate change, and nutrient deficiency are certainly two of the major threats for this sector resulting in large economic losses due to reduced yields. Silicon (Si), as a beneficial element for plants, is involved in plant tolerance and/or resistance to various abiotic and biotic stresses. The beneficial role of Si has been shown in various plant species and its accumulation relies on the root's uptake capacity. Si has been widely reported to alleviate plant drought stress in both Si-accumulating and non-accumulating plants. In contrast, the literature concerning Si potential in mitigating nutrient deficiencies is far scarcer. This chapter aims to outline several mechanisms regulated by Si in response to drought and nutrient deficiency and summarize with important agronomic crops in which its application has shown beneficial effects in alleviating these abiotic stresses.

1. Introduction

Climate change will increase drought in many regions of the world (Bista *et al.*, 2018). Agriculture is an important sector of the world's economy and is highly dependent on water availability, which ultimately constitutes an essential input to agricultural production. Furthermore, drought negatively affects root nutrient uptake due to the reduction in soil moisture which causes a decreased rate of nutrient diffusion from the soil matrix to the absorbing root surface and subsequent translocation to the leaves (Rouphael *et al.*, 2012). Ensuring optimal crop yields in the context of climate change while safeguarding natural resources is deemed to be one of the biggest challenges in the current century (Le Gouis *et al.*, 2020). Silicon (Si), the second most predominant element in the earth's crust (Liu *et al.*, 2019a; Liu *et al.*, 2019b), has a beneficial effect on plants playing a functional role in the alleviation of several abiotic and biotic stresses, which have been studied for more than two decades (Líška *et al.*, 2017; Lux *et al.*, 2020). Therefore, Si has been widely used in agriculture due to improved productivity of several crop species and, also, Si serves as a valued element to humans (Tubana *et al.*, 2016). In this chapter, we will outline several mechanisms regulated by Si in response to drought or nutrient deficiency, which are highly relevant under the current context of population growth and climate change, as well as summarize several studies with important

agronomic crops in which its application has shown beneficial effects in alleviating the effects of these abiotic stresses.

2. Drought

Water stress is one of the major environmental constraints limiting plant growth and agricultural productivity. Drought induces a range of physiological and biochemical processes in plants that can reduce crop yields by up to 70%, resulting in serious economic losses (Osakabe *et al.*, 2014; Siracusa *et al.*, 2018). Although plants have evolved different morpho-physiological and biochemical strategies to cope with drought stress (Lamaoui *et al.*, 2018), increasing evidence suggests that the detrimental effects of drought can be mitigated by an adequate and balanced supply of mineral nutrients (Waraich *et al.*, 2011). One of the methods involving the alleviation of negative water stress is the application of Si. The beneficial effect of Si on plant water status has been extensively studied in various plant species subjected to drought stress (Table 1). The improvements in relative water content and/or water potential by Si application occurred under both polyethylene glycol-induced osmotic stress (Hattori *et al.*, 2007; Ming *et al.*, 2012; Sonobe *et al.*, 2010) and potted soil drought conditions (Amin *et al.*, 2018; Gong *et al.,* 2003). Different compounds of Si, including Na_2SiO_3, K_2SiO_3, or H_2SiO_3 (Table 1), have been applied either in the soil or the nutrient solution, being the first two, the most studied compounds. Although in most of the studies, Si application took place after the seedling's emergence, the priming of seeds with Si has been suggested as an alternative method to improve the drought stress tolerance in plants. Some studies have shown that Si-seed priming enhances plant resistance against future stress events and propose it as a promising and cost-effective procedure in circumstances, where water scarcity might be a problem (Ahmed *et al.*, 2014; Biju *et al.*, 2017). Although the mechanisms behind the beneficial effects of Si are still largely unknown, Si-induced plants' resistance to drought seems to be primarily related to its role in increasing non-enzymatic antioxidant capacities, by activating antioxidant enzymes and decreasing $O_2^{\bullet-}$ production rate, H_2O_2 content, and malondialdehyde concentrations (Cao *et al.*, 2017; Gong *et al.*, 2003; Gunes *et al.*, 2007; Gunes *et al.*, 2008; Shi *et al.*, 2014); maintaining photosynthetic machinery, through the promotion of photosynthetic rate and increased activity of photosynthetic enzymes (Shen *et al.*, 2010) and delaying leaf senescence (Hosseini *et al.*, 2017). Wang *et al.* (2019b) observed that Si treatment might play roles in the absorption, transformation, and transfer of light energy by optimizing the thylakoid membrane protein components in rice seedlings under drought stress. The impact of Si on chlorophyll fluorescence and the ultrastructure of chloroplasts of drought-stressed

tomato was also reported (Cao *et al.*, 2015). Silicon played an important role in suppressing the decline of the activities of ROS scavenging enzymes in the chloroplast, therefore, protecting it from severe oxidative damage, such as the distortion of the grana lamellae and stroma lamellae. Zhang *et al.* (2018) also demonstrated the positive beneficial effects of exogenous Si on the chlorophyll fluorescence and expression of photosynthesis-related genes in tomato seedlings under water stress. Increased concentrations of chlorophyll and carotenoids were also observed, thus promoting photosynthesis. Silicon is also believed to be important in reducing evapotranspiration or controlling stomata conductance when taken up and deposited in leaf cuticle, thereby reducing transpiration (Gao *et al.*, 2006; Matoh *et al.*, 1991) and consequently improving drought tolerance. It has also been shown to enhance radial hydraulic conductivity and mediate stress tolerance in sorghum seedlings and tomato plants exposed to drought (Cao *et al.*, 2017; Liu *et al.*, 2014). In sorghum seedlings, the increased root hydraulic conductance after Si addition was attributed to Si-mediated transcriptional up-regulation of some aquaporin genes (Liu *et al.*, 2014).

Several key conjugated polyamines synthesis genes were shown to be up-regulated by Si under sorghum drought-stressed plants (Yin *et al.*, 2014). It is suggested that Si improves sorghum drought resistance by mediating the balance of conjugated polyamines and ethylene levels. In leaves, the increased conjugated polyamines and decreased 1-aminocyclopropane-1-carboxylic acid (ACC, the precursor of ethylene) help to retard leaf senescence. In roots, the balance between conjugated polyamines and ACC participates in the modulation of root plasticity, increasing the root/shoot ratio thus contributing to an increase in water uptake (Yin *et al.*, 2014).

Additionally, Si is pointed out as responsible for maintaining nutrient balance (Kaya *et al.*, 2006), stimulating osmolyte accumulation (Cao *et al.*, 2017; Ming *et al.*, 2012), regulating growth substance levels (Zhu and Gong, 2014) and sequestering toxic ions (Rizwan *et al.*, 2015) when plants are under drought stress.

Since water is a limited resource in many regions of the world and taking into account the increased demand for water and food production, to fulfill the need of an increased world population, sustainable approaches to promote water use efficiency (WUE) in agriculture are highly required. Therefore, the application of Si may be an important strategy to improve crop yield in arid or semi-arid areas under water-stress conditions. However, the pathways by which Si regulates the plant water status still need further investigation, especially regarding the molecular and biochemical mechanisms. The proper form of Si application (i.e. dosage, Si source and plant developmental stage) and its performance under field conditions, as most of the studies were conducted in

laboratory trials or hydroponic conditions, is another topic that still needs extensive investigation.

Table 1 – Overview of studies in which silicon (Si) application has shown beneficial effects alleviating drought stress

Plant species	Family	Si Source	Si Concentrations	Main Results	Reference
Brassica napus L. Rapeseed/ Canola	Brassicaceae	Na_2SiO_3	0.35, 2.73 mmol/kg soil	↑ growth parameters, net assimilation rate, CO_2 absorbance, relative water content, root amino acid content, antioxidant enzymes activity ↓ intracellular CO_2 concentration, oxidative damage	Habibi, 2014
		SiO_2	1 mM	↑ photosynthetic pigments, ROS scavenging capacity, leaf relative water content and proline content ↓ oxidative stress	Hasanuzzaman *et al.*, 2018
Cicer arietinum L Chickpea	Fabaceae	$Na_2Si_3O_7$	100 ppm	↑ growth, relative water content, non-enzymatic antioxidant activity ↓ oxidative damage, proline content, enzymatic antioxidant activity	Gunes *et al.*, 2007
Cucumis sativus L. Cucumber	Cucurbitaceae	K_2SiO_3	120, 240 ppm	↑ biomass, net photosynthetic rate, leaf water content, chlorophyll content, antioxidant enzymes activity ↓ stomatal conductance, oxidative damage, plasma membrane permeability	Ma *et al.*, 2004
Glycine max L. Merrill Soybean	Fabaceae	$Na_2SiO_3 \cdot 9H_2O$	1.70 mM	↑ growth, photosynthetic enzymes, chlorophyll and anthocyanin content, relative leaf water content ↓ oxidative damage, proline and antioxidant enzymes	Shen *et al.*, 2010
Helianthus annuus L Sunflower	Asteraceae	$Na_2Si_3O_7$	100 ppm	↓ stomatal resistance, oxidative damage, proline content, SOD and APX activity ↑ leaf relative water content, CAT activity	Gunes *et al.*, 2008

Lens culinaris Medik. Lentil	Fabaceae	$Na_2Si_3O_3$	2 mM	↑ germination and seedling vigour, Si content, antioxidants, hydrolytic enzymes activity ↓ concentration of osmolytes and ROS	Biju *et al.*, 2017
Mangifera indica L. Mango	Anacardiaceae	K_2SiO_3	1.5 mM	↑ growth, net assimilation rate, relative water content, photosynthetic pigments, N, P, K and protein content, IAA, GA, and CK (promoters) endogenous levels, yield and fruit quality ↓ ABA, accumulation of several osmolytes and antioxidant enzyme activity	Helaly *et al.*, 2017
Medicago sativa L. Alfafa	Fabaceae	K_2SiO_3	4 kg/pot	↑ forage biomass, number of branches, water use efficiency ↓ transpiration rate and stomatal conductance No effect on leaf area and photosynthetic rate	Liu and Guo 2013
Oryza sativa L. Rice	Poaceae	K_2SiO_3	0.5, 1.0, 1.5, 2.0 mM	↑ dry weight, root traits, chlorophyll content, water potential, photosynthetic rate, transpiration rate, basal quantum yield, and maximum quantum efficiency of PSII photochemistry ↓ K, Na, Ca, Mg, Fe content	Chen *et al.*, 2011
		Na_2SiO_3	2.5 mM	↑ growth parameters, total, free, and bound water contents, water and osmotic potential, turgor pressure, active accumulation of several osmolytes, net photosynthetic rate, transpiration, and water-use efficiency	Ming *et al.*, 2012
Poa pratensis L. Kentucky Bluegrass	Poaceae	$Na_2SiO_3 \cdot 9H_2O$	200, 400, 800 mg/L	↑ net photosynthesis, leaf water contents, relative growth rate, root/shoot ratio, instantaneous water use efficiency, leaf green color and turf quality. ↓ Stomatal conductance, C:N ratio	Saud *et al.*, 2014
Saccharum spp. L. Sugarcane	Poaceae	$CaO\text{-}SiO_2$	20, 40, 60, 80, 100 g/pot	↑ photosynthetic CO_2 assimilation, stomatal conductance, transpiration, chlorophyll fluorescence, plant growth, leaf relative water content, biomass-yield, photosynthetic pigments, antioxidant enzymes activity (CAT, POD, SOD), phytohormones content (ABA, IAA, GA_3)	Verma *et al.*, 2020

Solanum lycopersicum L. Tomato	Solanaceae	Na_2SiO_3	0.6, 1.2, 1.8 mM	↑ growth parameters, photosynthetic efficiency of PSII, electron transport rate, net photosynthetic rate, antioxidant enzymatic system ↓ oxidative damage, chlorophyll degradation	Cao *et al.*, 2017
		K_2SiO_3	2.5 mM	↑ tomato growth, photosynthetic and transpiration rate, water status, root hydraulic conductance, enzymatic and non-enzymatic antioxidant system, ↓ oxidative damage and ROS levels No effect on the transcription of plasma membrane aquaporin genes or proline levels	Shi *et al.*, 2016
		Na_2SiO_3	50 ppm	↑ K, Ca and Mg accumulation, plant growth, RWC, fruit yield ↓ Na+ uptake, electrolyte leakage, proline content	Ullah *et al.*, 2016
		$Si(OH)_4$	1.5 mM	↑ growth parameters, chlorophyll content, uptake of sulfur and ammonium, production of amino acids (arginine, methionine, serine, proline, alanine, threonine, glutamic acid, GABA and glycine), production of free polyamines (putrescine and spermidine ↓ GSSG to GSH ratio	Ali *et al.*, 2018
		Na_2SiO_3	1.2 mM	↑ growth parameters, hydraulic conductivity in radial direction, water uptake, solute accumulation (proline, soluble sugar and soluble protein) osmotic adjustment ability of root, water absorption and antioxidant activity, ↓ oxidative damage	Cao *et al.*, 2017
Solanum tuberosum L. Potato	Solanaceae	$CaMgSiO_4$	284.4 mg/dm^3	↑ Si concentration in potato leaves, mean tuber weight, tuber yield and proline content. ↓ total sugars and soluble proteins, stalk lodging	Crusciol *et al.*, 2009
Sorghum bicolor (L.) Moench Sorghum	Poaceae	Not specified	1.78 mM	↑ dry weight, root water uptake, root amino acid and sugar content ↓ osmotic potential No effect on root anatomical traits related to water transport	Sonobe *et al.*, 2010

		Na_2SiO_3	1. 67 mM	↑ plant biomass, Si concentration, photosynthetic rate, stomatal conductance, leaf and whole-plant transpiration, relative water content, whole-plant hydraulic conductance and expression of root aquaporin genes ↓ root hydraulic conductance, No effect on osmotic potential of root xylem sap, on leaf-specific conductivity of stem and on root surface area, vessel diameter and number.	Liu *et al.*, 2014
		H_2SiO_3	0.83 mM	↑ growth parameters, chlorophyll concentration, photosynthetic rate, leaf RWC, transpiration rate, soluble sugar contents, levels of both free and conjugated polyamines (putrescine, spermidine and spermine) and of several key polyamines (synthesis genes ↓ proline and 1-aminocyclopropane- 1-carboxylic acid (ACC) content and osmotic potential	Yin *et al.*, 2014
		Na_2SiO_3	7.14 mmol	↑ biomass, leaf weight ratio, RWC, water potential and leaf area ↓ specific leaf area	Gong *et al.*, 2003
Triticum aestivum L. Wheat	Poaceae	Na_2SiO_3	1, 2 mM	↑ growth parameters, chlorophyll content, RWC, Si, K and Ca concentration ↓ proline accumulation and oxidative damage	Kaya *et al.*, 2006
Zea mays L. Maize	Poaceae	Ca_2SiO_4	100 ppm	↑ growth parameters, photosynthetic rate, yield and yield attributes, ↓ transpiration rate	Amin *et al.*, 2018
		Na_2SiO_3	4, 6 mM	↑ plant growth parameters, photosynthetic pigments, antioxidant enzymatic activity ↓ oxidative damage, proline levels, accumulation of glycine betaine and total soluble sugars	Parveen *et al.*, 2019

↑ (increase) or ↓ (decrease) compared to water stress treatments without Si application

3. Nutrient Deficiency

Compared with the benefits of Si under drought stress, knowledge about the role of Si in alleviating nutrient deficiency is scarcer, as recently pointed out by Ali *et al.* (2020) and Pavlovic *et al.* (2021). Nevertheless, for several important agronomical crops, Si supplementation alleviates nutrient deficiency (Table 2) with most of these crops being Si accumulating species.

The literature is even scarcer concerning the Si benefits for N deficiency mitigation. Nevertheless, recent studies showed that the application of Si positively impacted N uptake, assimilation, and remobilization, improving N utilization within plant tissues (Minden *et al.*, 2020; Pavlovic *et al.*, 2021). For example Haddad *et al.*, 2018 showed a beneficial effect of Si in preventing N starvation in *Brassica napus*, coinciding with a decrease in oxidative stress markers (H_2O_2 and MDA) and a delay in leaf senescence. Wu *et al.* (2017) showed that in rice plants under low N supply, Si treatment increased the transcript levels of genes involved in N uptake and assimilation (*OsNRT1;1*, *OsGS2*, *OsFd-GOGAT*, *OsNADH-GOGAT2,* and *OsGDH2*). The authors suggest a positive feedback response mechanism operating under reduced N availability, that could explain the increases in plant dry weight and decreases in root-to-shoot ratio resulting from Si application.

Additionally, a possible explanation for the positive effect of Si on N stress mitigation is its role in the protection and alteration of photosynthetic mechanisms. Si might promote a decline in carbon (C) levels, partially replacing it in shoot tissues, favoring the C metabolism efficiency, and modulating the N and P use efficiency. Consequently, Si enhances photosynthetic rates, but mostly in Si-accumulating species such as quinoa (Lata-Tenesaca *et al.*, 2021), sorghum (de Carvalho *et al.*, 2022; Calero Hurtado *et al.*, 2020), sugarcane (Frazão *et al.*, 2020; de Oliveira Filho *et al.*, 2021; de Souza Júnior *et al.*, 2022), sunflower (Calero Hurtado *et al.*, 2020) and wheat (Neu *et al.*, 2017). Nevertheless, the positive effect of Si in photosynthesis might depend on the application method used as mentioned by Deus *et al.* (2020). These authors suggest that Si foliar application has a low impact on the physical mechanism of protection, as no alterations in the photosynthetic processes (net CO_2 assimilation) and transpiration of rice plants were found. Deus *et al.* (2020) propose that Si sprayed on the leaves might have related to lignin-carbohydrate complexes thus increasing lignification in plants under N starvation.

Nevertheless, although the underlying mechanisms are not yet well established, some modes of Si action have been suggested, as recently reviewed by Pavlovic *et al.*, (2021). Considering these studies, it is reasonable to hypothesize that N-deficient pants

could benefit from Si supplementation. However, more research on the impact of Si application on several mechanisms, such as the antioxidant system of plants under N deficiency, one of the key mechanisms proposed by Liang *et al*. (2007) for Si alleviating abiotic stresses, is needed.

4. Conclusion

To help mitigate climate change effects on plant growth and to guarantee enough food production for the growing population, innovative strategies need to be tested, including the application of novel compounds that help plants to cope with environmental hazards. In this chapter, we have clearly shown that various chemical forms of Si have mitigated the negative effects of drought and nutrient deficiency. However, the mechanisms underlying plant responses to Si application in response to the aforementioned stresses, especially regarding nutrient deficiency, are not yet well explored which requires future attention.

Table 2 – Overview of studies in which silicon (Si) application has shown beneficial effects alleviating nutrient deficiency stress.

Plant species	Family	Nutrient Deficiency	Si Source	Si Concentrations	Main Results	Reference
Brassica oleracea L. Cabbage	Brassicacea	Calcium	Na_2SiO	2.5 mM	↑ root and shoot dry matter, shoot Ca accumulation, root and shoot Si accumulation, leaf AsA content and leaf firmness index ↓ accumulated fresh matter loss and electrolyte leakage No effect on root Ca accumulation,	da Silva *et al.*, 2021
Cucumis sativus L. Cucumber	Cucurbitaceae	Iron	$Si(OH)_4$	1.5 mM	↑ chlorophyll content, root and shoot dry mass, leaf Fe and Si concentration, accumulation of Fe-mobilizing compounds in roots and root activity of Fe acquisition,	Pavlovic *et al.*, 2013
Eruca vesicaria Rocket	Brassicacea	Calcium	Na_2SiO	2.5 mM	↑ leaf area, leaf dry matter, shoot Ca accumulation, roots and shoot Si accumulation, phenol, carotenoids ascorbic acid and chlorophyll concentration, F_v/F_m ↓ electrolyte leakage and accumulated fresh matter loss No effect on root dry matter	da Silva *et al.*, 2021
Glycine max L. Merrill Soybean	Fabaceae	Potassium	Na_2SiO_3	2 mM	↑ length of primary and lateral roots, lateral root density, root, stem and leaf dry weight, root/shoot ratio and root, stem and leaf K content. ↓ electrolyte leakage, oxidative damage, reactive oxygen species and antioxidant enzymes activity	Miao *et al.*, 2010

Hordeum vulgare L. Barley	Poaceae	Potassium	Na_2SiO_3	0.5, 1 mM	↑ root and leaves Si concentration, total Si uptake per plant, glycolysis (3PGA and PEP) and TCA cycle (fumarate and citrate) in leaves ↓ of senescence marker gene HvS40, sugars (glucose and fructose), glycolysis (PEP) and TCA cycle (fumarate) in roots and sugars (glucose and fructose) in leaves No effect on root and shoot dry weight, chlorophyll content, starch metabolism and ABA concentration	Hosseini *et al.*, 2017
		Sulphur	$Si(OH)_4$	1.5 mM	↑ Si concentration in roots and shoots, NO_3^- concentration in shoots and primary metabolism (total amino acids, glucose, and sucrose) in roots and shoots, ↓ shoot fresh weight, root to shoot ratio, chlorophyll content, of senescence marker gene HvS40, S and SO_4^{2-} concentration in shoots, GSSG:GSH ratio in roots and ABA concentration in roots No effect on root fresh weight and relative water content	Maillard *et al.*, 2018
Oryza sativa L. Rice	Poaceae	Nitrogen	Not specified	3.45 g L^{-1}	↑ Si levels, dry biomass, lignin content in shoots and grain yield ↓ Leaf electrolyte leakage No effect on transpiration rate, net CO_2 assimilation rates and on the levels of P and N in shoots	Deus *et al.*, 2020
Saccharum officinarum L. Sugarcane	Poaceae	Manganese	Na_2SiO	2.0 mM	↑ Si accumulation, ↓ antioxidant enzymes activity No effect on leaf area, dry matter, Mn accumulation, Mn efficiency use, lipid peroxidation, protein, total phenol, carotenoid, chlorophyll a and b content, F_v/F_m	Oliveira *et al.*, 2021

Saccharum spontaneum L. Energy cane					↑ leaf area, dry matter, Si and Mn accumulation, Mn efficiency use, protein, total phenol, carotenoid, chlorophyll a and b content, F_v/F_m, antioxidant enzymes activity ↓ lipid peroxidation	
Sorghum bicolor (L.) Moench Sorghum	Poaceae	Potassium	H_2SiO_3	1 mM	↑ dry weight, stomatal conductance, transpiration rate, photosynthetic rate, leaf relative water content, leaf osmotic potential, total K^+uptake, whole-plant hydraulic conductance, Root hydraulic conductance, xylem sap K^+ concentration and K^+ osmotic potential contribution ↓ leaf water potential and osmotic potential in xylem sap i No effect on root/shoot ratio and K concentration,	Chen *et al.*, 2016b
		Potassium	H_2SiO_3	1 mM	↑ total dry weight, photosynthetic rate,F_v/F_m, soluble protein content, chlorophyll content and chlorophyll a/b, ↓ arginine accumulation, diamine oxidase and polyamine oxidase activity, reactive oxygen species and antioxidant enzyme activities No effect on K^+ concentration in leaves	Chen *et al.*, 2016a
		Phosphorus	Na_2SiO_3	1.5 mM	↑ Stem diameter, plant height and shot dry matter, Si and P accumulation, higher efficiency of P absorption, relative chlorophyll index, phenolic compounds and F_v/F_m ↓ electrolyte leakage	Silva and Prado, 2021
Triticum aestivum L. Wheat	Poaceae	Phosphorus	Na_2SiO_3	400 mg/kg	↑ Dry biomass, citrate and malate exudation, shoot P concentration, total P uptake and relative expression of genes encoding inorganic P transporters in roots	Kostic *et al.*, 2017

		Potassium	K_2SiO_3	2 mM (via nutrient solution), 5.3 mM (via foliar spray)	↑ shoot dry weight, total dry matter, root and leaf area, root and stem diameter, root and shoot length**,** K use efficiency, Si concentration, total chlorophyll and carotenoid content, photosynthesis, transpiration rate, stomatal conductance, relative water content and water use efficiency ↓ electrolyte leakage No effect on root dry matter and K concentration	dos Santos Sarah *et al.*, 2022
Zea mays L. Maize	Poaceae	Magnesium	$Si(OH)_4$	1.5, 5 mM	↑ shoot fresh weigh, chlorophyll levels, Si content, accumulation of soluble sugars in shoots, stress amin oacids (GABA, glycine, serine) in shoots, organic acids (isocitric acid and glutamic acid), polyamines synthesis (putrescine) and phytohormones levels (cytokinins and jasmonates) ↓ root fructose and sucrose concentration No effect on root dry weight, uptake and/or translocation of Mg, root glucose concentration and root GABA, glycine and serine concentration	Hosseini *et al.*, 2019

↑ (increase) or ↓ (decrease) compared to nutrient deficiency treatments without Si application.

CHAPTER 2

Unravelling the mechanisms involved in tomato responses to combined nitrogen and water deficit

Sub-chapter 2.1

Young tomato plants respond differently under single or combined mild nitrogen and water deficit: an insight on morpho-physiological responses and primary metabolism

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Young tomato plants respond differently under single or combined mild nitrogen and water deficit: an insight on morpho-physiological responses and primary metabolism

Abstract

This study aimed to understand the morphophysiological responses and primary metabolism of tomato seedlings subjected to mild levels of nitrogen and/or water deficit (50% N and/or 50% W). After 16 days of exposure, plants grown under the combined deficit showed similar behavior to the one found upon exposure to single N deficit. Both N deficit treatments resulted in a significantly lower dry weight, leaf area, chlorophyll content, and N accumulation but in a higher N use efficiency when compared to control (CTR) plants. Moreover, concerning plant metabolism, at the shoot level, these two treatments also responded similarly, inducing higher C/N ratio, nitrate reductase (NR) and glutamine synthetase (GS) activity, expression of RuBisCO encoding genes as well as a downregulation of *GS2.1* and *GS2.2* transcripts. Interestingly, plant metabolic responses at the root level did not follow the same pattern, with plants under combined deficit behaving similarly to W deficit plants, resulting in enhanced nitrate and proline concentrations, NR activity, and upregulation of *GS1* and *NR* genes than in CTR plants. Overall, our data suggest that the N remobilization and osmoregulation strategies play a relevant role in plant acclimation to these abiotic stresses and highlight the complexity of plant responses under a combined N+W deficit.

1. Introduction

Water scarcity and nitrogen (N) over usage are two of the most serious bottlenecks to the sustainability of the agricultural sector (Fernandes *et al.*, 2022). This sector uses about 80% of the total available freshwater (which is becoming increasingly scarce), as well as high amounts of N fertilizer, with their total required amounts projected to double by 2050 (Esteban *et al.*, 2016a; Liang *et al.*, 2019; Elbehri, 2015). However, in general, crops only take up 40–50% or less of the applied N (Coskun *et al.*, 2017a; Wang *et al.*, 2019; Coskun *et al.*, 2017b), with the rest being lost to the environment consequently leading to a negative impact on soil, groundwater and freshwater bodies, ecosystems, and climate (Liu *et al.*, 2016; Wang *et al.*, 2019). To enhance the sustainability of food production systems, the European Green Deal (2020) has established as targets for 2030 a reduction by at least 20% in the use of fertilizers and by 50% in nutrient losses in an attempt to contribute to reducing N leaching and improving water quality (Schebesta and Candel, 2020).

Tomato (*Solanum lycopersicum*) is one of the most cultivated vegetable crops worldwide (Heuvelink *et al.*, 2020). In Europe, tomato production was valued at Euro 7.3 billion in 2017, accounting for 21% of the value of the total fresh vegetable production (AD, 2019). However, its production relies on high fertilizer inputs and regular irrigation for maximum yield (Sandhu *et al.*, 2021), with most commercial tomato cultivars being quite sensitive to water deficit and N deficiency at all stages of plant development (Du *et al.*, 2018; Heuvelink *et al.*, 2020). Thus, considering the increased consumption of fresh tomato and tomato-derived products (AD, 2019), the development of strategies focused on the rational use of water and fertilizers in tomato production is highly required.

Several studies have demonstrated that both stresses, when individually imposed, negatively impact leaf and root morphological traits, affecting overall plant growth rates (recently reviewed by Machado *et al.*, 2022). Furthermore, both stresses are known to have a severe impact on metabolic pathways, including osmotic adjustment, decreased nutrient uptake, and photosynthesis, either due to stomatal closure and/or metabolic damage (Gonzalez-Dugo *et al.*, 2010; Ding *et al.*, 2018). Among the osmolytes produced, proline is known to accumulate at high levels in response to osmotic and many other abiotic stresses, including N deficiency (Szabados and Savouré, 2010; Hayat *et al.*, 2012; Bradford, 1976). Beyond that, several enzymes and genes involved in N metabolism and assimilation are known to be affected by N or water deficit (Kavoosi *et al.*, 2014; Lemaître *et al.*, 2008; Nacry *et al.*, 2013; Plett *et al.*, 2020). N assimilation in plant tissues requires the reduction of nitrate (NO_3^-) by nitrate reductase (NR; EC 1.7.1.1), generating nitrite (NO_2^-), which is further enzymatically reduced to ammonium (NH_4^+). This last form can be directly assimilated by glutamine synthetase (GS; EC 6.3.1.2) into glutamine or be oxidized back to NO_2^- (Cao *et al.*, 2018). The bioavailability of N in soil depends on the water status (Végh, 1991); therefore, under drought, N uptake can be reduced in some crops (reviewed by Gonzalez-Dugo *et al.*, 2010), including tomato (Sánchez-Rodríguez *et al.*, 2011c). In line with this, Sánchez-Rodríguez *et al.* (2011a) reported that moderate water deficit also decreased the NO_3^- content in tomato cultivars sensitive to drought, as well as NR activity. This decrease in tomato NR activity was also reported by Hayat *et al.* (2012), suggesting that both factors can interact with each other and that their combined action may induce a different plant response (Gonzalez-Dugo *et al.*, 2010; Ding *et al.*, 2018; Plett *et al.*, 2020).

Indeed, recent studies have shown that plants' physiological and molecular responses to several combined stresses (namely chilling and drought, drought and heat, or salinity and heat) can be unique and contrasting to those observed under single stresses (Zhou *et al.*, 2017; Hussain *et al.*, 2018; Sousa *et al.*, 2022). Machado *et al.* (2023) also found that

tomato plants were able to employ different acclimation strategies to cope with combined N and water deficits, resulting in a significantly higher total antioxidant capacity and enzymatic activity compared to single deficits. Nonetheless, although the effect of single N and water deficits have been broadly studied in different species, research focused on the effect of combined N and water deficit is still very scarce but urgently needed to promote the efficient use of these two highly valuable crop inputs (Machado *et al.*, 2023; Ruggiero *et al.*, 2022).

Based on these premises, the present study aims at contributing to a better understanding of the mechanisms behind the acclimation strategies of young tomato plants when subjected to single or combined N and water deficit. A focus was given to the morpho-physiological responses and the primary plant metabolism through the analysis of a large number of parameters, including (i) morphological traits; (ii) physiological responses (water relations, stomatal responsiveness; (iii) plant metabolism [concentration of proline, total soluble protein, N, C, and NO_3^- as well as N metabolism-related enzymes (NR and GS activity)]; and (iv) transcript accumulation of C (*rcbL* and *rcbS*) and N (*GS1, GS2.1, GS2.2,* and *NR*) metabolism-related genes. We hypothesize that there is a cross-talking between N and water uptake; therefore, plants will respond differently to single deficits compared to combined deficits.

2. Material and Methods

2.1. Plant material and growth conditions

The experiment was carried out in a growth chamber (temperature: 25 °C Day/23 °C night; relative humidity: 70%; photosynthetic photon flux density (PPFD): 300 µmol/m^2/s; photoperiod: 16 h light) according to a complete randomized design.

Seeds of *Solanum lycopersicum* cv. Micro-Tom were sown in trays filled with commercial germination potting substrate (SIRO, Portugal). At the third leaf stage (approximately three weeks after sowing), seedlings were selected for uniformity to be transplanted to single-plant pots (10 cm high, 10 cm diameter) filled with 0.1 to 1.5 mm-grade vermiculite (60 g). These tomato seedlings were divided into four groups for applying the following treatments: control (CTR; 10.5 mM N; 100% field capacity), nitrogen deficit (N; 5.3 mM N), water deficit (W; 50% field capacity), and combined stress (N+W; 5.3 mM N; 50% field capacity). Since we aimed at having a progressive water deficit, as seedling transplanting is a critical phase of the cultivation period, just prior to transplanting, all pots were irrigated to field capacity (FC), determined using the soil gravimetric water content method (Machado *et al.*, 2023). This corresponded to 300 mL of the respective nutrient

solution applied to each pot [CTR and W deficit treatments: 10 mM NO_3^-; 0.5 mM NH_4^+; 1.9 mM H_2PO_4; 6.1 mM K^+; 3.6 mM Ca^{2+}; 1.6 mM SO_4^2; 2.5 mM Mg^{2+}; 2.6 mM Cl^-; 0.5 mM HCO_3^- (pH of 6 and E.C. of 2.50 dS/m); N deficit and N+W deficit treatments: 5 mM NO_3^-; 0.3 mM NH_4^+; 1.9 mM H_2PO_4; 6.1 mM K^+; 3.6 mM Ca^{2+}; 4.8 mM SO_4^2; 2.8 mM Mg^{2+}; and 5.5 mM Cl^-; 0.5 mM HCO_3^- (pH 6 and E.C. of 2.80 dS/m)]. Both nutrient solutions had the following micronutrient composition: 35 µM Fe-EDDHA; 10 µM Mn^{2+}; 20.1 µM B; 0.9 µM Cu^{2+}; and 5.0 µM Zn^{2+}; 0.5 µM MoO_4^{2-}. Following seedling transplanting, all pots were covered to prevent evaporation, and no more nutrient solution was added until the end of the experimental period. Concerning further water supply, each pot from the CTR and N deficit treatments was daily weighted and rewatered (with distilled water) to maintain FC at 100% throughout the trial. In contrast, in W and N+W deficit treatments, no additional irrigation was given to the plants, resulting in a progressive decrease of substrate FC. Ten days after transplanting, five plants from each treatment were randomly collected. The average fresh weight of these plants (per treatment) was subtracted from the total pot weight (per treatment) to calculate the actual weight of the water/nutrient solution present in the pot (which corresponded to 75% FC for Treatment W deficit and N+W deficit). The experiment stopped when plants subjected to single or combined water deficits reached 50% of FC (corresponding to 16 days after transplanting).

At harvest, an analysis of the morphological and physiological parameters was performed (n = 6). The remaining nine plants were separated into roots and shoots and immediately frozen in liquid nitrogen, macerated (n = 3, each biological replicate consisting of a pool of three individual plants), and subsequently, stored at −80 °C for further use in biochemical and molecular analyses.

2.2. Plant growth and physiological parameters

At the end of the experimental period, several plant biometric traits were measured. The maximum root length was recorded, and the total leaf number only took into account leaves with more than 1 cm in length. The total leaf area was measured using an LI-3100C area meter (LI-COR, Lincoln, Nebraska, USA). The dry weight (DW) of leaves, stems, and roots were determined separately (48 h at 105 °C in a ventilated oven). Specific leaf area (ratio of leaf area to leaf dry weight; cm^2/g dw), leaf weight ratio (ratio of leaf dry weight to total dry weight; g/g dw), leaf area ratio (ratio of leaf area to total dry weight; cm^2/g dw), and shoot-to-root ratio (ratio of shoot-to-root dry weight; g/g dw) were calculated according to the “classical approach” described by Hunt (2012).

Leaf chlorophyll content was measured on the youngest fully expanded leaf using the non-destructive chlorophyll meter SPAD (Konica Minolta SPAD-502 model, Minolta, Tokyo, Japan).

N use efficiency and N accumulation were calculated as follows: ratio of total plant DW to total N applied (g dw/g N applied) and coefficient between the total N concentration in all plant tissues and the total DW of the plant (mg N).

Plant water status was estimated by measuring shoot water potential (Ψ; MPa) 2 h before the photoperiod, using a Scholander pressure chamber. Water use efficiency (mg/mL) was calculated as the ratio between total plant DW (mg) and water consumption per plant (mL).

Stomatal responsiveness to leaf desiccation was assessed gravimetrically following the method described by Carvalho *et al.* (2017). In short, the youngest fully expanded leaflet of each plant was detached at the onset of the light period. Immediately after excision, leaflet petioles were recut under water and placed in flasks filled with degassed water. Leaflets were further incubated for 2 h to establish their saturated fresh weight. After rehydration, the leaflets were allowed to desiccate for 4 h on a bench. Leaflet weight was gravimetrically recorded every 5–10 min for 4 h. Following the evaluation of stomatal responsiveness, leaflet area (using Image J; https://imagej.nih.gov/ij/, accessed on 15 December 2021) and dry weight were measured. The transpiration rate was calculated according to Equation (1), and for the calculation of the RWC, Equation (2) was employed. In both measurements, one leaflet per plant was evaluated for six plants per treatment.

$$\text{Transpiration rate (mmol m}^{-2}\text{ s}^{-1}) = \left(\frac{(\Delta leaflet\ fresh\ weight\ (g))}{molar\ mass\ water\ (g\ mol^{-1})} \times 1000\ (mmol\ mol^{-1})\right) / measurement\ interval\ (s) / leaflet\ \ area\ (m^2) \quad (1)$$

$$\text{RWC} = \frac{fresh\ weight - dry\ weight}{saturated\ fresh\ weight - dry\ weight} \times 100 \quad (2)$$

2.3. Plant Metabolism

2.3.1. Proline and Protein Quantification

Proline concentration was quantified spectrophotometrically in frozen roots and shoots (250 mg), according to the method of Bates *et al.* (1973). Briefly, after reading sample absorbance at 520 nm, proline levels were calculated through a standard curve previously prepared with known concentrations of proline and expressed in terms of fresh weight (FW).

The extraction of total soluble protein was performed under cold conditions according to Fidalgo *et al.* (2011), using 250 mg of frozen roots and shoots. After centrifugation, the supernatant was collected, and the soluble protein concentration of each sample was determined by the Bradford method (Bradford, 1976). Results were calculated from a standard curve prepared with solutions of known concentration of bovine serum albumin (BSA) and expressed on an FW basis.

2.3.2. Total nitrogen (N), carbon (C), nitrate (NO_3^-) quantification

These analyses were performed by an external lab (A2 Análises Químicas Lda, Guimarães, Portugal). The N and C concentrations were determined using the DUMAS method based on ISO-16634-1 (ISO, 2008). The NO_3^- quantification samples were macerated and extracted with water and quantified by the potentiometric method based on the methods described by Mills and Jones Jr (1996).

2.3.3. Extraction and quantification of N metabolism-related enzymes

The quantification of nitrate reductase (NR; EC 1.6.6.1.) activity was performed in roots and shoots by spectrophotometry according to the method of Kaiser and Brendle-Behnisch (1991). NR activity was estimated using NADH extinction coefficient (6,22 mM^{-1} cm^{-1}) and expressed as mmol/min for each mg of protein.

Glutamine synthetase (GS; EC 6.3.1.2.) activity was determined following the protocol of Shapiro and Stadtman (1970), using frozen roots and shoots. The activity was determined by the transferase assay, based on a colorimetric assay, by recording the absorbance at 500 nm. After measuring the absorbance, the activity of GS was calculated and expressed as a nkat/mg of protein.

2.3.4. Gene expression quantification analysis of RuBisCO and N metabolism-related genes

The transcript levels of *rcbS, rcbL, GS1, GS2.1, GS2.2,* and *NR* genes were evaluated by quantitative real-time PCR in roots and shoots. Total RNA was extracted from frozen roots and shoot tissues (ca. 100 and 50 mg, respectively) using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. RNA yield was quantified using a NanoPhotometer (Implen GmbH, München, Germany), and its integrity was checked by agarose gel electrophoresis. Single-stranded cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions in a Doppio Thermal Cycler (VWR, Oud-Heverlle, Belgium).

Primers for *GS1, GS2.1,* and *GS2.* genes were selected using Primer-BLAST from NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/, accessed on 15 January 2022.) for an expected PCR product of 70–150 bp and primer melting temperatures between 57 and 63 °C, whereas primer sequences for *rcbL, rcbS,* and *NR* genes were obtained from previous studies (Mariz-Ponte *et al.*, 2021; Jin *et al.*, 2009). The primer sequences for each gene are listed in Table 1.

Table 1 - Primer sequences (Forward and Reverse) and annealing temperature (T_{ann}) of target genes used for RT-qPCR analysis.

Gene (Accession number)	Primer sequence (5'-3')		$T_{ann.}$
	Forward	Reverse	
rcbL	ATC TTG CTC GGG AAG GTA ATG	TCT TTC CAT ACC TCA CAA GCA G	55.9
rcbS	TGA GAC TGA GCA CGG ATT TG	TTT AGC CTC TTG AAC CTC AGC	55.7
GS1 *NM_001319855.1*	ACA GCA CCA AGT CGA TGA GG	TGA TGT TGG CTG TTT CGT GC	58.8
GS2.1 *NM_001323669.1*	TGC ATT GTC CAC TTA GTT GGT T	TTC AGC ACC ACA GAG CTC CA	57.7
GS2.1 *NM_001323670.1*	TGC ATT GTC CAC TTA GGA GGT	CAC CAC AGA GCT CCA CAT CTT	56.2
NR	CAA GCA ATC CAT CTC CCA T	CAT CTC TGT ATC GTC TTC AGG A	53.3

Reverse transcription polymerase chain reactions (RT-qPCR) were performed on a thermal cycler CFX96 Touch Deep Well Real-Time PCR Detection System (Bio-Rad Laboratories, Inc, California, USA) and data visualized with the software Bio-Rad CFX Manager 3.1. The amplification protocol cycle was 95 °C for 3 min and 40 cycles at 95 °C for 15 s, 30 s at each primer pair optimal annealing temperature (Table 2), and 72 °C for 30 s. Amplifications were carried out using a final volume of 20 µL, which consisted of 1 µL of the primer forward and 1 µL of the primer reverse (both at 6 µM), 10 µL of 2× iQ SYBR® Green Supermix (Bio-Rad, CA, USA), and 8 µL of a 1:100 dilution of the template cDNA. Melt curve profiles were analysed for each tested gene. The comparative CT method (ΔΔCT, Livak and Schmittgen, 2001) was used for the relative quantification of gene expression values using ubiquitin (*UBI*) and TIP41-like protein (*TIP41*) genes as control transcript and the control plants as the reference sample. For relative gene expression analysis, the fold change with biological significance was considered to be lower than 0.5 (downregulation) or higher than 2 (upregulation) (Vaerman *et al.*, 2004), and for each of the three biological replicates and target genes, two technical replicates were analysed.

2.4. Statistical analysis

For each analysis performed, there was a different number of biological replicates (specified in section 2.1). Results were expressed as mean ± standard error of the mean (SEM). Data were subjected to a one-way analysis of variance (ANOVA), followed by Tukey's *post-hoc* test, whenever significant differences were found (p=0.05). Additionally, a Principal Component Analysis (PCA) with *Varimax* rotation was performed to establish the relationship among the variables which showed significant differences among treatments. Data included in the PCA (n=6 for plant growth and n=3 for plant metabolism) was mean-centered and weighed with the inverse of the standard deviation before performing this analysis to guarantee that all variables had the same weight when building the model. All statistical analyses were performed in IBM SPSS Statistics 26.

3. Results

3.1. Plant growth and physiological parameters

Exposing young tomato plants for 16 days to single or combined mild N and water (W) deficit significantly impacted several morphological traits (Table 2). For instance, total dry weight (DW) and shoot DW were significantly lower in plants subjected to single or combined N deficit (resulting in a 27% decrease in total DW and up to 32% decrease in shoot DW compared to CTR plants). Plants exposed to single W deficit did not differ significantly from the CTR plants in those traits. In terms of root DW, no significant effects were found when comparing the CTR plants with the ones grown under different deficits. Taking into account the dry matter partitioning, plants under W deficit (single or combined) significantly invested more in their roots (leading to a 30% and 33% lower shoot-to-root ratio compared to CTR plants) and reduced the partitioning towards their leaves (resulting in significantly lower leaf weight ratio). Moreover, plants grown under N deficiency (single or combined) showed a significantly lower leaf area (31 and 36% decrease) and leaf chlorophyll content (14% and 18% decrease) when compared to CTR plants (Table 1). In contrast, leaf area ratio and specific leaf area did not significantly change with any of the imposed deficits (Table 2). Additionally, plant height and its components (internode number and average internode length), as well as root length, did not respond to any of the studied deficits.

Finally, concerning the indicators of N and W use capacity, significant differences were found in the former. Plants subjected to N and N+W deficit had up to 50% lower N accumulation and up to 47% higher N use efficiency (NUE) than plants grown under CTR or W deficit conditions. The N accumulation of plants subjected to W deficit was 39% and 120%

higher than in CTR and N+W deficit plants (Table 2). Water use efficiency (WUE) and shoot water potential were not significantly influenced by the studied N and W deficits (Table 2).

Table *2* – Morphophysiological responses of tomato cv. Micro-Tom grown for 16 days under control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N+W; 5.3 mM N+ 50% W). Abbreviations: DW = dry weight, Av. = average; Ψshoot = shoot water potential.

Parameters	CTR	N deficit	W deficit	N+W deficit	*p*-value
Total DW (g)	0.774±0.051^{a}	0.567±0.040^{b}	0.750±0.028^{a}	0.566±0.037^{b}	**0.0010**
Shoot DW (g)	0.592±0.037^{a}	0.429±0.029^{b}	0.536±0.030^{ab}	0.402±0.029^{b}	**0.0009**
Root DW (g)	0.182±0.016 ^{ab}	0.138±0.012^{b}	0.214±0.013^{a}	0.164±0.011^{ab}	**0.0035**
Shoot-to-Root Ratio (g g^{-1} dw)	3.65±0.15^{a}	3.14±0.09^{ab}	2.55±0.22^{bc}	2.46±0.11^{c}	**<0.0001**
Leaf Weight Ratio (g g^{-1} dw)	0.62±0.01^{a}	0.60±0.01^{a}	0.55±0.02^{b}	0.55±0.01^{b}	**0.0012**
Leaf Area (cm^2)	61.29±2.94^{a}	42.08±2.50^{b}	58.94±2.24^{a}	39.40±2.76^{b}	**<0.0001**
Specific Leaf Area (cm^2 g^{-1} dw)	130±9.91	125±4.69	143±5.42	125±6.52	0.1092
Plant Height (cm)	9.00±0.73	8.83±0.48	10.67±0.61	9.33±0.56	0.1606
Internode Number	6.17±0.40	6.00±0.37	6.83±0.40	5.83±0.31	0.2728
Av.Internode Length (cm)	1.48±0.14	1.49±0.10	1.56±0.04	1.61±0.11	0.7753
Root Length (cm)	23.5±1.71	26.0±2.12	26.4±0.51	24.2±2.85	0.7162
Chlorophyll Content (SPAD)	61.7±0.42^{a}	53.0±1.28^{b}	61.04±0.55^{a}	50.82±0.98^{b}	**0.0888**
Nitrogen Use Efficiency (g dw g^{-1} N applied)	14.9±2.15^{b}	26.4±1.94^{a}	17.0±0.65 ^{b}	25.7±1.68 ^{a}	**0.0001**
Nitrogen accumulation (mg)	11.6±1.03b	7.58±0.79c	16.15±0.88 a	7.38±0.44c	**0.0002**

Water Use Efficiency (mg dw mL^{-1})	3.50±0.12	3.73±0.21	4.02±0.13	3.46±0.14	0.0682
Ψshoot (Bar)	-4.67±0.40	-5.50±0.18	-5.05±0.59	-4.67±0.42	0.4725

Data presented are mean ± SEM (n = 3 for nitrogen use efficiency and nitrogen accumulation and n =6 for all the other parameters). Different letters above indicate significant differences according to the Tukey's HSD test (p=0.05).

When analysing the transpiration rate dynamics during 4 h of leaflet desiccation, it was found that plants grown under water shortage conditions (single or combined) had a lower initial transpiration rate (up to 55%), particularly in comparison with plants grown under CTR conditions (Figure 1a). Nonetheless, the CTR plants showed a higher rate of stomatal closure in response to leaf desiccation (Figure 1b), which explains the lack of significant differences among treatments concerning the RWC after 4 h of leaf desiccation (Figure 1b, insert).

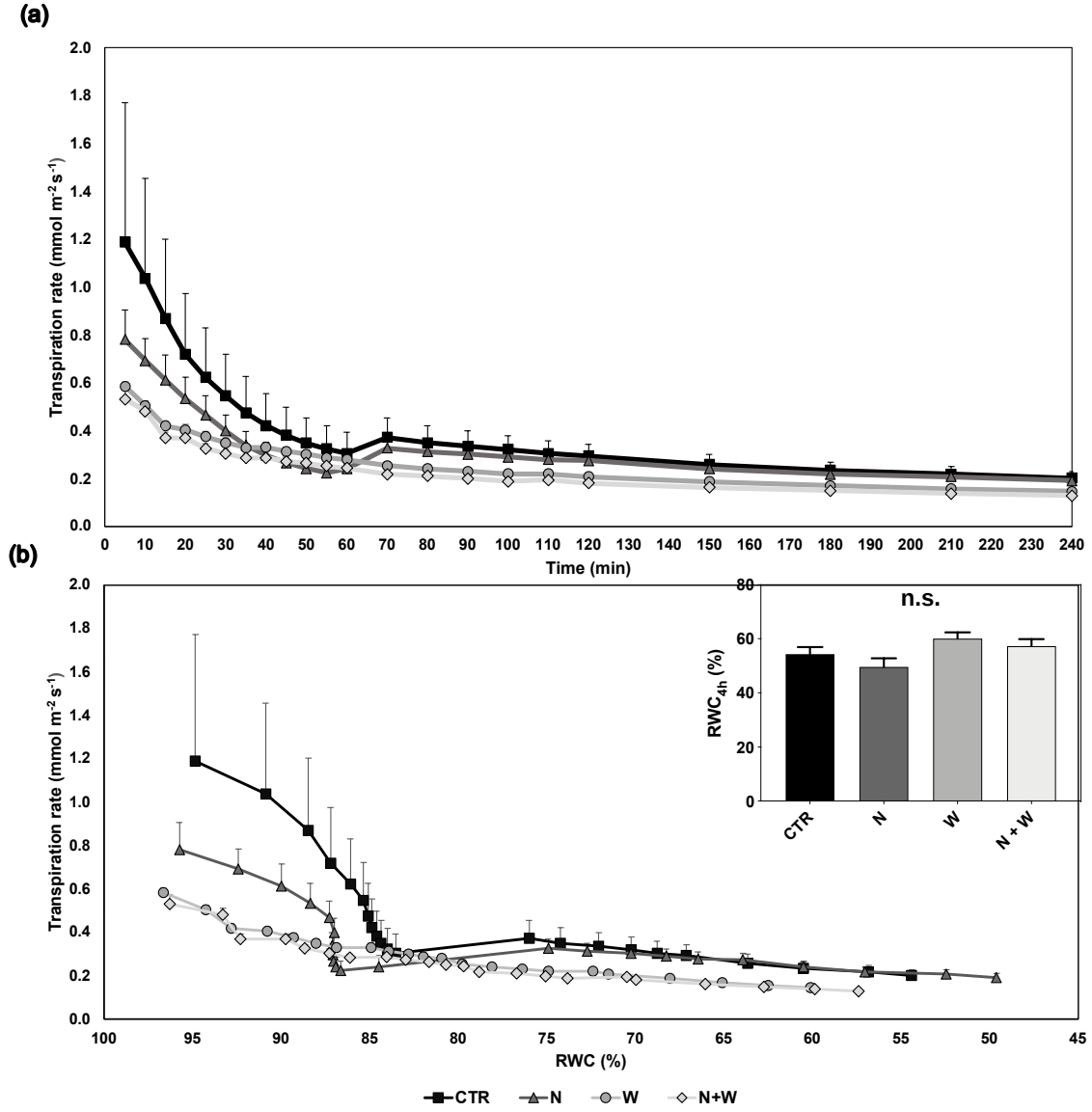


Figure 1 - Transpiration rate as a function of time **(a)** and of relative water content **(b)** (RWC; i**nsert**) in detached leaflets of tomato cv. Micro-Tom grown for 16 days under control (CTR; black; 10.5 mM N + 100% W), nitrogen deficit (N; dark grey; 50% N + 100% W), water deficit (W; light grey; 5.3 mM N + 100%W) or combined N and water deficit (N+W; white; 5.3 mM N+ 50% W). Data presented are mean ± SEM (n = 6). n.s. above bars indicate non-significant differences according to according to the Tukey's HSD test (p = 0.05).

3.2. Plant Metabolism

3.2.1. Proline and Protein concentration

Proline concentration in the roots was considerably higher in plants grown under single (504%) or combined (298%) W deficit in relation to CTR, but with no significant differences between them (Figure 2a). In the shoots, the concentration of this osmolyte was only significantly increased in plants subjected to single W deficit, resulting in up to 193% higher

levels compared to CTR or N+W deficit plants. In contrast, young tomato plants grown under N deficit showed 41% lower proline concentration compared to CTR conditions (Figure 2b).

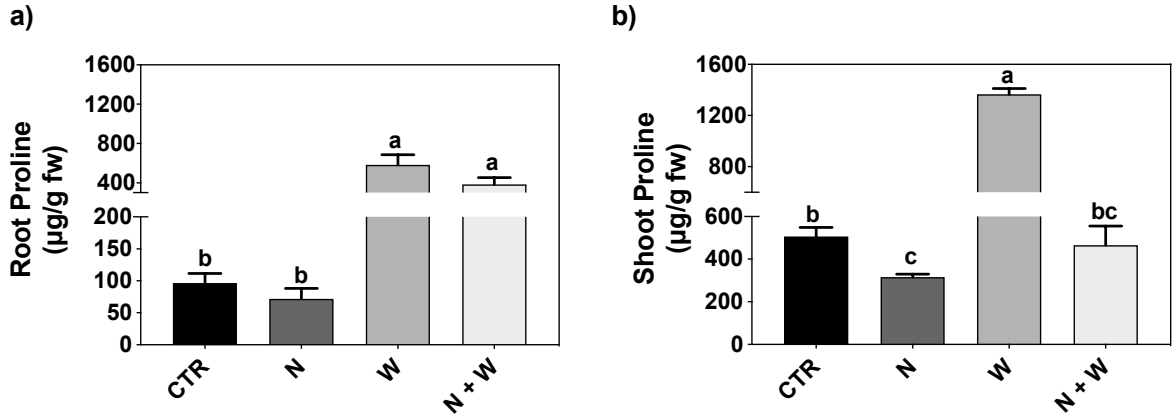


Figure 2 - Proline concentration in roots (a) and shoots (b) of tomato cv. Micro-Tom grown for 16 days under control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N+W; 5.3 mM N+ 50% W). Data presented are mean ± SEM (n = 3). Different letters above bars indicate significant differences according to the Tukey's HSD test (p = 0.05).

Total soluble protein concentration was significantly lower in the roots of plants exposed to N+W deficit compared to W deficit (30% decrease) (Figure 3a). Shoot protein concentration was significantly higher for plants grown under W deficit (70% and 113% higher compared to CTR and N+W-deficit plants, respectively), whereas the other treatments did not differ from each other (Figure 3b).

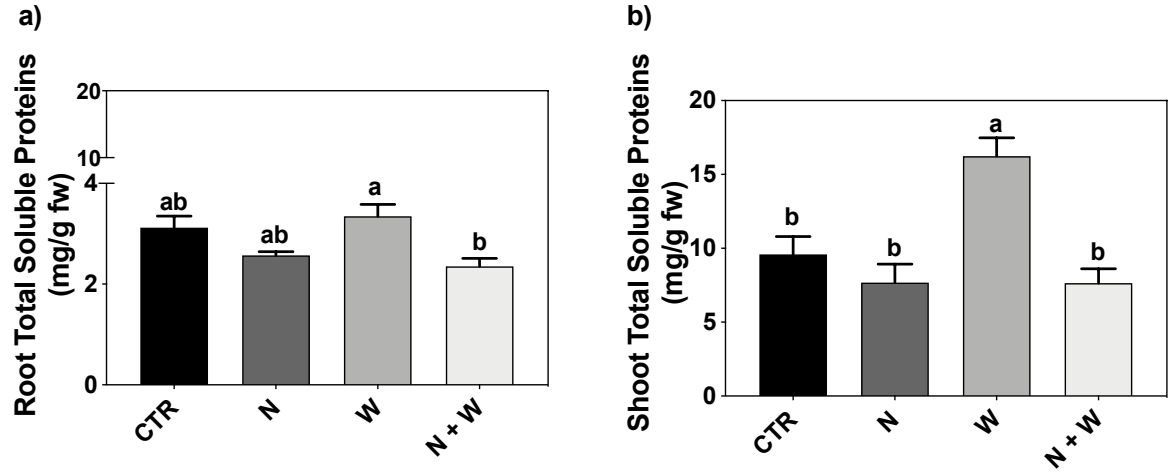


Figure 3 – Total soluble protein concentration of roots **(a)** and shoots **(b)** of tomato cv. Micro-Tom grown for 16 days under control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N+W; 5.3 mM N+ 50% W). Data presented are mean ± SEM (n = 3). Different letters above bars indicate significant differences according to the Tukey's HSD test (p = 0.05).

3.2.2. Total N, C, NO_3^- quantification

Root C concentration reached the lowest level in W-deficit plants, having 13% less C than CTR plants (Figure 4a). Contrarily, in the shoots, the highest C concentration was found in W deficit (Figure 4b). Single W deficit led to the highest levels of N concentration both in roots and shoots (18% and 33% higher, respectively, compared to CTR) (Figure 4c,d). Furthermore, when analysing C and N balance, it was shown that the C/N ratio reached the lowest values in the roots and shoots of W-deficit plants, decreasing by 28% and 39%, respectively, when compared to the combined N+W deficit (Figure 4e,f).

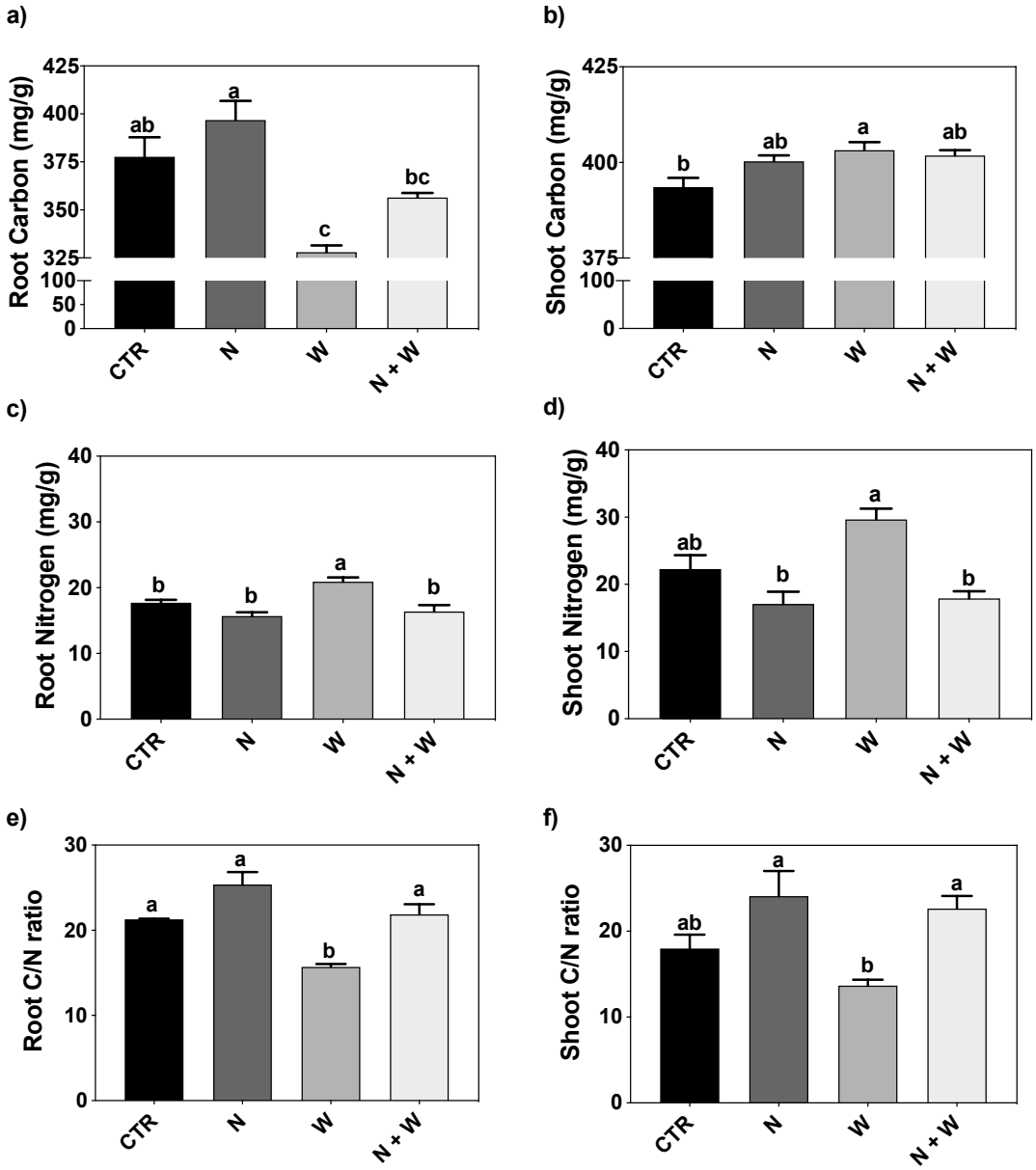


Figure 4 – Total carbon concentration in roots **(a)** and shoots **(b)**, total nitrogen concentration in roots **(c)** and shoots **(d)** and carbon to nitrogen (C/N) ratio of roots **(e)** and shoots **(f)**) of tomato cv. Micro-Tom grown for 16 days under control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N+W; 5.3 mM N+ 50% W). Data presented are mean ± SEM (n = 3). Different letters above bars indicate significant differences between treatments according to the Tukey's HSD test (p=0.05).

W deficit alone or combined with N deficit significantly increased root NO_3^- concentration (137 and 100% increase in relation to CTR, respectively). The differences in the NO_3^- concentration at the shoot level were far less pronounced, with no significant differences found between N+W treatment and the respective single deficits (Figure 5b).

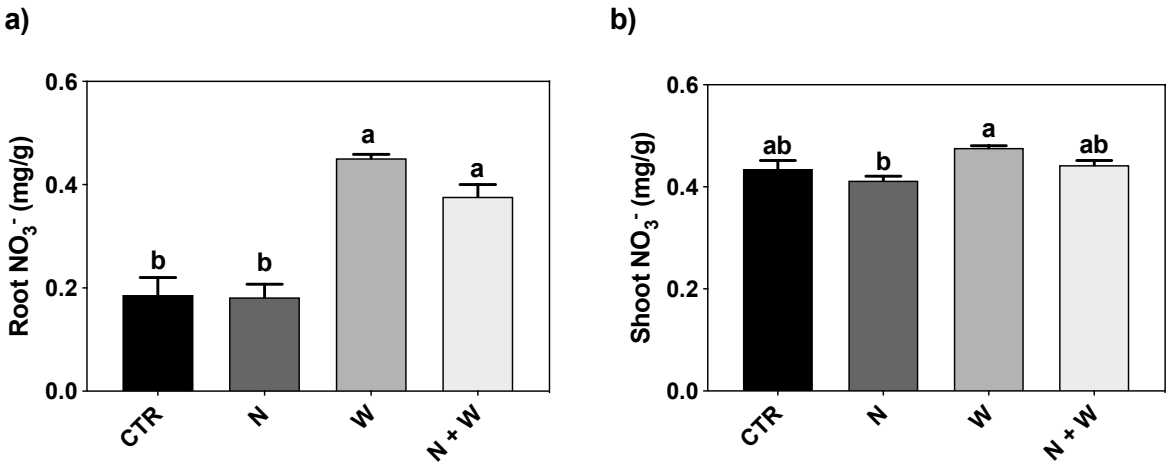


Figure 5 – Nitrate (NO_3^-) concentration in roots **(a)** and shoots **(b)** of tomato cv. Micro-Tom grown for 16 days under control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N+W; 5.3 mM N+ 50% W). Data presented are mean ± SEM (n = 3). Different letters above bars indicate significant differences according to the Tukey's HSD test (p=0.05).

3.2.3. Nitrate reductase and glutamine synthetase activity

N metabolism and assimilation were evaluated by the quantification of the total activity of NR and GS. Under N deficit, root NR activity was significantly lower as compared to all the other growth conditions (e.g., 29 and 37% decreases in relation to CTR and N+W, respectively; Figure 6a). However, in the shoots, the activity of this enzyme did not differ in plants grown under single or combined N deficit, although both treatments presented significantly higher enzyme activity (up to 119%) when compared to CTR and W-deficit plants (Figure 6b).

The GS activity in the roots was below the limit of detection for all treatments, whereas in the shoots, it was found the same pattern as the shoot NR activity, i.e., with the W-deficit plants being similar to CTR plants and N+W plants having a similar shoot GS activity compared to the N-deficit plants (Figure 6c).

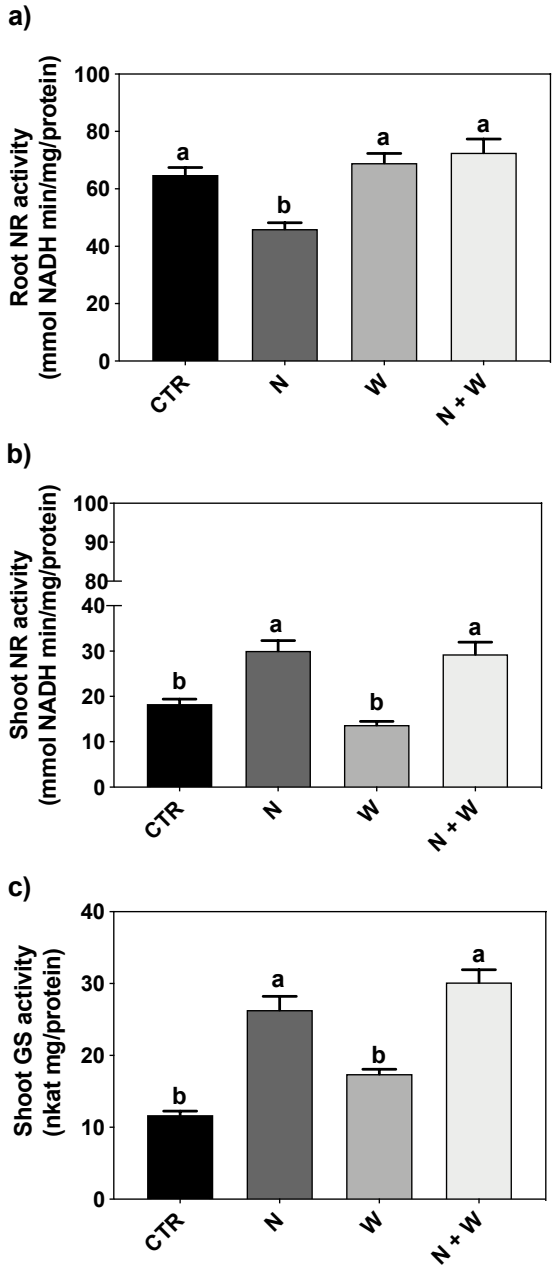


Figure 6 – Nitrate reductase (NR) activity in roots (**a**) and shoots (**b**) and glutamine synthase (GS) activity in shoots (**c**) of tomato cv. Micro-Tom grown for 16 days under control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100% W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N+W; 5.3 mM N + 50% W). Data presented are mean ± SEM (n = 3). Different letters above bars indicate significant differences according to Tukey's HSD test (p = 0.05). GS activity was not detectable in roots (i.e., below the limit of detection of the method).

3.2.4 RuBisCO and N metabolism-related genes

Among the two studied RuBisCO metabolism-related genes, *rcbS* was the most affected. Its expression level was upregulated by 5.7 and 5.8-fold in plants subjected to N or N+W deficit, respectively, compared to the CTR (Figure 7). *rcbL* expression followed the same pattern but with a lower magnitude of change, resulting in an upregulation of 2.3-fold and 2.5-

fold when plants were exposed to single or combined N deficit (Figure 7). In contrast, under W deficit none of these gene expression patterns were significantly impacted (Figure 7).

GS1 expression in the roots was significantly higher in plants grown under W and N+W deficit (2.7- and 2.5-fold, respectively). In shoots, *GS1* transcriptional levels were only significantly higher in plants exposed to W deficit (1.9-fold), but *GS2.1* was significantly downregulated in roots of plants grown under N shortage (0.4-fold for N and 0.7-fold for N+W plants). Moreover, W deficit upregulated *GS2.2* in roots (2.2-fold increase) (Figure 7), while in shoots it was downregulated in plants suffering from N and N+W deficit (0.4 and 0.3-fold). From the studied genes, *NR* was the most affected in roots (Figure 7). W deficit led to the highest upregulation (an increase of 40-fold), followed by N+W (23-fold increase) and N deficit (an increase of 4.9-fold).

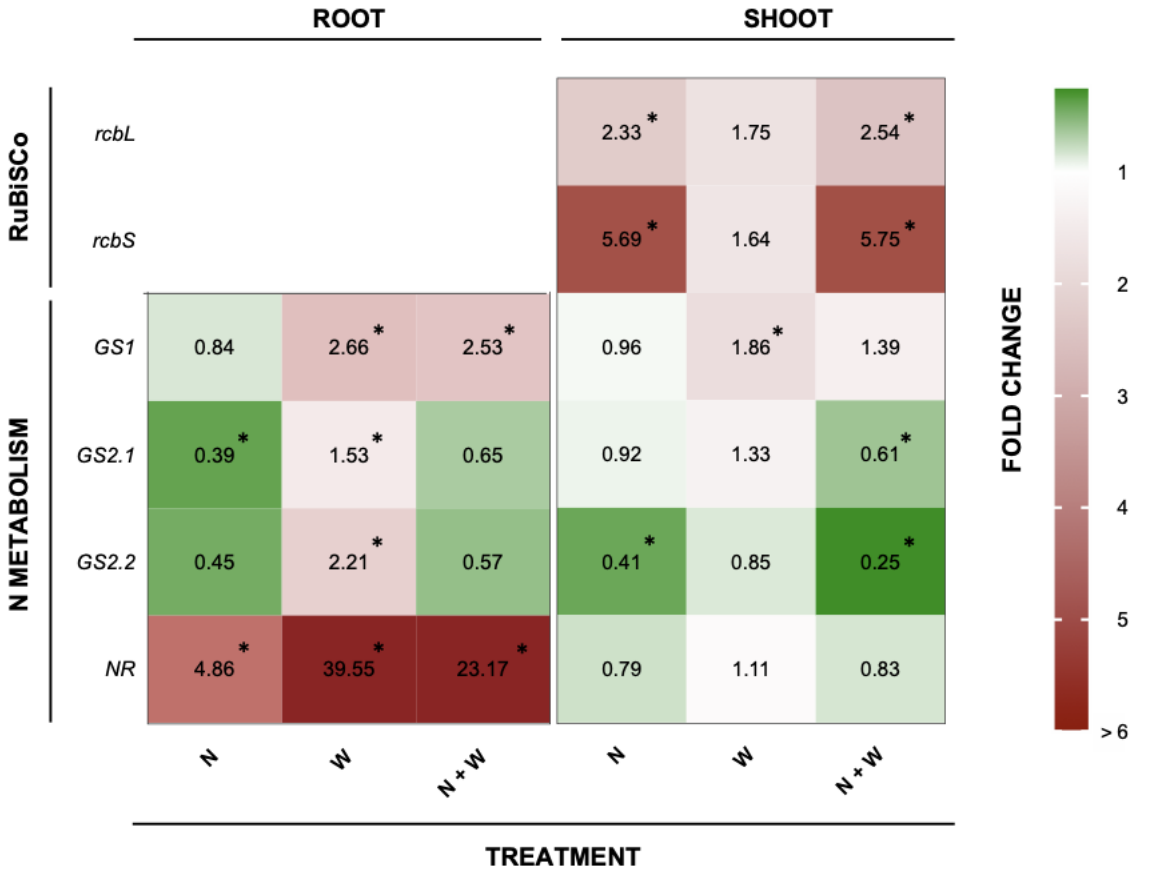


Figure 7 – Relative fold of expression of RuBisCO and N metabolism-related genes in tomato cv. Micro-Tom roots and shoots as affected by nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N+W; 5.3 mM N+ 50% W). The $2^{-\Delta\Delta Ct}$ values against the control are shown. * above mean values indicate significant differences compared to the control according to the t-Test (p = 0.05). Green indicates downregulation and red upregulation compared to the control plants. White indicates that gene expression was maintained at levels identical to the CTR.

3.3. Principal Component Analysis

In order to determine how all the analysed variables explain the differences among the four treatments, a PCA was performed (Figures 8 and 9). Results showed that, concerning plant growth, the two first components accounted for 56% and 25% of the variance. Moreover, N and N+W treatments shared more similarities among them than with plants from CTR and W treatments, with the former treatments being characterized by lower chlorophyll content (SPAD), shoot DW, leaf area, total DW, N assimilation, and root DW and by a higher NUE. Additionally, CTR and N deficit plants were mostly associated with higher leaf weight ratios and shoot-to-root ratios (Figure 8).

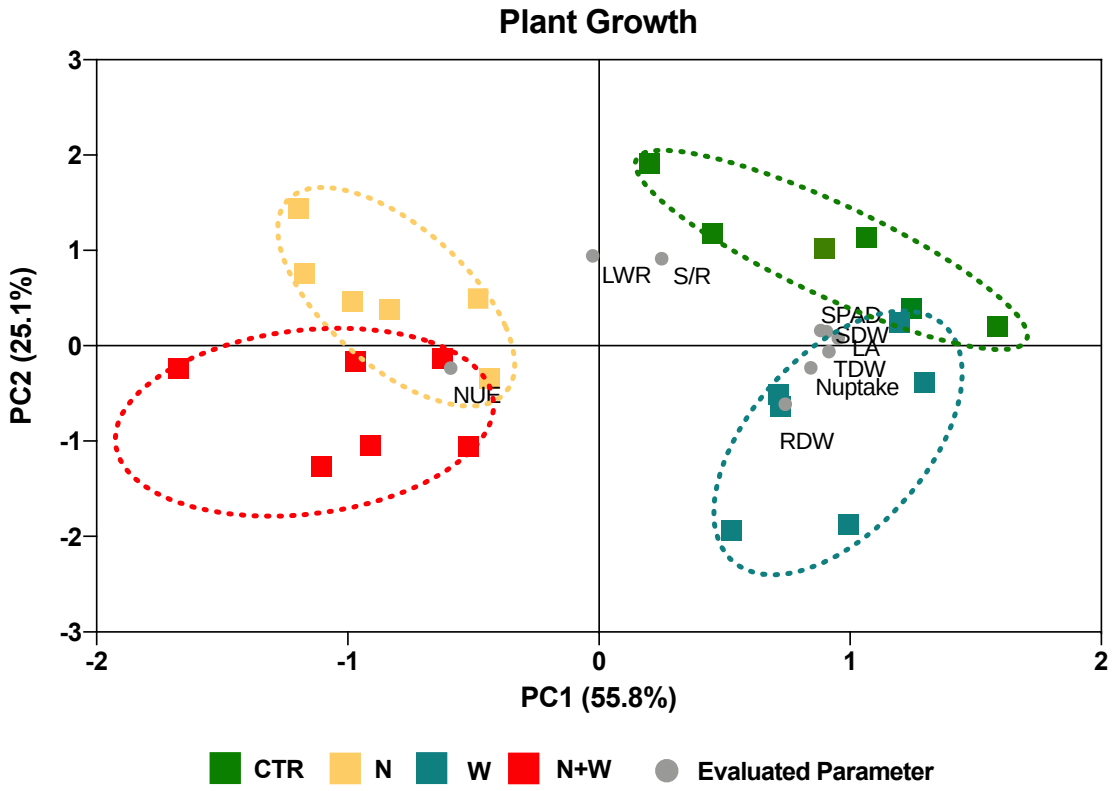


Figure 8 – Principal component analysis (PCA) of the plant growth parameters evaluated in tomato cv. Micro-Tom grown for 16 days under control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N+W; 5.3 mM N+ 50% W). The two principal components together (PC1, PC2) explained 80% of the total variance found among treatments. Details on the abbreviations are given in Material and Methods.

Concerning plant metabolism, the first component accounted for 70 and 59% of the variance in roots and shoots, respectively, and the second for 17 and 20%, respectively (Figures 9a and b). In the roots, CTR and N deficit were the treatments sharing more similarities, having an opposite behaviour to the W and N+W deficit groups. Indeed, roots from plants grown under single or combined W deficit were associated with enhanced NO_3^- and proline concentrations, NR activity, and *GS1* an *NR* expression, but with a lower C

concentration (Figure 9a). The CTR and W deficit were associated with a higher *GS2.1* and *GS2.2* transcript-levels and N concentration (Figure 9a). In shoots, N and N+W groups showed a higher C/N ratio, NR and GS activity, and a higher *rcbS* and *rcbL* transcript-levels together with a lower N concentration and expression of *GS2.1* and *GS2.2* genes. The W deficit was closely associated with higher NO_3^-, proline, and protein concentrations alongside increased transcript-levels of *GS1* (Figure 9b).

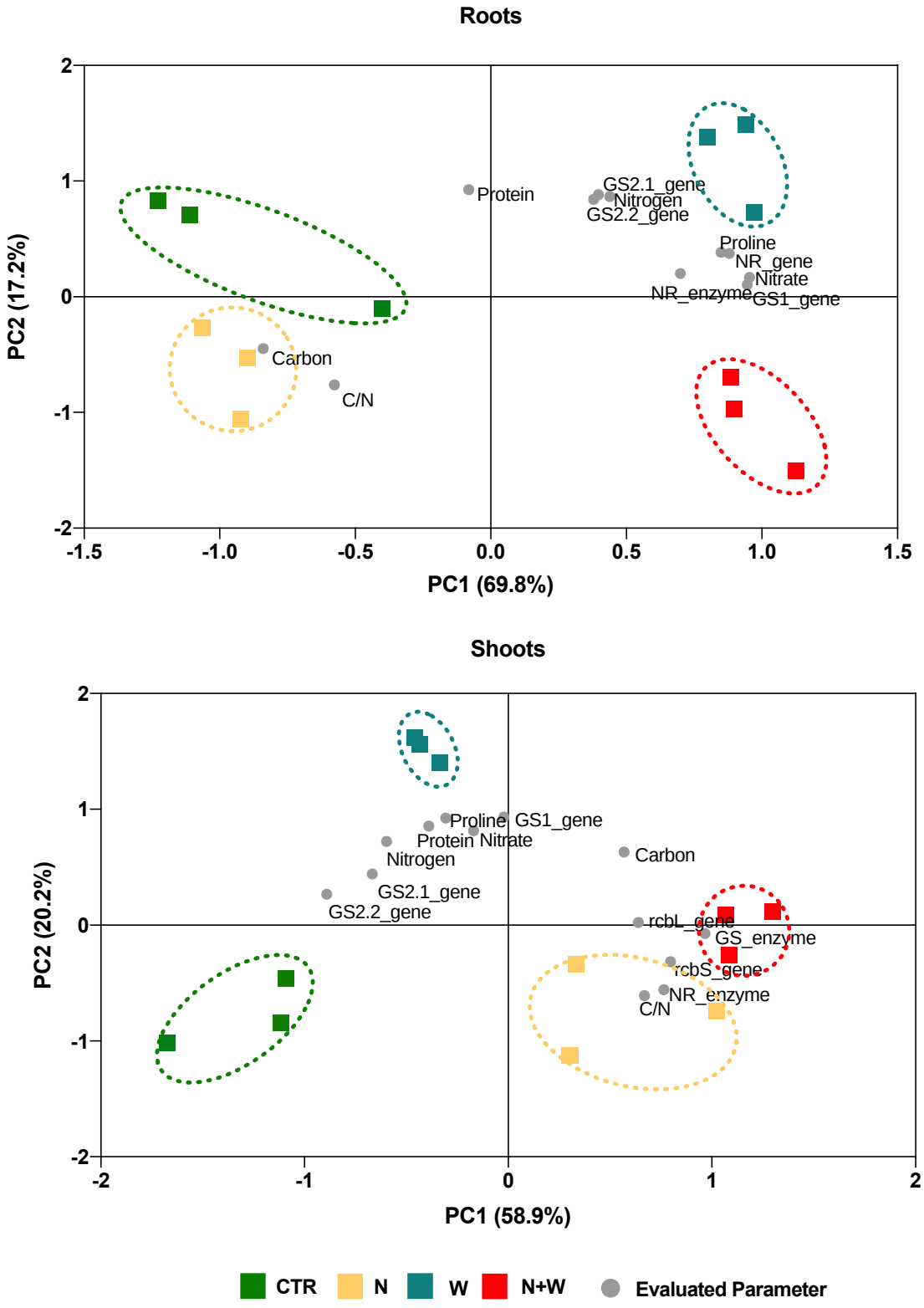


Figure 9 – Principal component analysis (PCA) of the plant metabolism in roots (A) and shoots (B) evaluated in tomato cv. Micro-Tom grown for 16 days under control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N+W; 5.3 mM N+ 50% W). The two principal components together (PC1, PC2) explained 87% and 79% of the total variance found among treatments in roots and shoots, respectively. Details on the abbreviations are given in Material and Methods.

4. Discussion

4.1. Effect of combined N and Water deficit on plant growth and physiological parameters

It is well known that plant responses to abiotic stresses significantly differ at various organizational levels, depending upon the intensity and the duration of the imposed stress, as well as on the plant species and its developmental stage (Shao *et al.*, 2008; Nemeskéri *et al.*, 2019). Although in our study we have applied a gradual and mild deficit throughout the vegetative cycle of tomato, several important growth-related traits, such as total DW, shoot DW, root DW, shoot-to-root ratio, leaf area, leaf weight ratio, chlorophyll content, NUE, and N accumulation were severely affected by at least one of the imposed deficits (Table 2, Figure 8). In contrast, some of the studied plant growth-related traits, such as plant height, root length, internode number, internode length, leaf area ratio, specific leaf area, WUE, and shoot water potential, known to be severely affected under N or drought stress (Khan *et al.*, 2020; Sibomana *et al.*, 2013; Khan *et al.*, 2015), were not significantly affected in this study (Table 2). Nonetheless, it is expected that if the trial had been prolonged, these deficits would have become more severe and treatment effects more evident due to the longer duration of the imposed stresses.

Integrating all the studied growth traits in a PCA revealed considerable variability among treatments, with the two principal components explaining 80% of the variability (Figure 8). The PCA showed that plants exposed to the N+W deficit behaved similarly to plants exposed to the single N deficit, having a lower total DW, shoot DW, root DW, leaf area, chlorophyll content, and N accumulation as well as a higher NUE. This suggests that in plants grown under a combined N+W deficit, the N deficit had a more predominant impact on the studied growth traits than the W deficit (Figure 8). Additionally, W-deficit plants were more similar to plants grown under CTR conditions (Figure 8), suggesting that the level of water deficit imposed in this study was not severe enough to negatively impact the growth of young tomato plants.

Due to the stronger negative effect of water deficit on shoots than root growth, the shoot-to-root ratio tends to decrease with drought (Moles *et al.*, 2018; Du *et al.*, 2018). Because roots contain much lower concentrations of N than aboveground biomass, Gonzalez-Dugo *et al.* (2010) suggest that this change in the allocation pathway diminishes N needs. Our results agree with such hypothesis, as the highest decreases in the shoot-to-root ratio were observed in plants subjected to combined N+W deficit. Additionally, several parameters related to leaf growth, namely leaf number, leaf area, leaf area ratio, and leaf weight ratio, were also reported to be significantly lower in tomato plants exposed to W deficit or N deficit (Selim and El-Nady, 2011; Pokluda *et al.*, 2010; Scholberg *et al.*, 2000; Machado *et al.*, 2022).

In our study, a decrease in leaf weight ratio was observed upon exposure to W and N+W deficit plants, while leaf area was reduced under N and N+W deficit (Table 2). When facing N limitation, plants can reduce their leaf area, paired with small changes in leaf N concentration, or alternatively, can maintain their leaf area and adapt leaf N concentration to N availability (Vos *et al.*, 2005). Based on the collected data, our results pointed out the first strategy, as a decrease in leaf area was observed, but no significant differences in leaf N concentration were found in plants subjected to N and N+W deficit compared to CTR plants (Table 2 and Figure 4b). Additionally, chlorophyll content, which is considered a key indicator of the photosynthetic capacity (Cannella *et al.*, 2016), is also often substantially decreased after drought exposure (Baquedano and Castillo, 2006) or when plants face N deficiency due to N partitioning dependence of the photosynthetic machinery (Bassi *et al.*, 2018). In the present work, total chlorophyll content was not significantly affected by the W deficit but was significantly decreased by the N deficit and N+W deficit (Table 2). Other studies also reported that, despite the stress condition, tomato plants did not significantly change their chlorophyll content, as observed in other drought-exposed species (Ashraf and Harris, 2013; Tamburino *et al.*, 2017; Nemeskéri *et al.*, 2019)). Moreover, no evident signs of leaf chlorosis were macroscopically detected, but the negative effects of N deficit on total chlorophyll content were evident (Table 2). Soval-Villa *et al.* (2002) reported that N status may be indirectly determined by the chlorophyll concentration present in leaves, even at early stages, as it is directly correlated to their N concentration. Thus, we can assume that in this study plants exposed to N deficit (single or combined) were starting to develop clear N deficiency symptoms.

Furthermore, NUE, an important parameter to benchmark N management, which is highly dependent on N-remobilization efficiency from senescing leaves (Safavi-Rizi *et al.*, 2018), and a crucial factor behind early leaf senescence (Zakari *et al.*, 2021), was significantly increased by N and N+W deficit (Table 2). This higher NUE could have resulted from the ability of the young tomato plants to efficiently remobilize N as suggested by the lower chlorophyll content (Table 2), lower N accumulation (Table 2), higher C/N ratio (Figure 4e,f; Figure 9a,b), and the higher activity of N metabolism-related enzymes in shoots (Figures 6b,d and 9b). Sánchez-Rodríguez *et al.* (2011b) reported that water deficit provokes a decline in the concentration and accumulation of N in four commercial cherry tomato cultivars grown under W deficit but not in the tolerant cv. Zarina, where an improvement was found. Our results are in accordance with those observations as we also found significantly higher N accumulation in plants exposed to W deficit (Table 1, Figure 4a,b). Furthermore, mild W deficit was reported to promote root growth and consequently nutrient uptake (Khapte *et al.*, 2019), which can be seen in this study as a tendency for longer roots and significantly higher root dry weight (Table 2). Actually, the stimulation of root length is a common response of plants to water deprivation

in an attempt to reach deeper soil layers to search for additional water and nutrients (Taiz *et al.*, 2015)

4.2. Effect of Combined N and W deficit on plant metabolism

The accumulation of solutes to decrease water potential (i.e., osmotic adjustment) is one of the most common responses of plants to maintain the water potential gradient as the soil becomes drier (Pirasteh-Anosheh *et al.*, 2016; Blum, 2017; Nahar and Ullah, 2017). Of these solutes, the most conspicuous accumulation is of proline. Furthermore, proline not only acts as an osmolyte but also as an antioxidant and a nutritional source, being used as a metabolic-compatible solute to store and transfer N and C after oxidation (Szabados and Savouré, 2010; Hayat *et al.*, 2012). In our study, proline levels were also significantly increased both in roots and shoots of plants grown under W deficit but decreased in shoots of plants subjected to single N deficit. When the N deficit was simultaneously applied with the water deficit, proline levels decreased in relation to the single W deficit yet presented higher levels than in plants only exposed to the N deficit (Figure 2). This result is in accordance with Szabados and Savouré (2010), who showed that proline accumulates as a compatible osmolyte during water deficit and then is used as a readily accessible source of N to compensate for the N deficit. Interestingly, while in roots of N+W deficit plants, the behavior was more similar to W deficit plants, in shoots, N+W deficit plants were more comparable to those grown under the single N deficit (Figures 2 and 9a,b).

Variation in protein content is another essential part of plants' acclimation to changes in environmental conditions (Kosová *et al.*, 2018; Qazi *et al.*, 2019). Often, to tolerate drought stress, plants accumulate soluble proteins which help to stabilize the structure of cells and proteins (Alscher *et al.*, 2002). In addition, drought stress often leads to altered water homeostasis within the plant, which can be balanced through the enhancement of water passage through protein membranes, in which aquaporins have a major role (Kapilan *et al.*, 2018; Afzal *et al.*, 2016). In our study, W deficit significantly increased shoot soluble protein concentration (Figure 3b). This suggests an accumulation of drought-induced proteins, which might be somehow related to the above-mentioned physiological processes as an acclimation response to water limitation, which could improve plant drought tolerance, thus contributing to the absence of severe effects on the morphological and physiological evaluated traits (Yang *et al.*, 2021). On the other hand, N deficiency accelerates leaf senescence and the production of reactive oxygen species, leading to the degradation of macromolecules like proteins and chlorophylls. Due to the pivotal role of N in protein biosynthesis, N deficiency can reduce amino acids and protein content, which has been observed in tomato (Urbanczyk-Wochniak and Fernie, 2005; Khavari-Nejad *et al.*, 2009). In our study, both roots and shoots of N and

N+W deficit plants tended to have a lower concentration of total soluble proteins (Figure 3). However, only in plant roots exposed to the combined N+W deficit, the total soluble protein content was significantly lower (Figure 3a), once again suggesting that stress combinations can induce unique responses that cannot be simply extrapolated from the exposure to the single deficits.

Although under drought conditions, N uptake usually diminishes (Gonzalez-Dugo *et al.*, 2010), in our study, surprisingly, the N accumulation and concentration increased both in roots and shoots of plants exposed to W deficit (Table 2, Figure 4c,d). This is in accordance with the higher amino acids (proline) and total soluble proteins that we also observed in these plants (Figures 2 and 3). Indeed, drought may increase soil solute concentration, which promotes nutrient uptake (Gonzalez-Dugo *et al.*, 2010; Plett *et al.*, 2020; Ding *et al.*, 2018). Furthermore, the availability of C and N is an important factor for the regulation of plant metabolism and development; however, the ratio of C to N metabolites in the cell has a higher role in the regulation of plant growth and development than C or N individually (Coruzzi and Zhou, 2001; Martin *et al.*, 2002). Leaf senescence can be triggered by high C and low N availability (a typical C/N imbalance) (Aoyama *et al.*, 2014). In our study, the C/N ratio significantly decreased under the W deficit, disrupting the C/N balance, especially in the roots (Figure 4a,b). However, the C/N ratio tended to increase in plants exposed to N or N+W stress (Figure 4e,f). This tendency to increase the C/N ratio suggests that leaf senescence may have occurred, as previously reported by Aoyama et al. (Aoyama *et al.*, 2014).

Furthermore, under drought conditions, transpiration and turgor losses may cause a decrease in NO_3^- absorption by the roots, as well as a reduction in the translocation from the roots to the leaves (Foyer *et al.*, 1998; Chen *et al.*, 2015). In our study, NO_3^- concentration increased in roots exposed to W deficit but not in shoots (Figure 5). Sánchez-Rodríguez *et al.* (2011a) reported that under moderate water stress conditions, NO_3^- significantly diminished in some tomato cultivars but increased in others, suggesting that this trait might follow a cultivar-dependent response. Moreover, drought stress also restricts the ability of plants to reduce and assimilate N through the inhibition of enzymes involved in N metabolism and assimilation, such as NR and GS (Ferrario-Méry *et al.*, 1998; Robredo *et al.*, 2011). However, as with NO_3^- concentration, we did not detect significant changes in NR activity in plants grown under W deficit (Figure 6b). Nevertheless, this group of plants presented a significantly higher NR transcript-level than the CTR plants (Figure 7).As the expression of NO_3^- assimilation genes is highly controlled by the NO_3^- concentration (Kavoosi *et al.*, 2014), the significant upregulation found in roots of W and N+W-deficit plants is probably correlated with the significant increase in NO_3^- concentration observed in roots of plants subjected to water shortage (Figures 5a and 7). However, as proteolytic activity increases during exposure to

water stress, enhanced protease activity has been implicated in the acceleration of protein turnover, with typical proteinogenic amino acids and proline accumulation occurring in water-stressed plants (Fukutoku and Yamada, 1984). Accordingly, in our study, the upregulation in the *NR* gene was not translated into a clear increase in the enzyme activity (Figures 6a and 7), but increased levels of proline were observed (Figure 3). Thus, we hypothesize that the increased NO_3^- concentration served as an intracellular signal to induce a higher expression of NR coding genes, but drought-induced effects, probably on enzyme activity and stability, did not allow a higher catalytic activity of this enzyme. Concerning plants grown under N shortage, NO_3^- concentration was not affected in plants grown under the single N deficit, but it was increased in roots of plants subjected to the combined N+W deficit, to the same extent as that of plants grown under single W deficit (Figure 5a). The activity of the NR enzyme is increased in the presence of low NO_3^- concentrations and starts to decrease at higher cytoplasmic NO_3^- levels (Kavoosi *et al.*, 2014). Therefore, it is not surprising that low levels of external NO_3^- have a positive effect on the NR activity (Kavoosi *et al.*, 2014). In line with this significantly lower NO_3^- concentration (Figure 5a), the upregulation of *NR* observed in roots of plants grown under N deficit was considerably lower than in plants grown under W and N+W combined deficit (Figure 7). In contrast, in plants grown under N deficit, loss of NR protein occurred even when an increase in *NR* transcript-level was found (Figures 6a and 7). This can be explained, for example, by the induction of NR proteases that can occur due to high levels of sugars (Kaiser and Huber, 2001), which are usually found under N deficiency conditions (Lacrampe *et al.*, 2020). In tomato and tobacco plants, high NO_3^- levels also induced an increase in the level of NR mRNA after N starvation, followed by a decrease in NR protein and NR activity (Galangau *et al.*, 1988), which agrees with our results (Figures 6a and 7). In shoots, NR regulation was maintained in the basal threshold, which is also in line with the shoot NO_3^- concentration that we found (Figures 5b and 7). In this organ, NR seems to be controlled post-transcriptionally as no alteration in its expression was detected, but there was an increased activity of the enzyme (Figures 6b and 7).

In line with NR, GS activity was not affected in plants subjected to W deficit (Figure 6d). The same was previously observed in some tomato cultivars subject to mild water deficit (Sánchez-Rodríguez *et al.*, 2011a). On the other hand, GS has an important role in senescence-induced nutrient remobilization (reviewed Masclaux-Daubresse *et al.*, 2008). In Arabidopsis and oilseed rape, it was shown that N can be remobilized from senescing leaves to expanding leaves at the vegetative stage (Diaz *et al.*, 2008; Malagoli *et al.*, 2005; Lemaître *et al.*, 2008). Thus, the increase in GS activity observed in shoots of plants exposed to N and combined N+W deficits suggests an N remobilization strategy (Figure 6c). Additionally, the increased level of *GS* transcripts found in roots of W and N+W stressed plants point to an

acclimation mechanism that plants used to increase GS activity, thus enhancing proline levels in response to the high levels of NO_3^-. Accordingly, although the GS activity in the roots was belong the limit of detection, when plants were grown under water shortage (W and N+W), increased levels of NO_3^- and proline were found (Figures 2a and 5a). As for *NR*, regarding N deficit in shoots, the regulation of *GS1*, which is known to be involved in N remobilization, seems to be post-transcriptional since there was no change in gene expression, but there was a greater activity of the enzyme when plants were grown under this deficit conditions (Figures 6c and 7).

RuBisCO degradation is a common response of plants exposed to abiotic stress. Together with other photosynthesis-related proteins, RuBisCO is a major source of N for remobilization. Transcriptome analysis has shown that many autophagy genes related to RuBisCO are upregulated during senescence and in response to N limitation (Thompson *et al.*, 2005; Wingler and Roitsch, 2008). In our study, *rcbS* and *rcbL* were upregulated in the presence of all stress treatments (Figure 7). However, N and N+W deficits had a higher impact, especially regarding *rcbS*, whose expression was considerably increased in both treatments. These results confirm the increase observed for C/N ratio and GS activity as well as the decrease observed in proline and total soluble protein concentration, which also points towards N remobilization. On the other hand, the evaluation of *rcbS* and *rcbL* genes also suggests that W deficit alone did not affect RubisCO activity which is aligned with the low level of stress imposed previously mentioned and pairs with the lack of differences found on the chlorophyll content (Figure 7 and Table 2).

The integration of all biochemical and gene traits linked to plant metabolism in a PCA revealed considerable variability among treatments, with the two principal components explaining 87% of the variability in roots and 79% in shoots (Figure 9a). The PCA showed that, in roots, plants under N+W deficit had a behavior similar to that of plants exposed to single W deficit, presenting higher NO_3^- and proline concentrations, as well as higher NR activity and *GS1* and *NR* expression (Figure 9a). This indicates that in roots from plants grown under combined deficit, W deprivation had a higher contribution to the changes in biochemical traits and gene expression levels than N deficit (Figure 9a). Contrarily, the PCA revealed that, in shoots, plants grown under combined N+W had a behavior similar to that of plants exposed to single N deficit, having higher C/N ratio, NR and GS activity, and *rcbS* and *rcbL* transcript-levels but lower N concentration and *GS2.1* and *GS2.2* levels (Figure 9b). The differential behavior between combined N and water deficit and single deficits, as well as between plant organs (shoots and roots) was already reported in terms of enzymatic and non-enzymatic antioxidant responses (Machado *et al.*, 2023) and highlights the need for more studies focused on plant responses to combined deficits.

5. Conclusion

Although the effects of single N and W deficits have been well documented on morphological, physiological, and molecular mechanisms of tomato, the impact of combined N and water deficit is still far from being understood and explored. In a recent study, we showed that the combination of N and W deficit clearly induces differential impacts on the redox homeostasis of young tomato plants, suggesting that significant metabolic alterations might be taking place. Here we moved one step forward, showing that tomato plants can employ N remobilization and osmoregulatory mechanisms as a coping strategy to combined N+W deficit. Additionally, the pattern of response seems to be quite distinct among different plant organs. Future work should address tomato responses to more severe levels of single and combined N+W stress, as well as explore more susceptible developmental stages.

Sub-chapter 2.2

Enzymatic and non-enzymatic antioxidant responses of young tomato plants (cv. Micro-tom) to single and combined mild nitrogen and water deficit: not the sum of the parts

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Enzymatic and non-enzymatic antioxidant responses of young tomato plants (cv. Micro-tom) to single and combined mild nitrogen and water deficit: not the sum of the parts

Abstract

This study aims to perform a broad analysis of the antioxidant (AOX) responses of young tomato plants exposed to single and combined mild nitrogen (N) and water deficits through the evaluation of oxidative biomarkers, non-enzymatic and enzymatic AOX components. 'Micro-Tom' seedlings were subjected to four treatments: control (CTR; 100%N + 100%W), N deficit (N; 50%N), water deficit (W; 50%W), and combined deficits (N + W; 50%N + 50%W). An enhancement of several non-enzymatic and enzymatic components were found in plants subjected to N + W deficit, which presented higher anthocyanins accumulation (up to 103%) as well as higher levels of superoxide dismutase (SOD) transcripts at the root level and of ascorbate peroxidase (APX) and catalase (CAT) transcripts at shoot level. This increase in the gene expression was also translated in augmented SOD (up to 202%), APX (up to 155%) and CAT (up to 108%) activity compared to CTR plants and the single deficits. Overall, tomato plants were able to employ defense strategies to cope with this combined deficit, as demonstrated by the higher total AOX capacity (up to 87%) compared to the single deficits, which contributed to the maintenance of their redox homeostasis, with unchanged values of lipid peroxidation and hydrogen peroxide compared with CTR plants.

1. Introduction

The agricultural sector globally uses about 80% of the total available freshwater, as well as high amounts of nitrogen (N) fertilizers, with total required amounts projected to double by 2050 (Elbehri, 2015; Esteban *et al.*, 2016b; Liang *et al.*, 2019). Therefore, new goals are being set to reduce the use of these inputs. This is the case of the recent European Green Deal (2020), which is defined as targeting a reduction of nutrient losses by at least 50% and the use of fertilizers by at least 20% by 2030 (Schebesta and Candel, 2020). Despite the need to use fewer resources, it is important to take into account that water and N deficits significantly impact plants' growth and metabolism, which will ultimately affect their productivity. Hence, it is important to understand the mechanisms involved in plant responses when subjected to these single or combined deficits to increase agricultural production in a sustainable way where the available resources are more efficiently used. Tomato has been often used as a

model system to study these processes when plants are exposed to single abiotic stresses (recently reviewed by Machado *et al.*, 2022)).

One of the most crucial consequences of abiotic stress is the disturbance of the equilibrium between the generation of reactive oxygen species (ROS) and antioxidant (AOX) defense systems, which could result in a loss of cell viability and death (Laxa *et al.*, 2019; Soares *et al.*, 2019a). Drought impairs plant growth, affecting several physiological and biochemical processes, such as photosynthesis, respiration, nutrient uptake, and photoassimilates translocation (Kumar *et al.*, 2018). N supply is crucial for plant development and growth, because it affects almost all cellular processes, from the production of metabolites, carbohydrates, fats, and pigments to general protein synthesis **(Sun *et al.*, 2020; Mu and Chen, 2021)**. Therefore, water, as well as N shortage, can ultimately lead to reduced yield, loss of viability, and redox status alterations (Mittler *et al.*, 2011). The cellular damage caused by water deficit has two primary causes: first, the overproduction of ROS, and second, the alteration of water relationships within the plant (Laxa *et al.*, 2019). Free ROS may then attack biological structures, damaging DNA, prompting the oxidation of amino acids and proteins, and provoking lipid peroxidation (Soares *et al.*, 2019a). To avoid such damage, plants have developed ROS-detoxification mechanisms that work in tandem to counteract ROS overproduction (Soares *et al.*, 2019a; Laxa *et al.*, 2019). Several studies using different tomato cultivars showed that drought stress increases the production of ROS and the oxidative load, including AOX substrates and AOX enzymes (Sánchez-Rodríguez *et al.*, 2010; Zhou *et al.*, 2019b). Little is known about the effects of N deficiency on ROS content and AOX enzyme activity in tomato plants, but some studies suggest that ROS content increases in tomato as a result of N starvation, as demonstrated by higher levels of malondialdehyde (MDA) in the root system of N-deficient tomato (Dong *et al.*, 2022).

Moreover, understanding how plants regulate their AOX defenses under a combined N and water deficit situation is important, as crops are often exposed to a combination of these two stresses under field conditions (Fernandes *et al.*, 2022). When two stresses occur concurrently, the plant's acclimation strategy is governed by the interaction of the two stresses, which is conceived by the plant as a new state of stress (Mittler, 2006). Rivero *et al.* (2014) and Sousa *et al.* (2022) demonstrated that tomato plants grown under the combination of salinity and heat stress showed a profile of accumulation of osmoprotectants and AOX metabolites that was not observed when these stresses were applied individually. Martinez et al. (2016) also demonstrated that the combination of salinity and heat increases tomato metabolomic profile differently from when these stresses were applied individually. This demonstrates that the combination of two or more abiotic stressors induces a unique plethora of responses that cannot be extrapolated from those observed under single exposure (García-

Martí *et al.*, 2019; Spormann *et al.*, 2022). Based on these premises, this study aimed to unravel the AOX responses of tomato seedlings to single and combined N and W deficits and to determine the differences when these stressors occur individually. To that end, oxidative stress biomarkers (lipid peroxidation (MDA equivalents) and hydrogen peroxide (H_2O_2) levels) were evaluated, along with the assessment of non-enzymatic (ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), phenolics, flavonoids, and anthocyanins) and enzymatic (superoxide dismutase—SOD; catalase—CAT; ascorbate peroxidase—APX) AOX components, both at transcript and enzyme activity levels.

2. Material and Methods

2.1. Plant material and growth conditions

The experiment was carried out in a growth chamber (temperature: 25 °C day/23 °C night; relative humidity: 70%; photosynthetic photon flux density (PPFD): 300 µmol s^{-1} m^{-2}; photoperiod: 16 h light).

Solanum lycopersicum cv. 'Micro-Tom' seeds were sown in trays filled with commercial germination potting substrate (SIRO, Portugal). At third leaf appearance (approximately three weeks after sowing), seedlings were selected for uniformity and transferred to single-plant pots (10 cm high, 10 cm diameter) filled with 0.1 to 1.5 mm grade vermiculite (60 g) and divided into four experimental groups: control (CTR; 10.5 mM N 100% field capacity), nitrogen deficit (N; 5.3 mM N), water deficit (W; 50% field capacity) and combined deficit (N + W; 5.3 mM N; 50% field capacity). Since we aimed at having a progressive water deficit, as seedling transplanting is a critical phase of the cultivation period, just prior to transplanting, all pots were irrigated to field capacity (FC), determined using the soil gravimetric water content method (Joshi *et al.*, 2021), by adding ~300 mL of the respective nutrient solution (CTR and W deficit treatments: 10 mM NO_3^-; 0.5 mM NH_4^+; 1.9 mM H_2PO_4; 6.1 mM K^+; 3.6 mM Ca^{2+}; 1.6 mM SO_4^{2}; 2.5 mM Mg^{2+}; 2.6 mM Cl^-; 0.5 mM HCO_3^- (pH 6 and electrical conductivity—EC—of 2.50 dSm^{-1}); N deficit and N + W deficit treatments: 5 mM NO_3^-; 0.3 mM NH_4^+; 1.9 mM H_2PO_4; 6.1 mM K^+; 3.6 mM Ca^{2+}; 4.8 mM SO_4^{2}; 2.8 mM Mg^{2+}; 5.5 mM Cl^-; 0.5 mM HCO_3^- (pH 6 and EC of 2.80 dSm^{-1}), with both solutions having the same micronutrients composition (35 µM Fe-EDDHA; 10 µM Mn^{2+}; 20.1 µM B; 0.9 µM Cu^{2+}; 5.0 µM Zn^{2+}; 0.5 µM MoO_4^{2})).

Following seedling transplantation, all pots were covered to prevent evaporation and no more nutrient solution was added until the end of the experiment. Concerning further irrigation, each pot from CTR and N deficit treatment was weighted and re-watered (with distilled H_2O) daily, to maintain FC at 100% during the experimental period, whereas in W and N + W deficit treatments, no additional irrigation was supplied, resulting in a progressive decrease in substrate FC. The experiment ended when plants from treatments W and N+W

reached 50% of FC (day 16). At this point, six plants from each treatment were harvested and used for the analysis of morphological and physiological parameters. The remaining plants were separated into roots and shoots, and immediately frozen in liquid nitrogen, macerated (n = 3, each biological replicate consisting of a pool of three individual plants), and, subsequently, stored at −80 °C for further use in biochemical and molecular analyses.

2.2. Biometric Traits

At the end of the experimental period (i.e., 16 days after transplantation), 6 plants per treatment were divided into roots, stems, and leaves. After carefully washing the roots, the dry weight of each organ was determined, by drying the samples for 48 h at 105 °C in a ventilated oven. Based on these data, the shoot–root ratio was further calculated (n = 6).

2.3. Analysis of Total Antioxidant Capacity and Non-enzymatic Component

2.3.1 Preparation of the extracts

For the analysis of the total antioxidant activity ($ABTS^{\bullet+}$ assay) and secondary metabolism (total soluble polyphenols, flavonoids, anthocyanins, and lignin), 300 or 250 mg of frozen grinded samples of roots and shoots, respectively, were extracted with 1.2 or 1.5 mL of 80% (*v*/*v*) methanol, respectively, under cold conditions. Samples were centrifuged at 15,000× *g* for 15 min at 4 °C. The resulting supernatant was used for all previously mentioned analyses, except lignin quantification, where the sediment was used.

2.3.2. Total Antioxidant Capacity: ABTS Assay

$ABTS^{\bullet+}$ (2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)) assay (n = 3) was performed following the protocol described by Gonçalves *et al.* (2009), with slight modifications for the 96-well microplate. Briefly, $ABTS^{\bullet+}$ solution was generated through a chemical oxidation reaction with potassium persulfate, and its concentration was adjusted with methanol to an initial absorbance of 0.700 (±0.020) at 734 nm (Multiskan GO Microplate Spectrophotometer, Thermo FisherScientific Inc., Waltham, MA, USA). To 20 µL of the sample or standard solution, 180 µL of the adjusted $ABTS^{\bullet+}$ solution was added. The mixture was then incubated for 5 min at 30 °C, the absorbance at 734 nm was measured and the results were obtained by calculating the reduction of percentage in sample absorbance (A_{SPL}) concerning the control (A_{CTL}) using Equation (1). Trolox was used as a standard to prepare a calibration curve, and the final results are expressed as mmol Trolox equivalents/g FW. All the analyses were performed in triplicate, and the radical stock solution was freshly prepared and filtered with a 0.45 µm syringe filter.

$$\text{ABTS scavenging (\%)} = (A_{CTL} - A_{SPL}) * \frac{100}{A_{CTL}} \quad (1)$$

2.3.3. Non-enzymatic Component: total phenolics, flavonoids, carotenoids, anthocyanins, and lignin

2.3.3.1. Determination of Total Phenolic Content (TPC)

Determination of TPC was performed using the Folin–Ciocalteu reagent following Ramos *et al.* (2019), with slight modifications. Briefly, 20 µL of extract or standard (gallic acid stock solutions) was mixed with 80 µL of Folin–Ciocalteu reagent (previously diluted 1:10 (*v*/*v*)) and 100 µL of 7.5% (*w*/*v*) sodium carbonate in a 96-well plate. After 60 min at room temperature under dark conditions, absorbance was measured at 750 nm. The results are expressed as mg gallic acid equivalents/g of fresh weight (mg GAE/g FW).

2.3.3.2. Determination of total flavonoids

Total flavonoids were quantified through the aluminum chloride method adapted from Zafar *et al.* (2016). Briefly, an aliquot of each extract (40 µL) was mixed with 20 µL of 10% (*w*/*v*) aluminum chloride and 20 µL of 1 M potassium acetate solutions. Thereafter, distilled water was added to obtain a final volume of 200 µL in a 96-well plate. After 30 min of incubation under dark conditions at room temperature, the absorbance at 415 nm was measured by using a microplate reader. The calibration curve was drawn by using quercetin as standard and the flavonoid concentration was established in µg quercetin equivalents/g FW (µg QE/g FW).

2.3.3.3. Determination of total monomeric anthocyanins

The spectrophotometric pH differential method was used to determine the total monomeric anthocyanins (TMA) in the methanol extracts, following Nicoue *et al.* (2007) with some modifications for the 96-well microplate. Briefly, 240 µL of 0.025 M potassium chloride solution or 0.4 M sodium acetate solution adjusted to pH 1.0 and 4.5, respectively, with hydrochloric acid were added to 60 µL of extracted sample. The absorbance was measured at 520 nm and 700 nm and the absorbance (*A*) was calculated as described in Equation (2):

$$A = (A_{\lambda max} - A_{700})_{pH=1.0} - (A_{\lambda max} - A_{700})_{pH=4.5} \quad (2)$$

TMA concentration was then obtained from equation 3:

$$\text{TMA(mg/L)} = (A_{CTL} - A_{SPL}) * \frac{A * MW * DF * 1000}{\epsilon * l} \quad (3)$$

where,

λ_{max} = 520 nm

MW of cyanidin 3-glucoside = 449.2 g mol^{-1}

DF= dilution factor

ε of cyanidin 3-glucoside = 26900 L mol^{-1} cm^{-1}

l = optical path length

2.3.3.4. Determination of lignin

Lignin quantification was performed according to a protocol adapted to 96-well microplate from Fukushima and Hatfield (2001). Briefly, the sedimented biomass that remained in the microcentrifuge tube after methanol extraction was subjected to three 20 min washes in an ultrasound bath first with distilled water, then with acetone, and finally with hexane. Afterward, samples were dried at 60 °C for 72 h, and to 10 mg of sample, 1 mL of 12.5% acetyl bromide (prepared in acetic acid) was added, followed by incubation at 50 °C for 2 h with vigorous stirring. The digested samples were centrifuged for 5 min at 15,000× *g*, and 100 µL of the supernatant was combined with 200 µL of acetic acid, 150 µL of 0.3 M sodium hydroxide, 50 µL of 0.5 M hydroxylamine hydrochloride and 500 µL of acetic acid. The absorbance of each sample was recorded at 280 nm, and the lignin concentration in each sample was quantified taking into consideration the lignin calibration curve, and the lignin concentration was established in µg quercetin equivalents/g FW (µg/g FW).

2.4. Oxidative Stress Biomarkers

2.4.1. Lipid peroxidation

Lipid peroxidation (LP) was estimated in frozen ground samples of roots and shoots (ca. 200 mg FW) and quantified following the procedure of Heath and Packer (1968). After homogenizing the material in 0.1% (*w*/*v*) trichloroacetic acid, malondialdehyde (MDA) content was determined using the molar extinction coefficient (ε) of 155 mM^{-1} cm^{-1} and expressed in $nmol^{-1}$ g FW. Absorbances were read at 532 and 600 nm, with the latter being subtracted from the first to avoid the effects of non-specific turbidity.

2.4.2. Hydrogen peroxide (H_2O_2) quantification

The quantification of H_2O_2 levels was performed by a spectrophotometric method according to Alexieva et al. (2001). Briefly, frozen ground samples of roots and shoots (ca. 200 mg FW) were extracted on ice, with 1.4 mL of 0.1% (*w*/*v*) trichloroacetic acid, and centrifuged for 15 min at 12,000× g at 4 °C. The homogenate was then mixed with 250 µL of 100 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide. The reaction was developed for 1 h in the dark and absorbance was measured at 390 nm. H_2O_2 levels were estimated from a standard curve prepared with solutions of known concentration of H_2O_2. The results are expressed as nmol H_2O_2 g^{-1} FW.

2.5. Enzymatic Component

2.5.1. Enzyme extraction and quantification: SOD (EC.1.15.1.1), CAT (EC.1.11.1.6) and APX (EC.1.11.1.11)

The extraction of the AOX enzymes was performed under cold conditions according to Fidalgo *et al.* (2011), using frozen samples of around 250 mg. After centrifugation, the supernatants were collected and used for both protein and enzyme quantification following the protocols described by Soares *et al.* (2019b). Briefly, protein quantification was performed as described by Bradford (1976), using bovine serum albumin as standard. Superoxide dismutase (SOD, EC.1.15.1.1) activity was quantified with a spectrophotometric assay based on the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) (Donahue *et al.*, 1997). SOD activity was expressed as units of SOD mg^{-1} protein, in which one unit of SOD corresponds to the amount of enzyme needed to inhibit the photochemical reduction of NBT by 50% (Spormann *et al.*, 2022). Absorbance was read at 560 nm and results are expressed as units of SOD/mg of protein. Catalase (CAT, EC.1.11.1.6) activity was determined spectrophotometrically by measuring CAT-mediated H_2O_2 degradation (ε_{240nm} = 43.6 mM^{-1} cm^{-1}) (Aebi, 1984). Absorbance measurements were recorded every 5 s for 60 s, and results are expressed as mmol H_2O_2 min^{-1} mg of protein. Ascorbate peroxidase (APX, EC.1.11.1.11) activity was determined by monitoring the oxidation of ascorbic acid (AsA) for 1 min, with readings every 5 s (ε_{290nm} = 0.49 M^{-1} cm^{-1}) (Nakano and Asada, 1981); results are expressed as mmol dehydroascorbate (DHA) min/mg of protein. The protocols for quantifying CAT and APX activity were adapted to UV microplates following the adaptation from Soares *et al.* (2019b).

2.5.2. SOD, APX, and CAT expression profile

The transcript levels of *Fe-SOD*, *Cu,Zn-SOD*, *APX*, *CAT1,* and *CAT2* were evaluated using quantitative real-time PCR.

Total RNA was extracted from frozen ground roots and shoot tissues (c.a. 100 and 50 mg) using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions for each tissue type. Afterward, RNA yield was quantified using a NanoPhotometer (Implen GmbH, Germany), and its integrity was checked by agarose gel electrophoresis. Single-stranded cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad, California, USA) according to the manufacturer's instructions in a Doppio Thermal Cycler (VWR, Oud-Heverlle, Belgium).

Primers for *APX*, *CAT1,* and *CAT2* were selected using Primer-BLAST from NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/ - accessed on 15 May 2021) for an expected

PCR product of 70–150 bp and primer melting temperatures between 57 and 63 °C, whereas primer sequences for *Fe-SOD* and *Cu-Zn-SOD* were obtained from previous studies (Martinez *et al.*, 2018). The primer sequences for each gene are listed in Table 1.

Table 1 - Primer sequences (Forward and Reverse) and annealing temperature ($T_{ann.}$) of target genes used for RT-qPCR analysis.

Gene (Accession number)	Primer sequence (5'-3')		$T_{ann.}$
	Forward	Reverse	
Fe-SOD	TAA ATA GAG ACT TTG GTT CC	TAT ATT TGC CTC TTA ACC CT	45.6
Cu,Zn-SOD	GGC CAA TCT TTG ACC CTT TA	AGT CCA GGA GCA AGT CCA GT	54.7
APX	TCT GAA TTG GGA TTT GCT GA	CGT CTA ACG TAG CTG CCA AA	55.4
CAT 1	CAA ACA ATG GAC CCC GAG GA	ACT GGG ATC AAC GGC AAG AG	60.3
CAT 2	GGG TCT GGT GTC CAC ACA TT	GCA TGG CTG TGA TTT GCT CC	59.0

Real-time polymerase chain reactions (RT-qPCR) were performed on a StepOne™ Real-Time PCR System (Applied Biosystems, California, USA) with the following reaction conditions: 2 min at 50 °C, 2 min at 95 °C and 40 cycles, with 15 s at 95 °C, 15 s at each primer pair optimal annealing temperature (Table 1) and 1 s at 72 °C. Amplifications were carried out using a final volume of 20 µL, which consisted of 1 µL of the primer forward and 1 µL of the primer reverse (both at 6 µM), 10 µL of 2× iQ SYBR® Green Supermix (Bio-Rad, California, USA), and 8 µL of a 1:100 dilution of the template cDNA. Melt curve profiles were analyzed for each tested gene. The comparative CT method ($^{\Delta\Delta}$CT - Livak and Schmittgen, 2001) was used for the relative quantification of gene expression values using ubiquitin (UBI) and TIP41-like protein (TIP41) genes as control transcript and the control plants as the reference sample. For relative gene expression analysis, the fold change with biological significance was considered to be lower than 0.5 (downregulation) or higher than 2 (upregulation) (Vaerman *et al.*, 2004), and for each of the three biological replicates and target genes, two technical replicates were analyzed.

2.6. Statistical analysis

Data were subjected to analyses of variance (ANOVA), followed by Tukey's post hoc test, whenever significant differences were found (p = 0.05). The results are expressed as mean ± standard error of the mean (SEM). Additionally, a Principal Component Analysis

(PCA) with Varimax rotation was used to establish the relationship among the different quantitative variables with the data set including only the parameters that were shown to be significantly different among treatments. The data included was mean-centered and weighed with the inverse of the standard deviation before applying the PCA to guarantee that all variables had the same weight in influencing the model. All statistical analyses were performed in IBM SPSS Statistics 26.

3. Results

3.1. Plant Growth

The total dry weight (TDW) of plants grown under single or combined N deficit was significantly lower (30%) when compared to CTR and W-deficit plants. (Figure 1a). The shoot–root ratio of N + W deficit plants was 22% lower compared to plants grown under single N deficit. However, it did not significantly differ from plants grown under single W deficit. For the single deficits, this ratio was only significantly changed under W shortage, which led to a 30% decrease compared to CTR plants (Figure 1b).

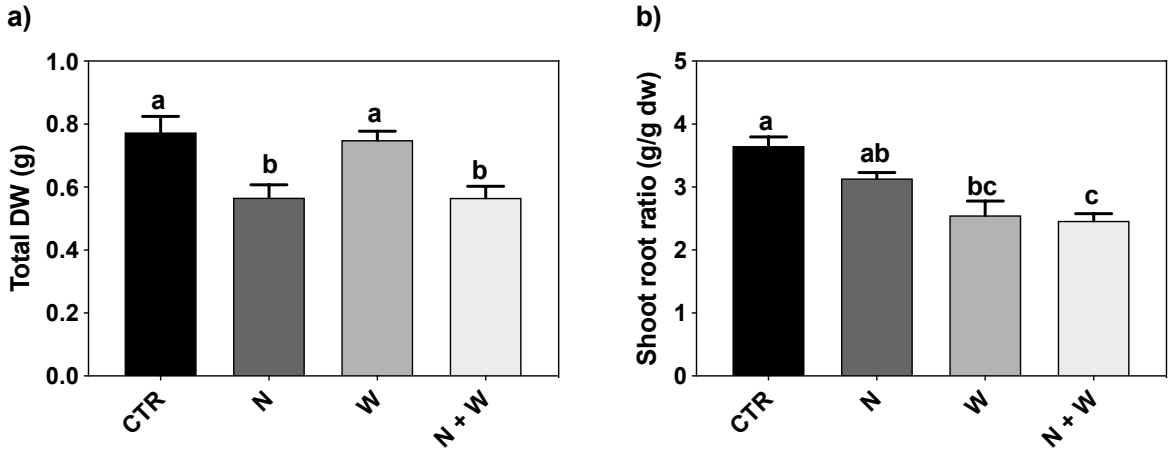


Figure 1 - Total dry weight **(a)** and shoot root ratio (**b)** of tomato plants cv. Micro-Tom grown for 16 days under control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N+W; 5.3 mM N+ 50% W). Data presented are mean ± SEM (n = 6). Different letters above bars indicate significant differences according to the Tukey's HSD test (p = 0.05).

3.2. AOX Activity

Total AOX activity, measured using the ABTS assay, was significantly increased by about 90% in plants exposed to combined N + W deficit, in both roots and shoots, compared to all other experimental conditions, which displayed no differences among them (Figure 2a,b).

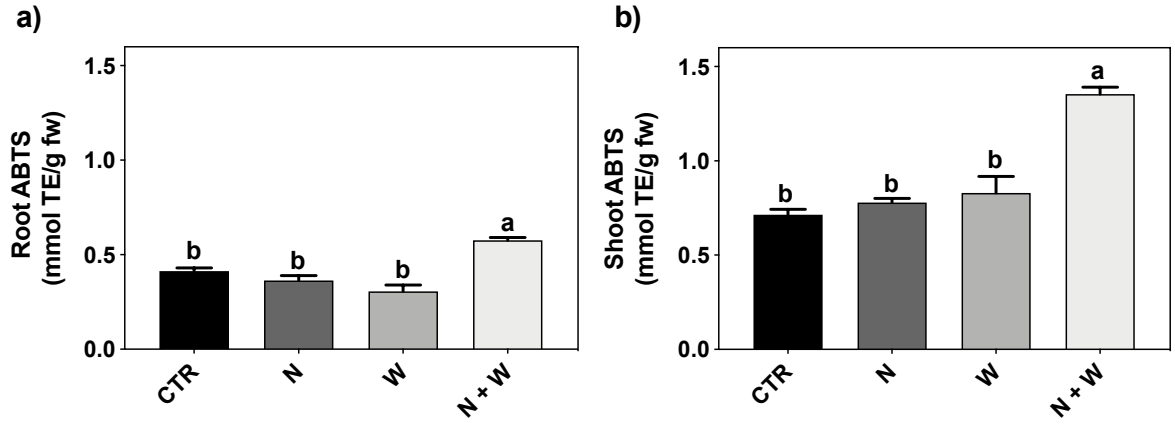


Figure 2 - Total antioxidant capacity measured by ABTS in roots **(a)** and shoots **(b)** of tomato plants cv. Micro-Tom grown for 16 days under control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N+W; 5.3 mM N+ 50% W). Data presented are mean ± SEM (n = 3). Different letters above bars indicate significant differences according to the Tukey's HSD test (p = 0.05).

3.3. Non-enzymatic component: total phenolics, flavonoids, carotenoids, anthocyanins, and lignin

The concentration of total phenolics in roots and shoots was not affected by single or combined N + W deficit (Figure 3a,b). However**,** flavonoid concentration was reduced by around 20% in the roots of plants exposed to combined N + W deficit in relation to CTR or to single deficits (Figure 3c). Interestingly, shoot flavonoid concentration was significantly higher in single W deficit plants, as compared to the remaining experimental conditions, whereas plants subjected to the combined deficit had intermediate levels (17% lower compared to plants grown under W deficit and 15% higher compared to single N deficit) (Figure 3d). The levels of shoot carotenoids were not significantly affected. Still, a clear tendency could be observed, with W-deficit plants having the highest levels of carotenoids (Figure 3e). The concentration of total monomeric anthocyanins reached the highest level in plants grown under combined N + W deficit, compared to all the other experimental groups (92%—CTR; 89%—N; 103%—W increase) (Figure 3f). Lignin concentration in the roots was not affected by any treatment (Figure 3g). In the shoots, it was significantly higher in plants exposed to W (by 35%) and N + W (by 26%) deficits compared to the CTR plants, but no significant differences between single and combined deficits were found (Figure 3h).

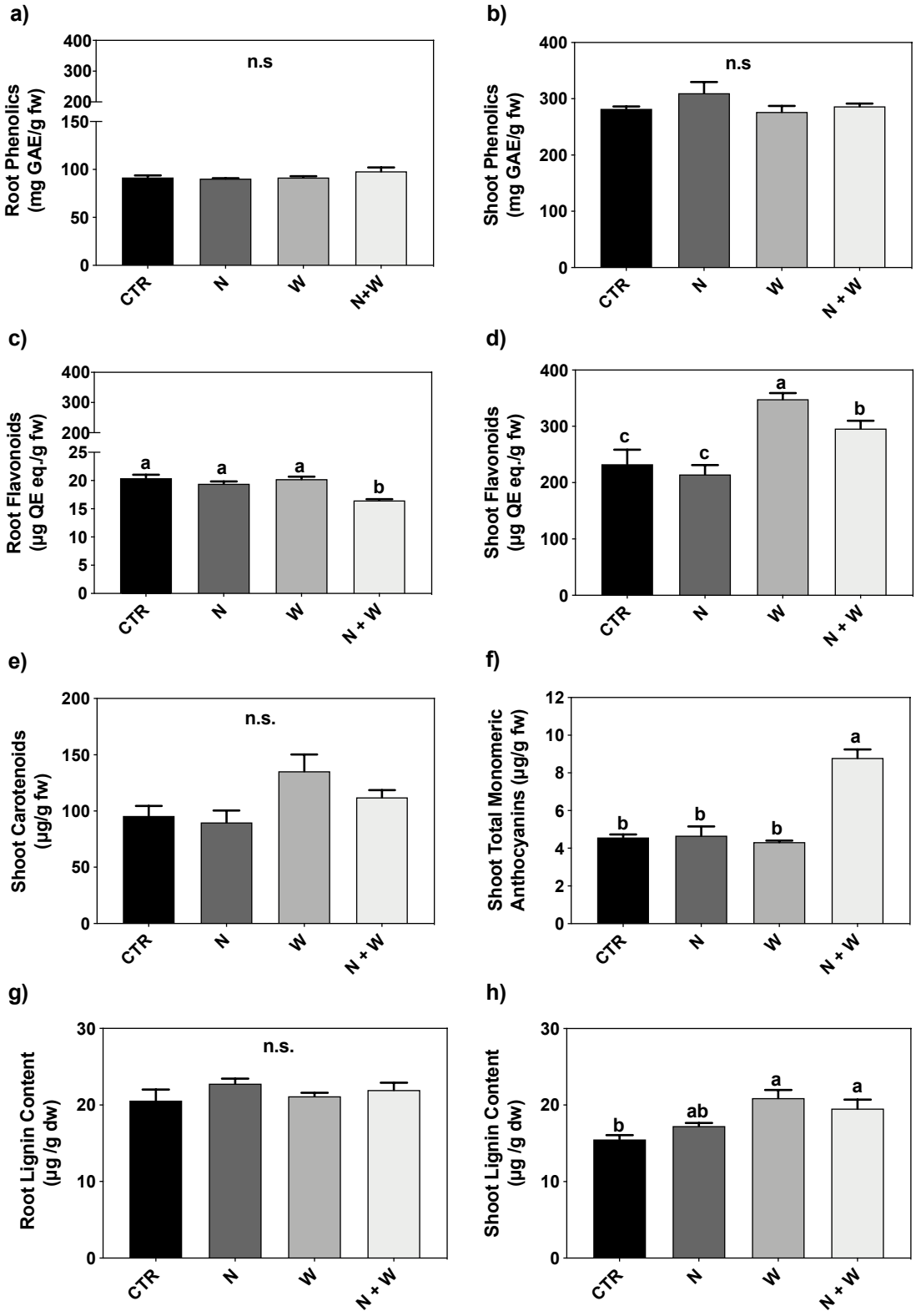


Figure 3 - Total phenolics in roots **(a)** and shoots **(b)**, total flavonoids in roots **(c)** and shoots **(d)**, total monomeric anthocyanin in roots **(e)** and shoots **(f)** and lignin concentration in roots **(g)** and shoots **(h)** of tomato plants cv. Micro-Tom grown for 16 days under control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N+W; 5.3 mM N+ 50% W). Data presented are mean ± SEM (n = 3). Different letters above bars indicate significant differences and n.s. correspond to non-significant differences between treatments according to the Tukey's HSD test (p = 0.05).

3.4. Oxidative stress biomarkers – MDA and H_2O_2 levels

The MDA concentration in roots and shoots was not significantly influenced by the imposed deficits (Figure 4a,b). H_2O_2 concentration was not affected in roots (Figure 4c); however, in shoots, H_2O_2 concentration was 40% higher in plants exposed to N + W deficit than in plants subjected to W deficit (Figure 4d).

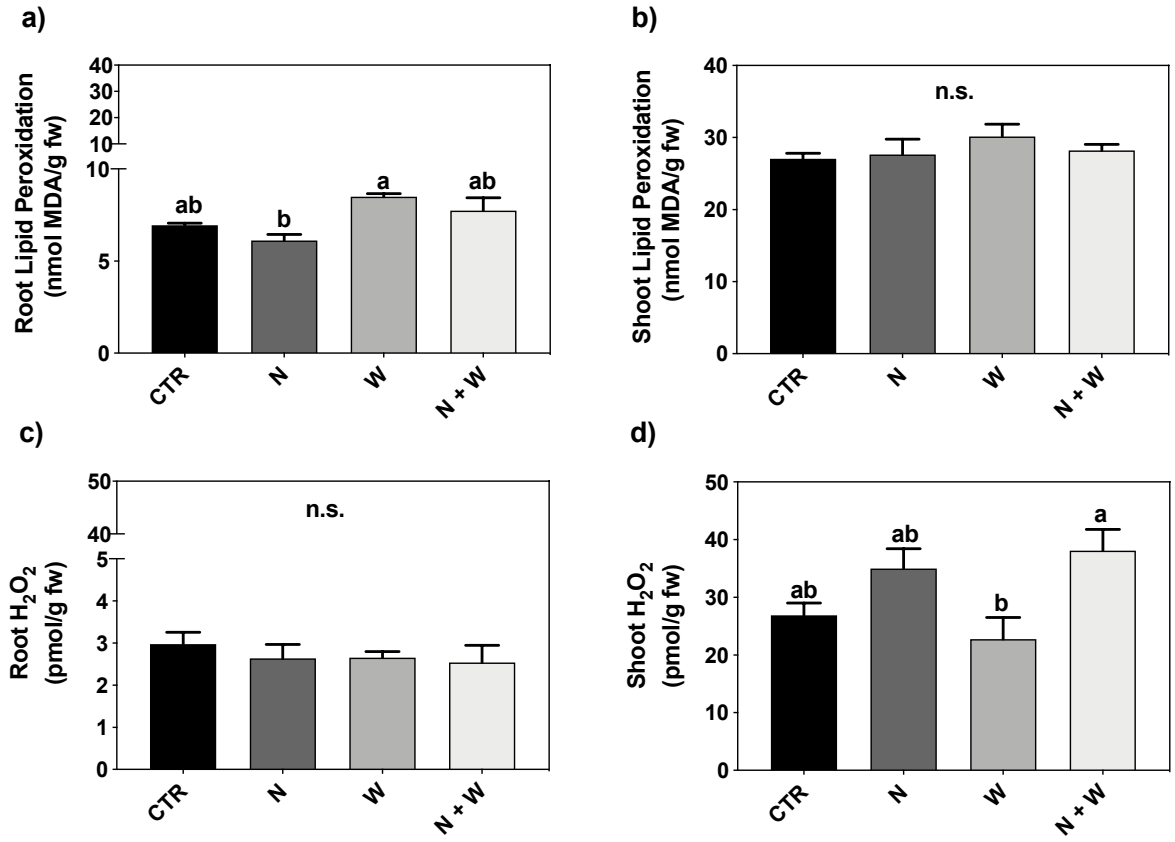


Figure 4 - Lipid peroxidation of roots (a) and shoots **(b)** and H_2O_2 content of roots **(c)** and shoots **(d)** of tomato plants cv. Micro-Tom grown for 16 days under control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N+W; 5.3 mM N+ 50% W). Data presented are mean ± SEM (n = 3). Different letters above bars indicate significant differences and n.s. correspond to non-significant differences between treatments according to the Tukey's HSD test (p=0.05).

3.5. Enzymatic component: SOD, CAT, APX

In roots, SOD activity was not significantly affected by the imposed stresses (Figure 5a). In shoots, significant differences were found among all the treatments. N + W plants had the highest SOD activity (77%—CTR; 32%—N; 202%—W increase), and W deficit plants had the lowest SOD activity (Figure 5b). APX activity was significantly higher (up to 57%) in the roots of W and N + W deficit plants compared to CTR and single N deficit (Figure 5c). In shoots, APX activity was higher in N + W plants compared to CTR (by 198%), N (by 128%), and W (by 155%) (Figure 5d). CAT activity followed the same trend as APX, both in roots and shoots (Figure 5e,f).

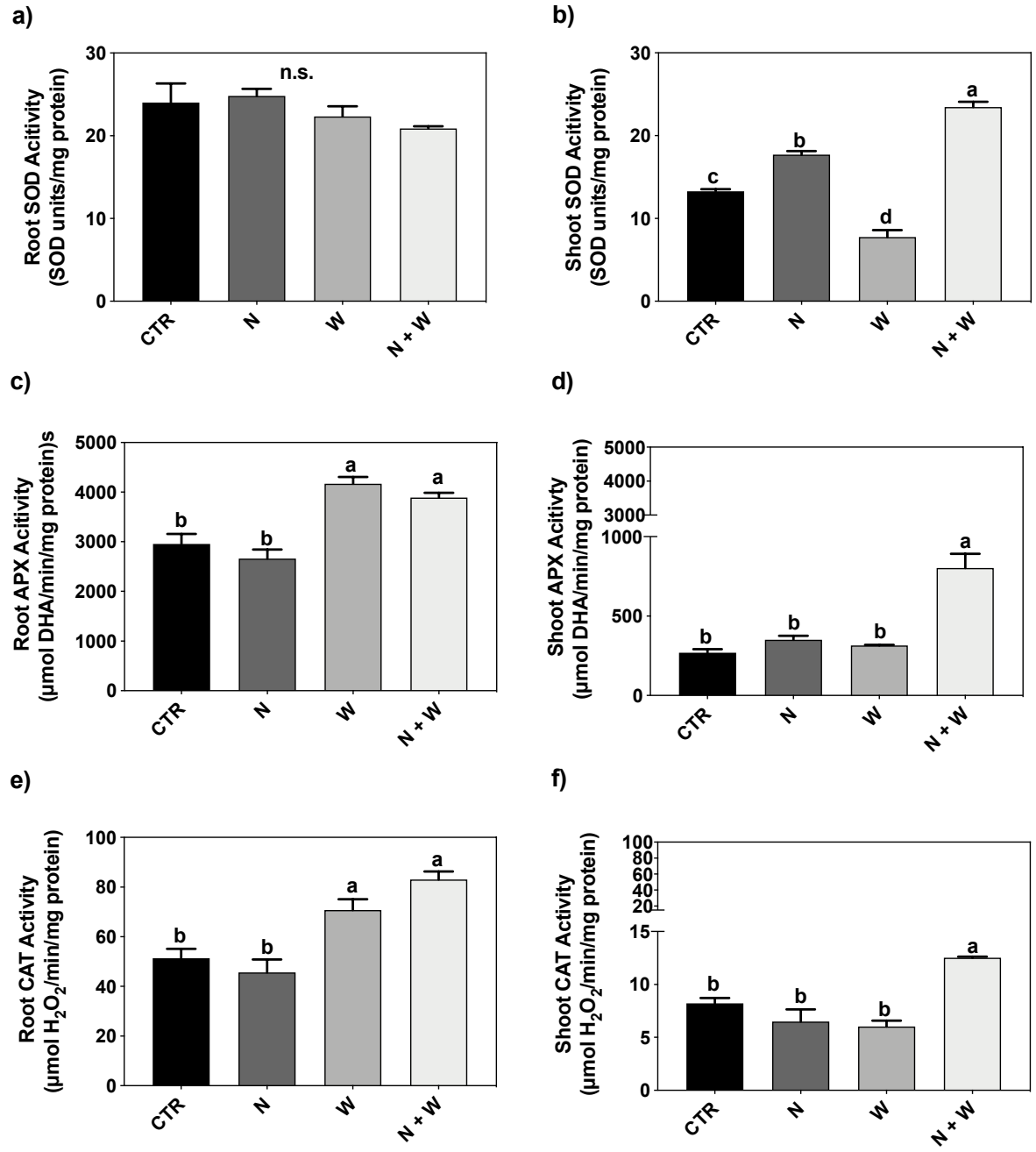


Figure 5 - SOD activity in roots **(a)** and shoots **(b)**, APX activity in roots **(c)** and shoots **(d)** and CAT activity in roots **(e)** and shoots **(f)** of tomato plants cv. Micro-Tom grown for 16 days under control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N+W; 5.3 mM N+ 50% W). Different letters above bars indicate significant differences and n.s. correspond to non-significant differences between treatments according to the Tukey's HSD test (p = 0.05).

3.6. SOD, APX, and CAT expression profile

Fe-SOD expression levels, in both roots and shoots of all treatments, were maintained within the basal threshold (with a fold of expression between 0.9 and 1.3) (Figure 6). However, in roots, a significant upregulation of *Cu,Zn-SOD* was observed for all deficits,

with the levels being higher in the combined N + W deficit group (4.6-fold upregulation). In shoots, no significant differences were found in the expression of this gene compared to CTR plants (Figure 6). Single as well as combined deficit resulted in a significant increase in root *APX* expression, with the highest being in W (by 2.9-fold) and N + W (by 2.7-fold) plants. In shoots, N + W showed the highest expression of APX (by 3.3-fold), followed by N deficit plants (upregulation of 1.7-fold) (Figure 6). *CAT1* expression was maintained at basal levels in all deficit treatments both in the roots and the shoot, whereas *CAT2* expression was significantly downregulated in roots to N deficit plants (by 0.3-fold), but upregulated in shoots of all the treatments when compared to CTR. This was more pronounced in the N + W plants, which showed a 3.4-fold upregulation (Figure 6).

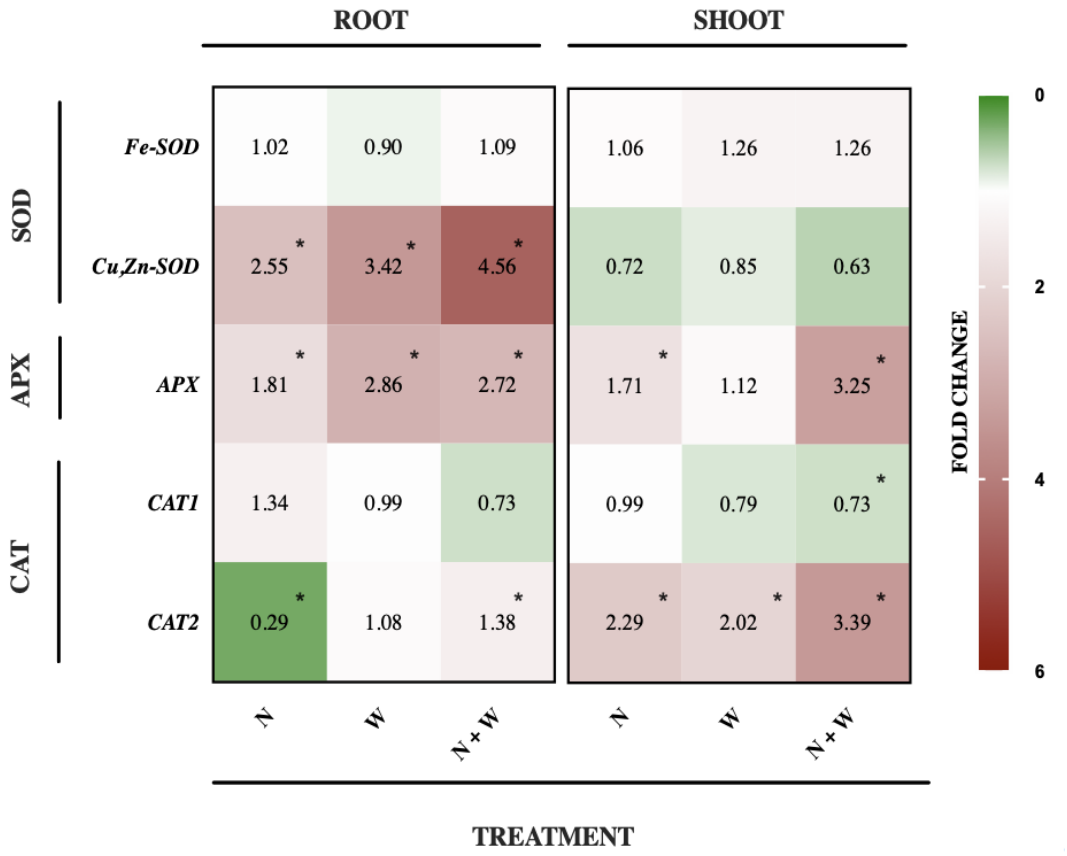


Figure 6 - Relative fold of expression of antioxidant system-related genes in tomato cv. Micro-Tom roots and shoots as affected by nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N+W; 5.3 mM N+ 50% W)). The $2^{-\Delta\Delta Ct}$ values against the control are shown. *above The $2^{-\Delta\Delta Ct}$ values against the control are shown (n = 3). *above mean values indicate significant differences compared to the control according to the t-Test (p = 0.05). Green colour indicates downregulation and red colour upregulation compared to the control plants.

3.7. Principal Component Analysis (PCA)

A PCA was performed to establish the relationship among the AOX parameters that were significantly affected by the single or combined N and water deficit treatments. The

results yielded two main factors (those with an Eigenvalue > 1), explaining over 81% or 87% of the total variance at root and shoot level, respectively (Figure 7). The first component accounted for 62% and 64% of the variance in roots and shoots, respectively, and the second for 19% and 23%, respectively (Figure 7a,b). At the root level, CTR and N-deficit plants behaved similarly, and these showed an opposite behavior to single or combined water deficit plants, mostly because the latter group had a higher activity of CAT and APX enzymes and *CAT2*, *Cu,ZnSOD*, and *APX* expression profiles (Figure 7a). Still, at the root level, the W deficit plants were associated with higher flavonoid concentration and lower antioxidant activity (ABTS), with N + W plants having the opposite behavior regarding these variables (Figure 7a). Interestingly, when analyzing plant responses at the shoot level, plants subjected to combined N + W deficit were different from all the other groups, mainly due to higher anthocyanins concentration, higher activity of CAT, SOD, and APX enzymes, and *CAT2* and *APX* expression (Figure 7b). However, they shared some similarities with W deficit plants regarding lignin and flavonoid levels, which were higher in these groups, and *CAT1* expression, which was downregulated (Figure 7b).

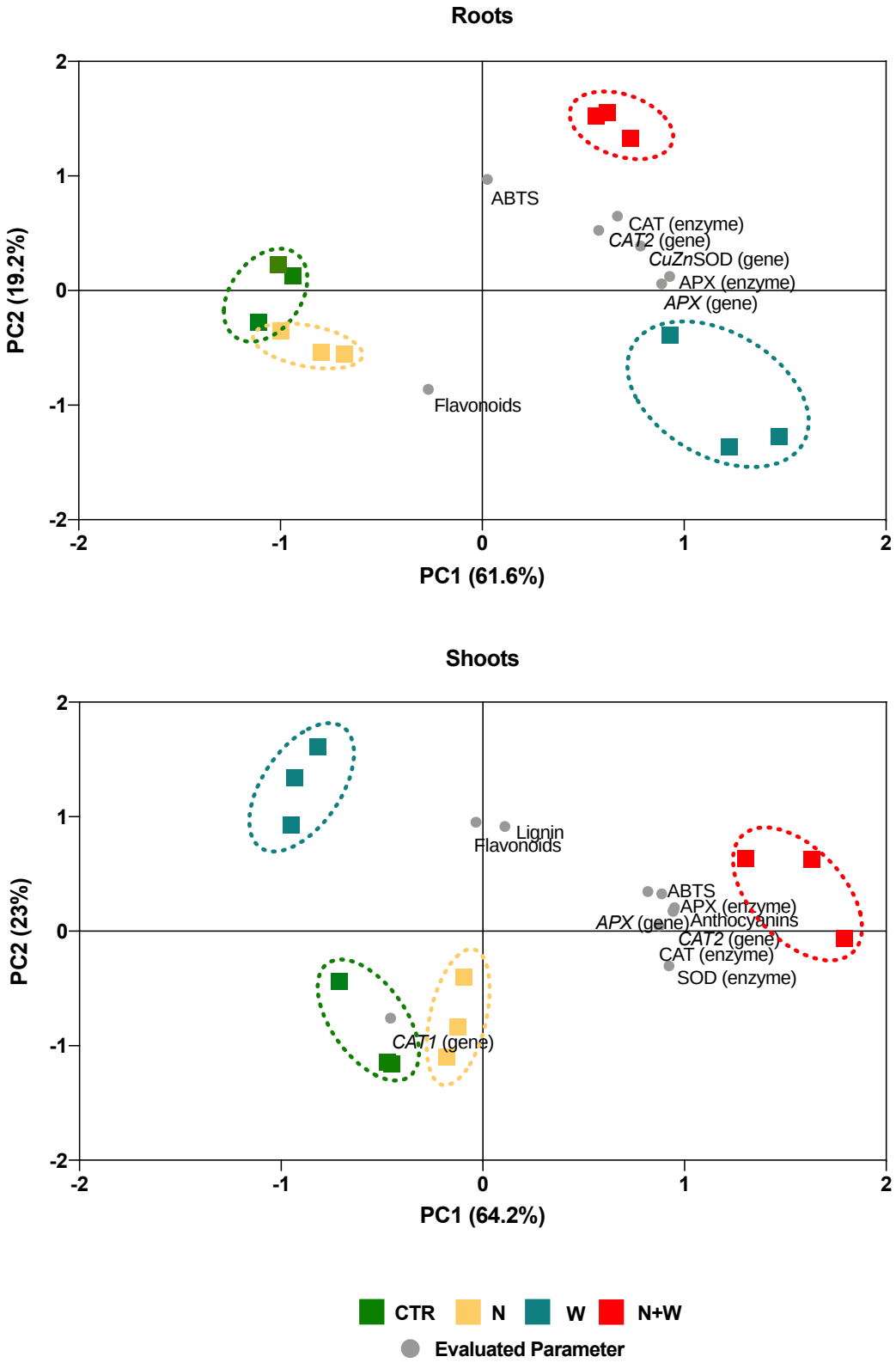


Figure 7 - Principal component analysis (PCA) of the antioxidant system in roots and shoots evaluated of tomato cv. Micro-Tom grown for 16 days under control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N+W; 5.3 mM N+ 50% W). The two principal components together (PC1, PC2) explained 81%, of roots, and 88%, in shoots, of the total variance found among treatments. Details on the abbreviations are given in Material and Methods

4. Discussion

More often than not, plants do not face single abiotic stresses, but rather a combination of several adverse growth conditions, especially under the current context of climate change and environmental instability. Still, literature describing the physiological and biochemical responses of plants simultaneously exposed to different stresses is scarce. Works focusing on the effects of combined N + W deficit on tomato plants can provide important and practical data for understanding impactful stress responses in likely environmental scenarios. To the best of our knowledge, this is the first study that focuses on a broad analysis of the AOX responses of young tomato plants subjected to single and combined N and W deficits. By studying a set of AOX biochemical endpoints, we showed that tomato plants can modulate their AOX network to ensure redox homeostasis, at least in some situations of N and water limitation (Figure 7).

Previous reports have demonstrated that water and/or N deficit severely affect plant performance, leading to a growth reduction in most crops, including tomato (recently reviewed by Machado *et al.*, 2022). In our study, W deficit per se did not induce an evident effect on TDW, whereas, in the single or combined N deficit plants, a negative effect was found (Figure 1a), which suggests a higher impact of the N deficit on this trait. Interestingly, dry matter partitioning only responded significantly under water deficit conditions (single or combined), resulting in a decreased shoot–root ratio (Figure 1b), representing a greater investment in the roots to handle the water shortage.

When facing stressful environments, plants often experience a general redox disbalance, capable of downstream affecting other metabolic and physiological processes. It has been reported that ROS can play a dual role in plant metabolism (Soares *et al.*, 2019a): while at high concentrations, ROS function as a toxic by-product, causing negative lipid membrane damage, at low concentrations, they might be acting as a signal transduction molecule to trigger a stress response, therefore improving non-enzymatic and enzymatic components (Laxa *et al.*, 2019; Soares *et al.*, 2019a; Mittler *et al.*, 2011)). Drought stress has been reported to result in higher MDA content (related to lipid peroxidation) in tomato plants, indicating that under drought stress, the balance of the redox status is disrupted, resulting in ROS accumulation and increased lipid peroxidation of membrane lipids, eventually causing membrane damage (Zhou *et al.*, 2019a; Zhou *et al.*, 2019b; Liang *et al.*, 2020; Raja *et al.*, 2020). The same was reported for the N deficit (Rubio-Wilhelmi *et al.*, 2011). In the present study, although plants under combined N+W deficit showed a significantly lower TDW and shoot–root ratio (Figure 1) compared to CTR and W deficit plants, ROS content and oxidative damage were not significantly affected by any of the applied treatments, as demonstrated by the unchanged values of LP and H_2O_2 (Figure 4). Based on this set of results, it seems that

mild N and W deficits were perceived by the plant as a signal, helping them to employ defence strategies to better cope with potential future stress episodes. In line with the low levels of H_2O_2 found, an increase in the total AOX activity (evaluated by the ABTS Assay) was observed under combined deficit, which might be related to the improvement found in different non-enzymatic factors (such as flavonoids and anthocyanins) (Figure 3) and enzymatic components (SOD, CAT, and APX transcripts and activity) players (Figures 2, 5 and 6).

It is known that non-enzymatic mechanisms play a vital role in counteracting oxidative stress and improving plant stress tolerance (Laxa *et al.*, 2019; Soares *et al.*, 2019a). Specialized metabolites often display potent AOX activity which allows more efficient scavenging of toxic ROS (Kubalt, 2016). In tomato, Sánchez-Rodríguez *et al.* (2011b) demonstrated that, after water deficit, cv. “Zarina” increased its AOX activity by upregulating the phenylpropanoids and flavonoids synthesis pathway, together with a lower oxidation index. Our results are in accordance with these findings, as a significantly higher shoot flavonoid content was observed in plants subjected to W deficit (Figures 3d and 7). Previously, it has been suggested that N deficiency also triggers the accumulation of flavonoids via increases in phenylalanine ammonia-lyase activity (Koleška *et al.*, 2017). However, here, no significant differences were found upon exposure to individual N deficit (Figure 3c,d). Interestingly, in shoots, flavonoid accumulation upon exposure to combined N + W deficit reached intermediate levels compared to the single deficits. Thus, it is possible that when facing the combined deficit plants responded by regulating the accumulation of other metabolites. It is known that the production of other secondary metabolites with a photoprotective role, such as anthocyanins and carotenoids, is important in plants’ response to oxidative stress caused by N or water deficit (Machado *et al.*, 2022). Accordingly, a higher concentration of anthocyanins was found in plants under N + W (Figure 3f), which may have functioned as ROS scavengers, photoprotectants, and/or stress signals in those plants subjected to the combined deficit (Kovinich *et al.*, 2015).

Besides the non-enzymatic mechanisms, the enzymatic component is another crucial part of the defence mechanisms against oxidative stress. SOD, as the first frontline defence against oxidative injury, catalyses the dismutation of O_2^- and generates H_2O_2, which is converted to H_2O and O_2 by CAT, with both usually increasing in plants grown under drought stress (Sanchez-Casas and Klessig, 1994). In tomato plants, studies have shown that the activity of SOD gradually increases in different tomato cultivars subjected to drought stress (Aghaie *et al.*, 2018; Hayat *et al.*, 2012; Yuan *et al.*, 2016; Zhou *et al.*, 2019a; Zhou *et al.*, 2019b; Liang *et al.*, 2020; Raja *et al.*, 2020). Besides SOD, the activity of CAT and APX has also been shown to increase in tomato plants under prolonged drought stress, from the

seedling to the anthesis stage (Rai *et al.*, 2021; Yuan *et al.*, 2016). In the present study, APX and CAT activity were only slightly increased in the roots of plants subjected to W deficit, demonstrating a faint response of the enzymatic AOX system associated with mild water deficit. Concerning N deficiency, its effect on the enzymatic AOX system has scarcely been studied in tomato. In general, it has been shown that N deficiency can lead to an overproduction of ROS, particularly of H_2O_2 and O_2^-, through the excitation of the photosystem II, consequently enhancing the activity of enzymes like POD and SOD (Machado *et al.*, 2022). In cucumber leaves of N-deficient plants, the activity of SOD increased by 37% compared to the control, a higher increase compared to CAT and POD (Hasanuzzaman *et al.*, 2020). In wheat, plants subjected to N deficiency also exhibited a significant increase in SOD activity (by 205%), with this being the most upregulated enzyme (Polesskaya *et al.*, 2004). This set of results agrees with the observed increase in SOD activity of our tomato shoots subjected to N deficiency (Figure 5b). Concerning plants subjected to combined N + W deficit, in roots, CAT and APX showed the same pattern of response observed in plants grown under single W deficit, suggesting that at the root level, the W deficit was the main factor contributing to this response (Figure 5a,c,e). Interestingly, at the shoot level, plants grown under co-exposure to both deficits presented the highest activity of SOD, APX, and CAT, which contributed to the enhancement of the total AOX activity (Figure 2).

In general, an upregulation of SOD and APX transcripts in roots of plants subjected to N deficiency did not seem to translate into an enhanced enzyme activity, which can suggest the involvement of post-translational modifications of the enzyme's status. However, it cannot be excluded that, after a longer period of exposure, these increased transcript levels could result in higher enzyme activity to ensure cell homeostasis, providing the cell with a large amount of mRNA ready to be translated into proteins. Regarding combined deficit plants, in general, the activity of all the studied enzymes increased both in roots and shoots, along with an upregulation of *Cu,Zn-SOD*, *APX,* and *CAT2* genes in roots and *APX* and *CAT2* genes in shoots.

An integration of all the AOX system-affected traits resulted in a PCA that revealed considerable variability among treatments, especially between tissues (Figure 7). The PCA showed that in roots, N + W deficit plants had a behaviour similar to that of plants exposed only to mild W deficit, showing higher activity of CAT and APX enzymes and *CAT2*, *Cu*, *ZnSOD*, and *APX* expression (Figure 7a). In shoots, the N + W deficit had a distinct and unique response, having, in general, a higher non-enzymatic and enzymatic activity (Figure 7b). The accumulation of AOX metabolites (e.g., ascorbate and glutathione) alongside the activation of several AOX enzymes (e.g., APX, CAT) was also shown to increase upon the

co-exposure of tomato plants to salinity and heat stress (Sousa *et al.*, 2022) or after the exposure of citrus plants to combined drought and heat stress (Zandalinas *et al.*, 2017). The differential behaviour between combined N and water deficit and single deficits, as well as between plant organs (shoots and roots) observed in our study, highlights that plants grown under combined stresses generate a specific AOX response that cannot be elucidated from the responses obtained when stressors are applied individually (García-Martí *et al.*, 2019; Pandey *et al.*, 2015; Hussain *et al.*, 2018).

Moreover, the mild water deficit level imposed in this study did not impact plant biomass, but it was enough to activate multiple AOX responses in plants grown under combined deficit, possibly making the plants more suited to handling subsequent abiotic stresses. Indeed, a recent study with maize showed beneficial stress memory responses induced by priming the seedlings with combined heat and drought stress, which prompt their defence system to trigger more effective clearance mechanisms against subsequent exposure to these combined stresses (Ru *et al.*, 2022). However, the intensity of the N-deficiency needs to be thoroughly adjusted to avoid, as in this study, impairments in plant growth. Flowering and fruit development have been described as highly sensitive stages to water deficits (Khapte *et al.*, 2019; Machado *et al.*, 2022). Therefore, complementary studies are necessary to address tomato responses to more severe stress events, as well as to explore other developmental stages.

5. Conclusions

This study suggests that tomato plants can employ defense strategies to cope with combined N+W deficit, as demonstrated by the maintenance of the redox homeostasis, with unchanged values of LP and H_2O_2 compared to control plants. Accordingly, an enhancement of different non-enzymatic (such as flavonoids and anthocyanins) and enzymatic (SOD, CAT, and APX transcripts and activity) players was observed, contributing to an increased AOX activity, especially in plants under the co-exposure to N+W deficit. In conclusion, tomato plants seem to have the ability to modulate their AOX network to ensure redox homeostasis in a situation of mild N and water limitation, and the pattern of response is quite distinct among different plant organs and to the one observed in plants exposed to these single deficits. Additionally, our results raise the hypothesis that mild N and water deficits, at least at the beginning of the tomato plant´s vegetative stage, activate relevant AOX responses which in turn might act as a priming strategy for preparing the plants to cope with future abiotic or biotic stresses. However, understanding the full mechanisms involved in the complex interactions between combined stress factors is still in its infancy. Future studies could test the aforementioned hypothesis and test whether the current findings can be extrapolated to more

susceptible tomato cultivars, more critical developmental stages as well as more severe or prolonged stress levels.

Sub-chapter 2.3

Combined nitrogen and water deficit impact on tomato plant anatomy, macronutrient accumulation and partitioning, and amino acids profile.

The content of this sub-chapter will be submitted as:

J. Machado, A.P.G. Fernandes, B. Bokor, M. Vaculík, D. Kostoláni, A. Kokavcová, E. Heuvelink, M.W. Vasconcelos, S.M.P. Carvalho. (2023). Combined nitrogen and water deficit impact on tomato plant anatomy, macronutrient accumulation and partitioning, and amino acids profile. *Environmental and Experimental Botany* (in prep.)

Combined nitrogen and water deficit impact on tomato plant anatomy, macronutrient accumulation and partitioning, and amino acids profile

Abstract

Crops are often exposed to multiple abiotic stresses and possible interactions between them may occur. Despite this, studies on combined nitrogen (N) and water (W) deficit still need to be conducted. This study explored the differences between single and combined N+W deficit in young tomato plants. For that, seedlings from cv. Micro-Tom were subjected to four treatments: control (CTR; 100%N+100%W), N deficit (50%N), W deficit (50%W), or combined deficits (50%N+50%W). The single deficits impacted anatomical traits, similarly, reducing cross-sectional hypocotyl, phloem, and xylem areas. The N+W deficit had a higher negative impact on tomato biometric traits, decreasing root dry weight (by up to 50%), stem dry weight (by up to 60%), and leaf dry weight (by up to 47%). Plants grown under the N+W deficit increased root synthesis of asparagine and arginine (up to 230% compared to the W deficit and 66% compared to the N deficit). This led to lower N remobilization from the photosynthetic machinery, as demonstrated by the lower decreases in chlorophyll content and photosynthetic rate compared to plants under single N deficit. Additionally, plants grown under N+W deficit have increased the reallocation and synthesis of osmolytes such as K^+ and proline, respectively. This together with the specific increase observed for other amino acids related to osmoregulation (alanine, tyrosine, and phenylalanine) contributed to a higher stomatal closure and lower transpiration rate compared to W deficit plants. On the other hand, N deficit plants responded mainly through N remobilization from the photosynthetic machinery, as demonstrated by the decreased chlorophyll content (up to 32%) and photosynthetic rate (up to 57%). Under single W deficit, plants invested more in the root system, as a strategy to increase uptake of water and nutrients, compared to plants grown under N+W deficit, being able to maintain physiological processes such as photosynthesis at CTR plants level. Hence tomato plants used different N remobilization and osmoregulatory mechanisms to respond to the single or combined deficits.

1. Introduction

Tomato (*Solanum lycopersicum*) is one of the most economically important vegetables for the fresh and processing markets, with global annual production reaching 180 million tons (Wu *et al.*, 2022). This crop has high requirements in nitrogen (N) fertilization and water supply (Du *et al.*, 2018). Therefore, suboptimal amounts of these resources cause severe constraints on plant growth (Machado *et al.*, 2022).

Several studies have demonstrated that N and water deficits are known to cause a severe impact on metabolic pathways (Ding *et al.*, 2018; Gonzalez-Dugo *et al.*, 2010), thus altering anatomical characteristics of vascular tissues responsible for meeting plant water and nutrient demands (Kapoor *et al.*, 2020; Song *et al.*, 2019). For instance, narrower xylem vessels under lower water potential could help to avoid cavitation (Knipfer et al., 2018). On the other hand, narrow xylem vessels together with a lower total xylem area reduce internal water transport (Hernandez-Espinoza & Barrios-Masias, 2020). Furthermore, water deficit is reported to have a negative effect on root nutrient uptake due to the reduction in soil moisture (Rouphael *et al.*, 2012). In tomato, drought reduces nitrate (NO_3^-) uptake, imposing further constraints on plant growth (Sánchez-Rodríguez *et al.*, 2011c) which can be aggravated in a situation where plants are facing a combination of both deficits. Additionally, since N is a dominant macronutrient, its deficit also impacts the accumulation of other nutrients, with its ionic form controlling cation and anion accumulation through the mechanism of charge balance in the ion uptake (Borgognone *et al.*, 2013). For example, under NO_3^- deficiency, cations like potassium (K^+), magnesium (Mg^{2+}), and calcium (Ca^{2+}) usually present lower accumulation while anions such as phosphate (PO_4^{3-}), sulfate (SO_2^{-4}), and most micronutrients were reported as having higher accumulation (Machado *et al.*, 2022).

Amino acids are the building blocks of proteins and have various prominent functions in plants (Hildebrandt *et al.*, 2015; Kumar *et al.*, 2017). Apart from their role in proteins, they are involved in several physiological processes such as plant growth and development, intracellular pH control, generation of metabolic energy or redox power, and resistance to abiotic and biotic stress, acting as signalling molecules (Guo et al., 2021; Hildebrandt et al., 2015). Therefore, under stressful conditions, such as those caused by N or water deficit, the amino acid pool is adjusted by amino acid catabolism and synthesis (Yang et al., 2020). For instance, proline can play an important role as osmolyte, being often increased in plants subjected to water deficit (Florencio-Ortiz *et al.*, 2018; Živanović *et al.*, 2020), but can also be used as a metabolic compatible solute to store and transfer N and C after oxidation often decreasing under N deficit (Hayat *et al.*, 2012; Szabados and Savouré, 2010). Also, N availability influences the C/N ratio, which consequently affects the amino acid profile of plants (for example, greater N availability promotes the production of amino acids with low C/N ratios

like arginine and glutamine) (Ruan *et al.*, 2010). Several studies, including a few using tomato plants, demonstrated that N deficiency often leads to lower total amino acids content since N is a central nutrient for their formation (Ková ik *et al.*, 2006; Ruan *et al.*, 2010; Sung *et al.*, 2015; Yang *et al.*, 2020). On the other hand, under water deficit, the content of free amino acids is increased in tomato plants (Živanović *et al.*, 2020). Therefore, free amino acids may also be differentially adjusted in a situation of combined N and water deficits. For instance, Jin *et al.* (2015) found that in *Portulaca oleracea* L. plants there was a specifically increased accumulation of certain amino acids, such as glutamine, ornithine, tyrosine, valine, and tryptophan, when subjected to drought and heat stress acting in combination, which suggests that the plant is making a cellular osmotic adjustment aimed at maintaining leaf turgor during this particular stress condition. Additionally, the metabolite profile of plants subjected to combined drought and heat was more similar to that of heat-treated plants than to that of the control or drought-treated plants (Jin *et al.*, 2015).

Based on the findings above reported, it is expected that the impact of combined N and water deficit on morphophysiological traits, anatomy, macronutrient content, and amino acids profile may be different from the one observed in plants grown under single exposure to N or water deficit. Therefore, due to the increased frequency of the co-occurrence of N and water deficit under field conditions, and the scarce literature concerning the subject, it is important to understand how these traits are affected in tomato plants subjected to the co-exposure to both deficits, which was the aim of this study. To that end, anatomical and morphological traits [hypocotyl, phloem and xylem area, dry weight (root, stem, leaf, and total)], physiological parameters (chlorophyll content, photosynthetic rate, stomatal conductance, and transpiration rate), macronutrient content and partitioning (N, K, P, Ca, and Mg), N utilization efficiency as well as amino acids concentration (alanine, asparagine, aspartic acid, arginine, glutamate, glutamine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tyrosine, tryptophane, and valine) were evaluated.

2. Material and Methods

2.1. Plant material and growth conditions

The experiment was carried out in a growth chamber under controlled conditions (temperature: 25 ºC day/ 23 ºC night; relative humidity: 70%; CO_2 concentration: 400 µmol mol^{-1}; photosynthetic photon flux density (PPFD): 150 µmol m^{-2} s^{-1}; photoperiod: 16 h light [Fluorescent lamps (Osram L58W/840, Lumilux, Cool White, Munich, Germany]))

Seeds from tomato cv. Micro-Tom were sown in trays filled with commercial germination potting substrate (SIRO, Portugal). At third leaf appearance (approximately three weeks after sowing) seedlings were selected for uniformity, transferred to single-plant pots

(10.5 cm high, 10.5 cm diameter) filled with 50 g of 0.1 to 1.5 mm-grade vermiculite and into four groups for applying the studied abiotic stresses: control (CTR; 10.5 mM N; 100% field capacity), nitrogen deficit (N; 5.3 mM N), water deficit (W; 50% field capacity) and combined deficit (N+W; 5.3 mM N; 50% field capacity). Since we aimed at having a progressive water deficit, as seedling transplanting is a critical phase of the cultivation period, just prior to transplanting, all pots were irrigated to field capacity (FC), determined using the soil gravimetric water content method (Joshi *et al.*, 2021), by adding ~230 mL of 100% of the respective nutrient solution [CTR and W deficit treatments:10 mM NO_3^-; 0.5 mM NH_4^+; 1.9 mM H_2PO_4; 6.1 mM K^+; 3.6 mM Ca^{2+}; 1.6 mM SO_4^{2}; 2.5 mM Mg^{2+}; 2.6 mM Cl^-; 0.5 mM HCO_3^- (pH of 5.8 and E.C. of 2.0 dSm^{-1}); N deficit and N+W deficit treatments: 5 mM NO_3^-; 0.3 mM NH_4^+; 1.9 mM H_2PO_4; 6.1 mM K^+; 3.6 mM Ca^{2+}; 4.8 mM SO_4^{2}; 2.8 mM Mg^{2+}; 5.5 mM Cl^-; 0.5 mM HCO_3^- (pH of 5.8 and E.C. of 2.1 dSm^{-1}) both having the same micronutrients composition (35 µM Fe-EDDHA; 10 µM Mn^{2+}; 20.1 µM B; 0.9 µM Cu^{2+}; 5.0 µM Zn^{2+}; 0.5 µM MoO_4^{2-}).

Following seedling transplanting all pots were covered with black plastic to prevent evaporation and no more nutrient solution was added until the end of the experiment. Concerning further irrigation, each pot from CTR and N deficit treatment was weighted and re-watered (with distilled water) daily, to maintain FC at 100% during the experimental period, whereas in W and N+W deficits treatment no additional irrigation was supplied until the day 10, resulting in a progressive decrease of substrate FC. At that point, three plants from each treatment were randomly collected. The average fresh weight of these plants (per treatment) was subtracted from the total pot weight (per treatment) to calculate the actual weight of the water/nutrient solution present in the pot (which corresponded to 50% FC for W deficit and N+W deficit treatments). After day 10, plants were kept at 100% or 50% FC, according to the respective treatment, for 17 more days by rewatering all treatments daily with distilled water.

2.2 Anatomical traits

At the end of the experimental period (i.e. 27 days after transplantation) the roots of six plants per treatment were carefully washed. The plants were quickly separated into roots and shoots and then immediately stored in methanol (n = 6) for subsequent analyses. For visualization of anatomical structures, thin cross-sections from the middle part of the hypocotyls were prepared by hand. The sections were placed on clean slides with a drop of distilled water. The cross sections were stained with 2% phloroglucinol (2% dissolved in 96% ethanol) and 25% hydrochloric acid to visualize lignin in the samples. All microscopic slides were observed with an epifluorescence microscope Axioskop 2 plus Zeiss, equipped with an Olympus DP72 camera (Olympus, Japan), and Quick PHOTO MICRO 3.0 software was used for image analysis.

2.3. Plant growth and physiological traits

Additionally, gas exchange analysis was performed with a portable infrared gas analyzer, model LI-COR 6400 IRGA (LI-COR, Lincoln, NE, USA), at harvest, in the morning period, on the top leaflet of the youngest fully expanded leaf (n=6). The light intensity, CO_2 concentration, leaf temperature, and relative humidity conditions were 150 ± 1 µmol m^{-2} s^{-1}, 400 ± 2 µmol mol^{-1}, 25 ± 1 °C, and 67 ± 1%, respectively. The photosynthetic rate (A, µmol m^{-2} s^{-1}), stomatal conductance (gs, mol m^{-2} s^{-1}) and transpiration rate (E, mmol m^{-2} s^{-1}) were determined. Leaf chlorophyll content was measured on the youngest fully expanded leaf using the non-destructive chlorophyll meter SPAD (Konica Minolta SPAD-502 model, Minolta, Tokyo, Japan).

The six plants were then divided into roots, stems, and leaves. After carefully washing the roots, the dry weight (DW) of each organ was determined, by drying the samples for 48 h at 105 °C in a ventilated oven. Based on these data, the root-to-shoot ratio was calculated (n=6).

2.4 Digestion of plant material and estimation of minerals

For the estimation of macronutrients, the dried plant tissues were separately ground (n=3, each biological replicate consisting of a pool of two individual plants).

The N quantification was performed using the Dumas method. Briefly, about 100 mg ground dried plant tissues (roots and shoots) were weighed in an aluminum crucible and analyzed on the Dumatec™ 8000/FOSS equipment (He flow rate: 195.0 mL min^{-1} and O_2: 300 mL min^{-1}, pressure: 1200 mBar). Ethylenediaminetetraacetic acid was used as a standard for constructing the N calibration curve.

For K, P, Ca, and Mg quantification), 250 mg of the ground dried plant tissues (root and shoots) were mixed with 12.5 mL of 65% nitric acid and 2.5 mL of hydrofluoric acid in a Teflon reaction vessel (MARSXpress, CEM Corporation) and heated in a microwave digestor system (Mars ONE, CEM Corporation). Each biological replicate resulted from a pool of two plants, that were ground together. The digestion procedure was conducted in one step, consisting of 175°C, 15 min ramp, and 15 min hold at a pressure of 800 psi and power of 900 W. The resulting clear solution after the digestion procedure was then brought to 50 mL with ultrapure water for further analysis and filtered with 0.45 µm filters. Mineral concentration determination was performed using the ICP-OES Optima 7000 DV (PerkinElmer, USA) with a radial configuration.

The macronutrient accumulation (mg) was estimated as the coefficient between the total concentration of a given mineral in all plant tissues (mg/g DW) and the TDW of the

plant (g). Mineral allocation was calculated as a ratio of the root or shoot content of a given mineral by the total content of that specific mineral. N utilization efficiency (NUtE) was calculated by dividing the TDW by the N concentration quantified in the plant.

2.5. Quantification of free amino acids and proline concentration

The free amino acids were extracted using an adapted protocol from Zheng *et al.* (2017). Briefly, 800 µL of water acidified with trifluoroacetic acid (pH 3.7) was added to 20 mg (DW) of a lyophilized powder from roots or leaves (n=3, each biological replicate consisting of a pool of three individual plants). After 15 min of ultrasonic extraction at low temperature, the samples were centrifuged at 15 000 g for 20 min (at 4 ◦C). The gathered supernatant containing the target metabolites was filtered through a 0.22-micron PTFE syringe filter to amber autosampler vials.

The identification and quantification of the individual amino acids in roots and leaves (n=3) subjected to the different treatments were determined with high-performance liquid chromatography using HPLC Varian ProStar (Varian Inc., Walnut Creek, CA, USA) which comprised a ProStar 240 ternary pump, a ProStar 410 autosampler, a ProStar 330 photodiode array detector and a ProStar 363 fluorescence detector. Briefly, a concentrated 100 mg/L stock solution containing each analyzed amino acid was prepared in 0.1 M hydrochloric acid. Calibration standards (ranging from (0.8 to 20 mg/L)) were prepared in 0.1 M hydrochloric acid from the concentrated standard solution and filtered through a 0.45µm filter. Internal Standard (IS) stock solution containing homoserine and norvaline at 20 mg/L was prepared in 0.1 M chloride hydroxide, with both stock solutions being stored in a refrigerator and protected from light. Additionally, for the precolumn derivatization, three reagents were prepared: 1) reagent A: 120 µl of β-mercaptoethanol was mixed with 3 mL of IS stock solution and 500 mg of sodium tetraphenylborate, and the final volume was adjusted to 25 mL with 0.1 M borate buffer (pH 9.5); 2) reagent B: 3.5g of indoleacetic acid were mixed with 50 mL 0.1 M borate buffer (pH 9.5) with the pH being adjusted to 9.5 with 4 M sodium hydroxide, and again with 0.1 M borate buffer (pH 9.5) until a final volume of 100 mL; 3) reagent C: 225 mg 2-O-phthaldialdehyde were diluted in 5 mL of methanol and 500 µl of β-mercaptoethanol until a final volume of 50 mL made up with 0.1 M borate buffer (pH 9.5).

For quantification, the standards or samples were pre-column derivatized with 2-O-phthaldialdehyde reagent solution. The derivatization reaction was performed in the autosampler where 250 µl of reagent A and 250 µl of reagent B were added to 100 µl of standard or sample. After 3 min of reaction, 250 µl of reagent C was added waiting another 3.5 minutes before the reaction mixture was injected (10 µL) onto the HPLC where the chromatographic separation was done on a Chromolith® Performance RP18 encapped

column (4.6x100/mm, 5/µm particle size, Merck, Darmstadt, Germany) at room temperature. The fluorescence detector was set at an excitation wavelength of 356 nm and an emission wavelength of 445 nm. The chromatographic separation was obtained using a binary mobile-phase gradient elution at a flow rate of 0.8 mL m^{-1} (Table 1). Mobile phase A: 0.06 M disodium hydrogen phosphate, 0.05 M propionic acid in water-acetonitrile-dimethyl sulfoxide (91.5/6.5/2 v/v/v) adjusted to 6.6 pH (with 0.4 M sodium hydroxide); and mobile phase B: acetonitrile/methanol/dimethyl sulfoxide/water (40/33/20/7 v/v/v/v), were degassed in an ultrasonic bath and vacuum filtered through a 0.45-micron membrane filter before use. To maintain column integrity, a mixture of 60% methanol and 40% water was run for 60 min to clean the column at the end of each batch of samples. 32 Karat software version 8.0 was used for instrumental control and data acquisition.

Proline quantification was performed spectrophotometrically using around 200 mg of frozen roots or leaves (n=3), according to the method of Bates *et al.* (1973). Briefly, after reading samples absorbance at 520 nm, proline levels were calculated through a calibration curve, previously prepared with known concentrations of proline, and expressed in terms of fresh weight (fw).

Table 1 - Mobile phases and gradient elution conditions used for amino acids identification

Time (min)	Phase A (%)	Phase B (%)
0	100	0
30	90	10
40	80	20
45	75	25
80	60	40
110	15	85
120	50	50
123	95	5
125	100	0

2.6. Statistical analysis

The experimental design was a completely randomized design with twenty-one biological replicates. The number of replicates used for each specific analysis are described above. Data were subjected to analyses of variance (ANOVA), followed by Tukey's *post-hoc* test, whenever significant differences were found (p=0.05). Results were expressed as

mean ± standard error (SEM). All statistical analyses were performed in IBM SPSS Statistics 26.

3. Results

3.1. Anatomical traits

Compared to CTR plants, the hypocotyl area on the cross-section decreased in all deficit treatments ranging from 22% for the N deficit to 30% for the N+W deficit (Figure 1a). The phloem area (Figure 1b and Figure 2) was significantly affected by single W and N+W deficit, showing a decrease of 34% and 51%, respectively, compared to CTR plants. A decrease in the xylem area (Figure 1c and Figure 2) concerning CTR plants was observed for all deficit treatments and varied between 29% for the W deficit and 40% for the N+W deficit.

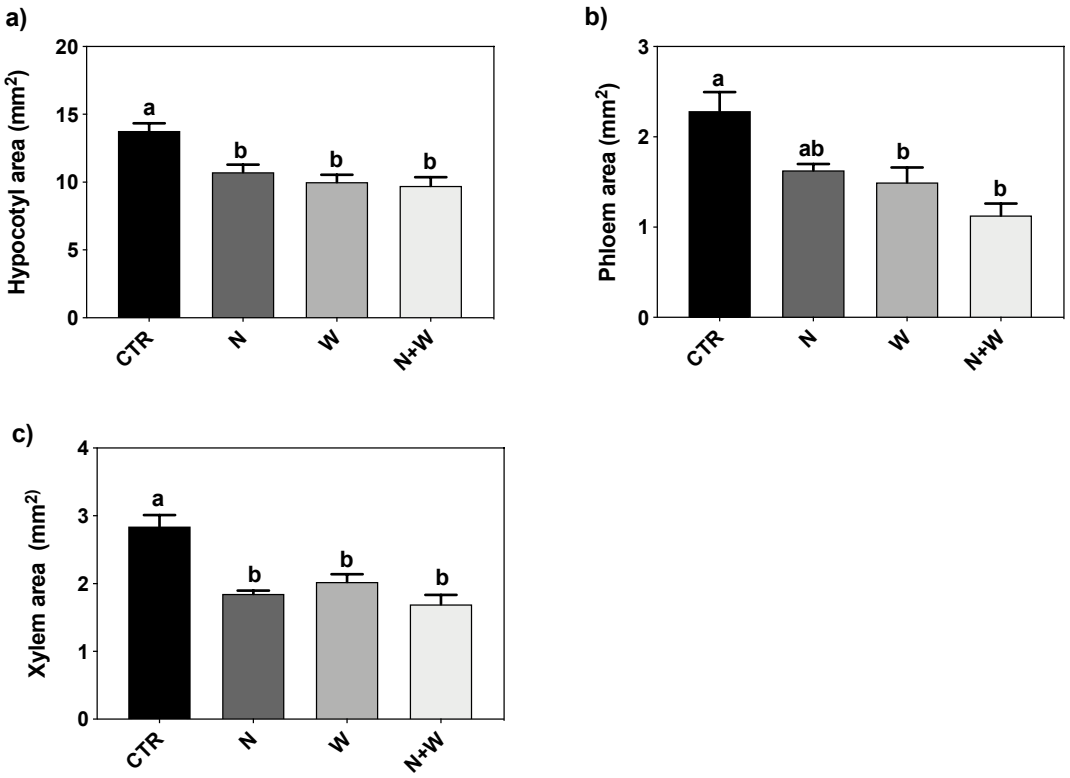


Figure 1 - Hypocotyl area **(a)** phloem area **(b)** and xylem area **(c)** on cross-section of the hypocotyl of tomato plants cv Micro-Tom grown for 27 days under control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N+W; 5.3 mM N + 50% W). Data presented are mean ± SEM (n = 3). Different letters above bars indicate significant differences according to the Tukey's HSD test (p=0.05)

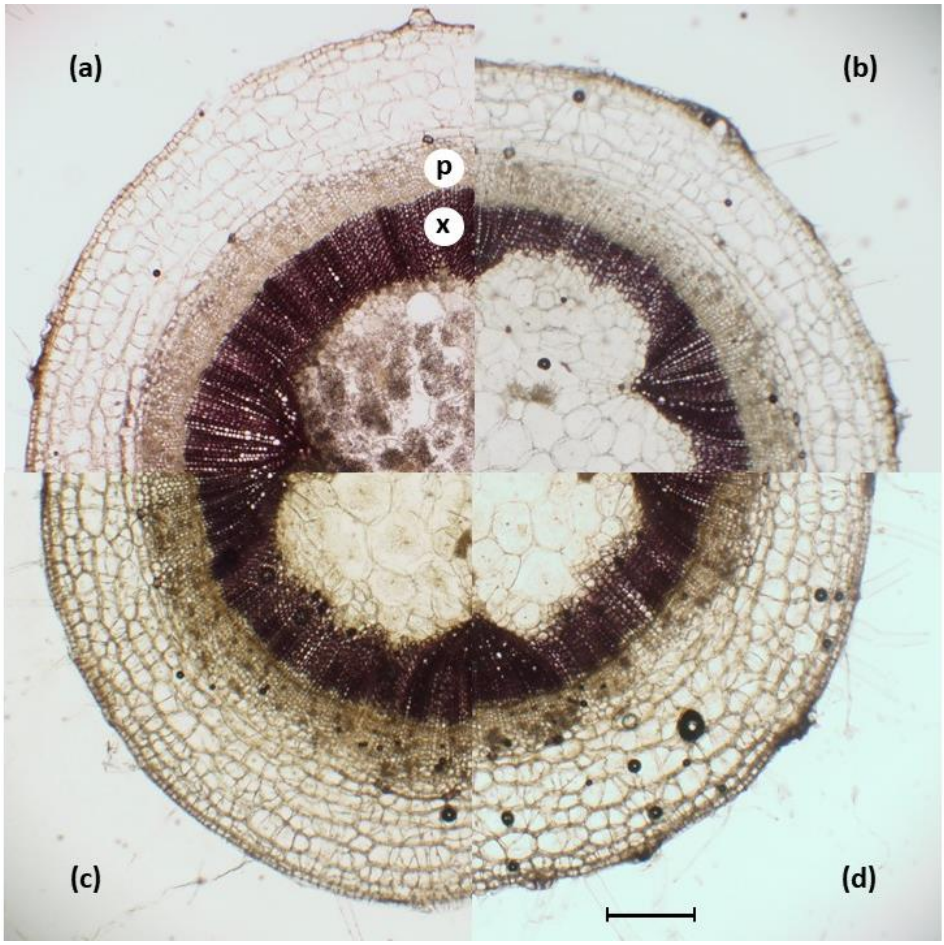


Figure 2 - Cross sections of tomato hypocotyls with histochemical visualization of lignin. Plants were grown for 27 days under control (CTR; 10.5 mM N + 100% W) **(a)**, nitrogen deficit (N; 5.3 mM N + 100%W) **(b)**, water deficit (W; 10.5 mM N + 50% W) **(c)** or combined nitrogen and water deficit (N+W; 5.3 mM N + 50% W) **(d)**. Abbreviations: p – secondary phloem, x – secondary xylem. Scale bar 500 µm

3.2. Plant growth and physiological traits

All the imposed deficits affected plant growth (Figure 3). RDW was not affected in plants subjected to W deficit, but it was decreased by 25% by N deficit and by 50% by N+W deficit, compared to CTR plants (Figure 3a). Single N deficit or W deficit decreased SDW by 31%, but this decrease was 60% for N+W deficit, compared to CTR plants, and 42%, compared to the single deficits (Figure 3b). LDW responded in a similar way as SDW when compared to CTR plants, however, no significant differences were found between N deficit and N+W deficit plants (Figure 3c). The root-to-shoot ratio was about 24% higher in W deficit plants compared to CTR plants and about 17% higher compared to N or N+W deficit plants (Figure 3d).

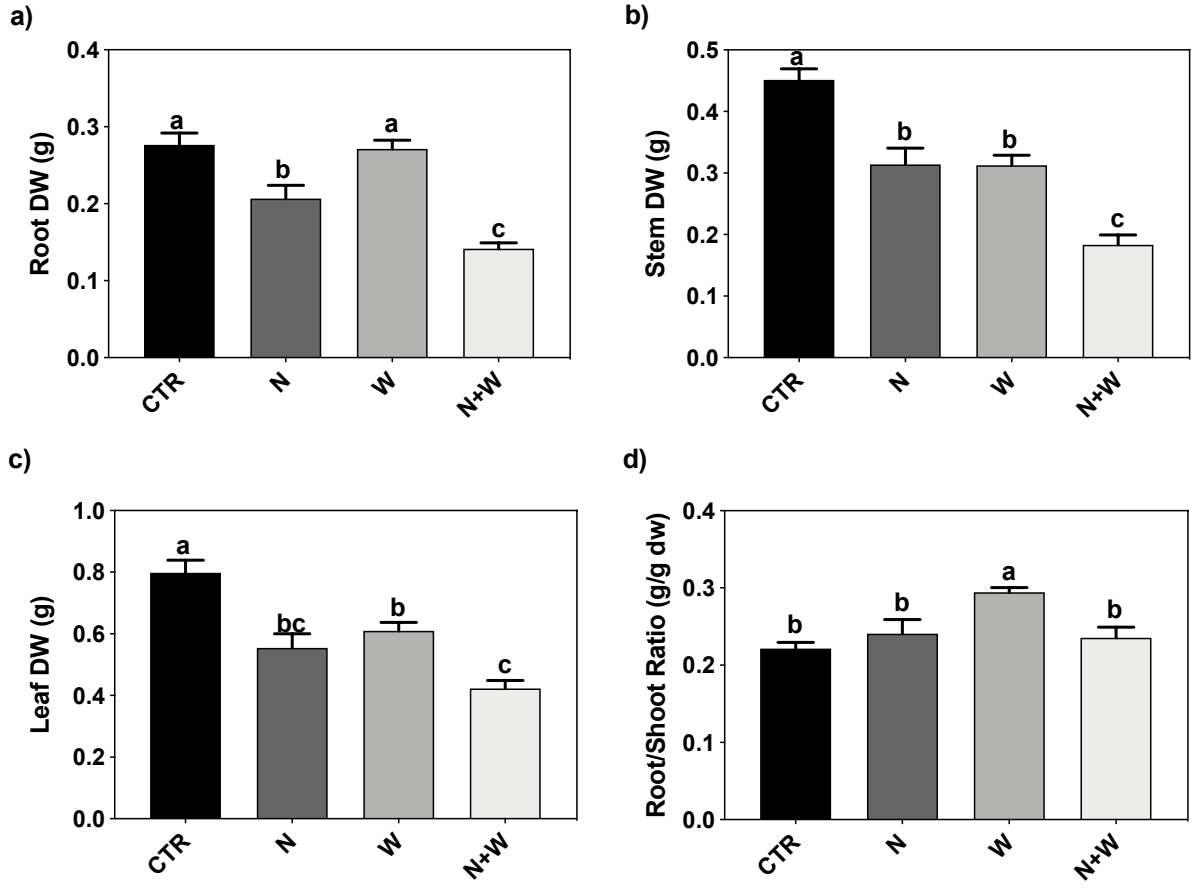


Figure 3 - Root **(a)** stem **(b)** and leaf dry weight **(c)** and root-to-shoot ratio **(d)** of tomato plants cv Micro-Tom grown for 27 days under control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N+W; 5.3 mM N + 50% W). Data presented are mean ± SEM (n = 6). Different letters above bars indicate significant differences according to the Tukey's HSD test (p=0.05).

Single N deficit and N+W deficits led to 32% lower chlorophyll content compared to CTR plants. Interestingly, plants subjected to the combined N+W deficit had intermediate levels compared to the single deficits (14% decrease compared to plants grown under the W deficit and 26% increase compared to single N deficit), showing that single N deficit had a stronger effect on these traits (Figure 4a). Photosynthetic rate responded in a similar way as chlorophyll content when compared to CTR plants, however, no significant differences were found between W deficit and N+W deficit plants (Figure 3c). On the other hand, stomatal conductance and transpiration rate were significantly affected in plants grown under combined N+W deficit, decreasing its values by up to 68% and 70%, respectively, compared to all the other experimental conditions (Figure 4c and d).

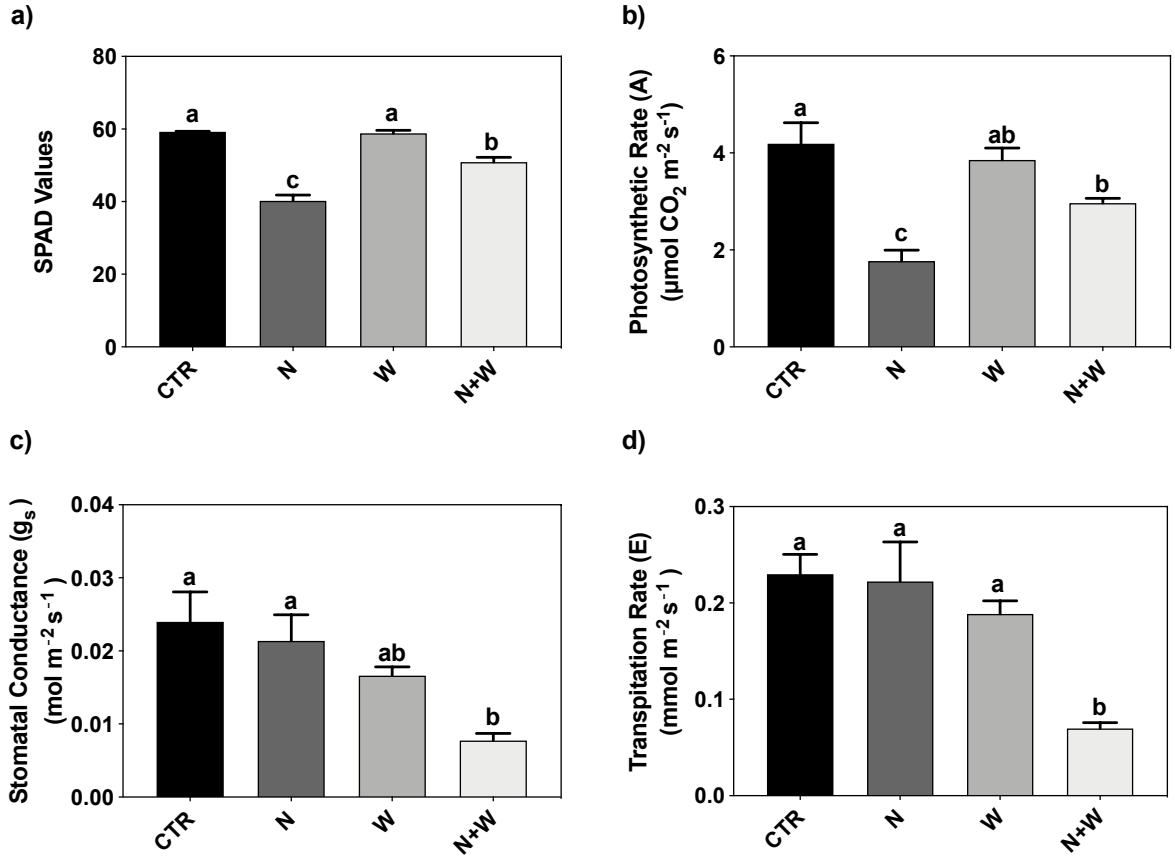


Figure 4 - Chlorophyll content (SPAD values) **(a)** photosynthetic rate (**b)** stomatal conductance **(c)** and transpiration rate (**d)** of tomato plants cv Micro-Tom grown for 27 days under control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N+W; 5.3 mM N + 50% W).Data presented are mean ± SEM (n = 3). Different letters above bars indicate significant differences according to the Tukey's HSD test (p=0.05)

3.3. Mineral composition of plant tissue

Except for N, Ca, and Mg accumulation, which were not affected by the W deficit, the accumulation of all macronutrients was affected by the imposed deficits (Figure 5). N accumulation was diminished to the same extent by N deficit or N+W deficit (up to 51%) compared to CTR or W deficit plants (Figure 5a). K accumulation was significantly lower for all deficit plants compared to CTR plants. (Figure 5b). When comparing the N+W deficit with the single deficits significantly lower levels were found: 46% for the N deficit and 58% for the W deficit (Figure 5c). P accumulation was also significantly decreased, especially under the N+W deficit, where a reduction of up to 78%, compared to all other experimental conditions, was observed (Figure 5c). Ca accumulated up to 40% less under N+W deficit compared to CTR or W deficit plants, but its accumulation was similar compared to plants grown under single N deficit (Figure 5d). Mg accumulation followed the same trend as K, being significantly reduced (up to 55%) (Figure 5e).

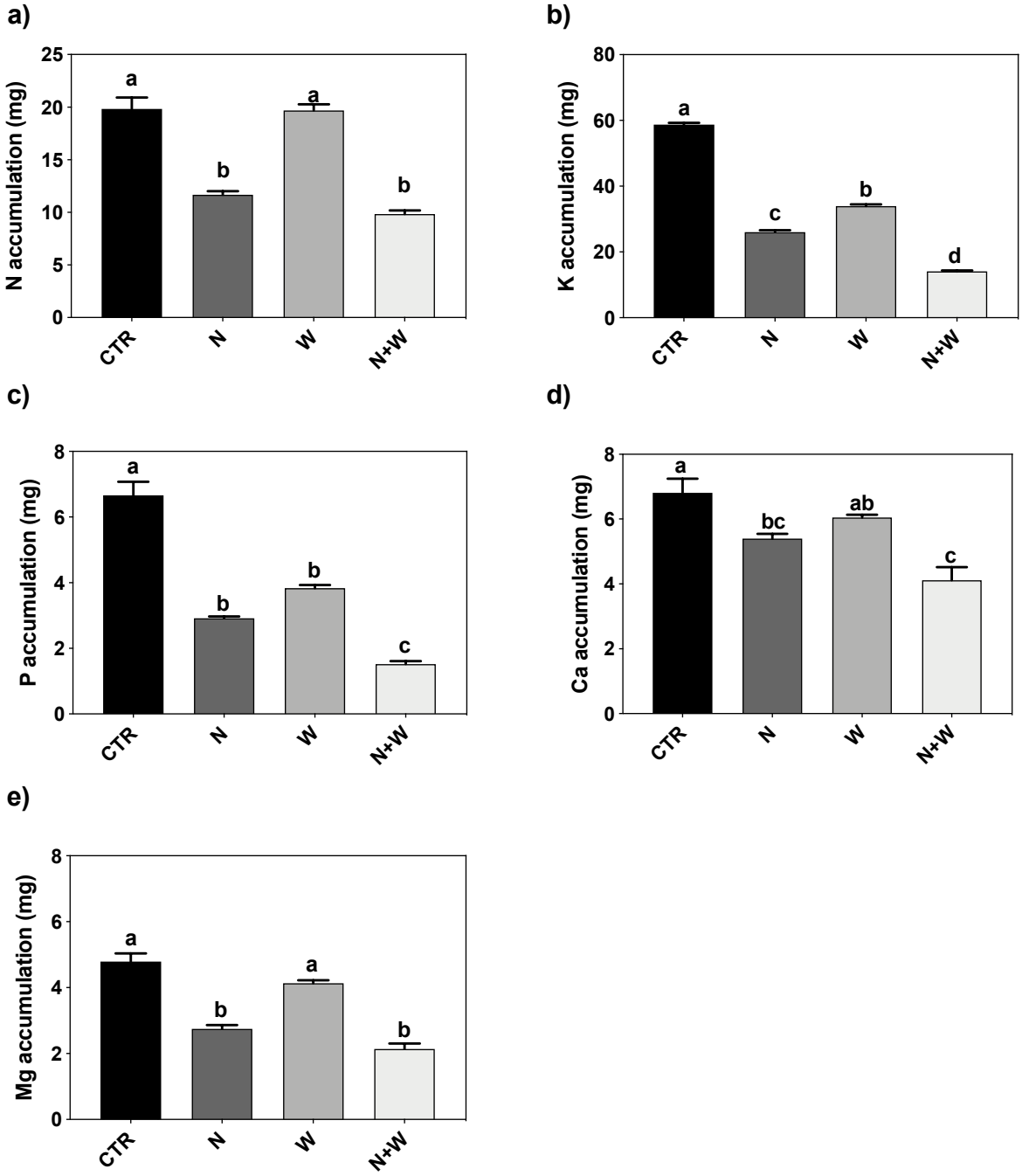


Figure 5 - Nitrogen (N) **(a)** potassium (K) (**b)** phosphorus (P) **(c)** calcium (Ca) **(d)** and magnesium (Mg) (**d)** accumulation per tomato plant cv Micro-Tom grown for 27 days under control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N+W; 5.3 mM N + 50% W). Data presented are mean ± SEM (n = 3). Different letters above bars indicate significant differences according to Tukey's HSD test (p=0.05).

The imposed deficits affected the partitioning of all macronutrients except for N (Figure 6a). Plants grown under the N+W deficit increased K allocation to shoot up to 23% in comparison to plants grown under single deficit (Figure 6b). However, for P, an increased

allocation to roots of up to 16% was observed in N-deficit plants compared with the other treatments, while for Ca an increased allocation to shoots of up to 9% was observed in N-deficit plants compared with the other treatments (Figure 6c and d). On the other hand, Mg allocation to roots increased by up to 85% in plants grown under W deficit compared with all the other treatments (Figure 6e).

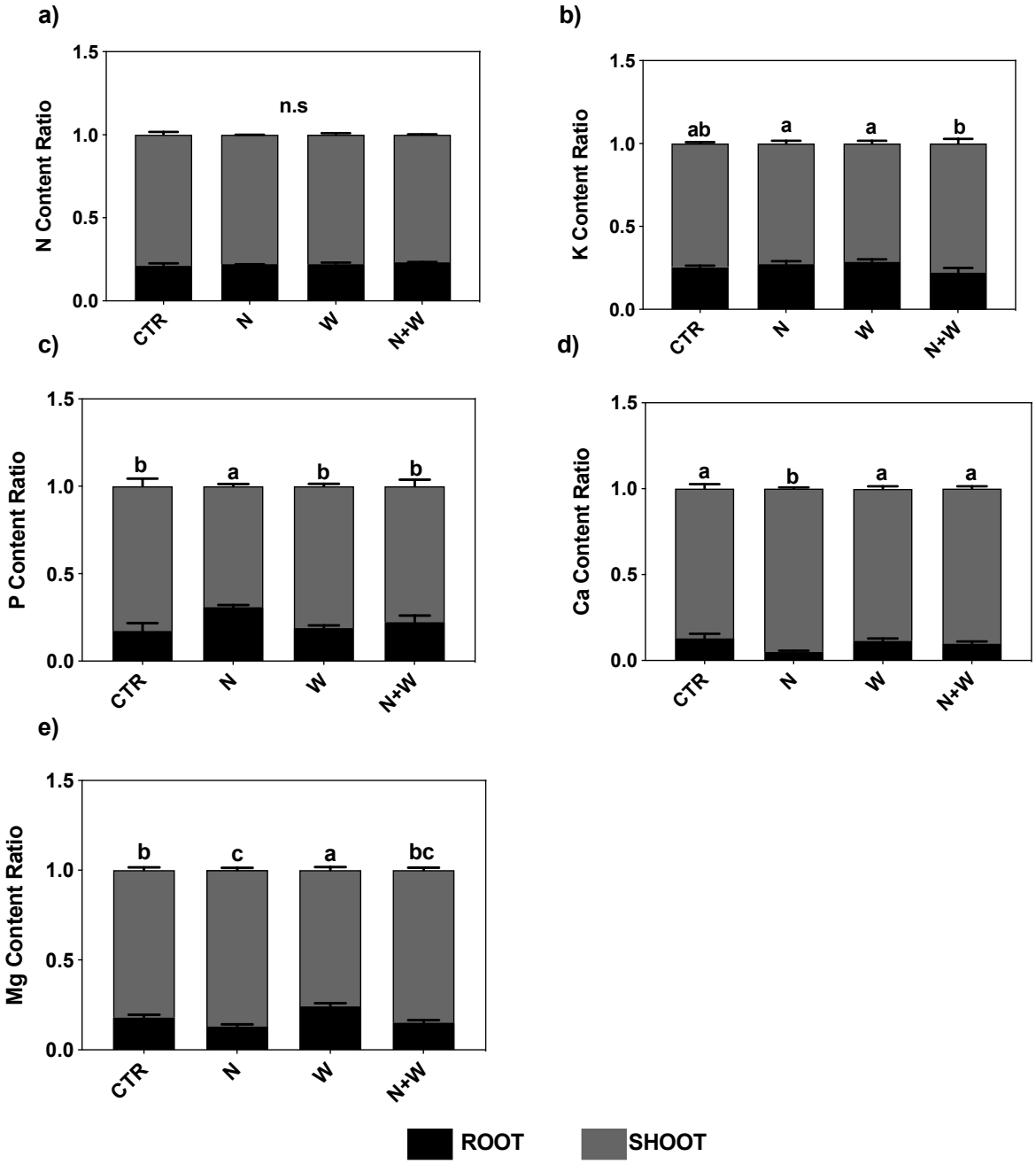


Figure 6 - Nitrogen (N) **(a),** potassium (K) (**b)** phosphorus (P) **(c)** calcium (Ca) **(d),** and magnesium (Mg) (**d)** content ratio of tomato plants cv. Micro-Tom grown for 27 days under control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N+W; 5.3 mM N + 50% W).Data presented are mean ± SEM (n = 3). Different letters above bars indicate significant differences according to the Tukey's HSD test (p=0.05

NUtE was significantly lower for plants grown at W or N+W deficit (up to 22%) (Figure 7).

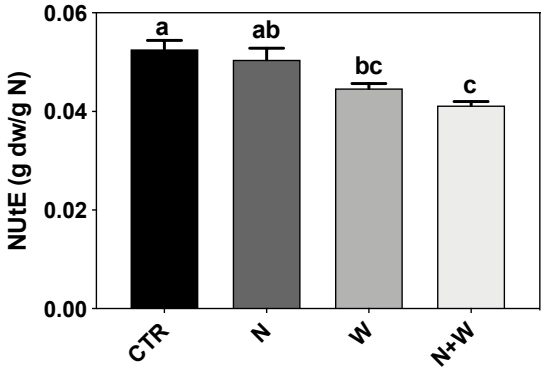


Figure 7 - Nitrogen utilization efficiency (NUtE) of tomato plants cv. Micro-Tom grown for 27 days under control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N+W; 5.3 mM N + 50% W). Data presented are mean ± SEM (n = 3). Different letters above bars indicate significant differences according to the Tukey's HSD test (p=0.05).

3.4. Free amino acids and proline composition of plant tissue

In general, the amino acid concentration was lower in roots than in leaves. In roots, from the 16 quantified amino acids, only tryptophane was not significantly affected by the imposed deficits (Table 1). Most of them were significantly lower when plants were grown under single N deficit than when they were grown under the N+W deficit [alanine (52%), asparagine (89%), aspartic acid (54%), glutamine (75%), leucine (17%), methionine (29%), phenylalanine (29%), proline (78%), serine (45%), threonine (24%), tyrosine (75%) and valine (26%)]. Interestingly, in general, N+W deficit plants had an amino acid profile like W deficit plants, except for arginine, alanine, tyrosine, and phenylalanine, where compared to this group an increase of 25%, 51%, 81%, and 23%, respectively, was observed (Table 1).

In the leaves, only 5 amino acids were not significantly affected by the imposed deficits (isoleucine, leucine, methionine, phenylalanine, and threonine; Table 1). Comparing CTR plants to plants grown under N deficit or N+W deficit, the levels of asparagine (up to 75%), aspartic acid (up to 31%), glutamine (up to 40%), serine (up to 34%), and valine (up to 35%) were significantly lower, while proline concentration was significantly higher (up to 77%) (Table 1). On the other hand, single W deficit only affected proline and tryptophane, which were significantly higher (96% and 14%, respectively) compared to CTR plants. In contrast to roots, leaves of plants grown under N+W showed, in general, an amino acid profile like both single deficits, except for asparagine, aspartic acid, arginine, glutamine, and serine which were more similar to N deficit leaves (Table 1).

Table 2 - Free amino acids and proline concentration of tomato plants cv Micro-Tom grown for 27 days under control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N+W; 5.3 mM N+ 50% W).

	ROOT					LEAVES				
Amino acid (µg/g DW)	**CTR**	**N deficit**	**W deficit**	**N+W Deficit**	***p*-value**	**CTR**	**N deficit**	**W deficit**	**N+W Deficit**	***p*-value**
Alanine	73.0±5.4^{a}	28.3±6.8^{c}	39.2±6.0^{b}	59.3±7.5^{a}	**0.0051**	263±25^{ab}	144±24^{b}	317±36^{a}	196±29^{ab}	**0.0130**
Asparagine	135±12^{b}	47.4±4.1^{c}	406±5.0^{a}	446±46^{a}	**<0.0001**	66.3±6.3^{a}	16.3±1.2^{b}	63.2±6.3^{a}	22.6±5.9^{b}	**0.0002**
Aspartic Acid	412±44^{a}	204±21^{b}	359±14^{a}	440±15^{a}	**0.0009**	858±23^{a}	595±34^{b}	875±59^{a}	595±20^{b}	**0.0007**
Arginine	38.6±5.4^{bc}	29.1±3.3^{c}	51.1±5.7^{b}	63.8±3.4^{a}	**0.0035**	87.3±9.3^{ab}	49.0±7.9^{b}	112±8.7^{a}	61.2±9.5^{b}	**0.0042**
Glutamate	369±27^{a}	206±20^{b}	214±5.7^{b}	304±43^{ab}	**0.0083**	469±31^{ab}	346±35^{a}	557±44^{b}	383±47^{ab}	**0.0236**
Glutamine	541±40^{b}	228±23.6^{c}	911±3.3^{a}	906±63^{a}	**<0.0001**	428±21^{a}	260±14^{b}	507±23^{a}	257.0±25^{b}	**<0.0001**
Isoleucine	77.9±4.2^{a}	45.10±6.8^{b}	76.0±4.5^{a}	57.3±3.5^{ab}	**0.0041**	107±9.9	78.2±8.1	100±0.8	93.6±5.8	0.0958
Leucine	102±9.8^{a}	65.6±5.8^{b}	94.8±7.0^{a}	78.8±5.1^{a}	**0.0269**	116±20	123±7.9	175±17	149±10	0.0749
Methionine	45.4±2.2^{ab}	36.5±1.8^{b}	50.8±1.7^{a}	51.4±3.3^{a}	**0.0068**	39.6±3.0	38.0±2.2	40.2±0.8	39.3±3.2	0.9299

Phenylalanine	72.4±2.2^{ab}	59.1±1.6^{c}	68.1±0.7^{bc}	83.5±4.2^{a}	**0.0009**	144±13	133±7.5	155±20	137±16	0.7404
Proline	69.5±12^{b}	76.7±20^{b}	427±35^{a}	341±18^{a}	**<0.0001**	118±12^{c}	175±4.1^{b}	232±2.4^{a}	209±21^{ab}	**0.0009**
Serine	126±7.1^{a}	68.1±5.8^{c}	102±3.9^{b}	123±1.6^{ab}	**0.0001**	173±6.9^{a}	121±7.1^{b}	200±3.5^{a}	115±10^{b}	**<0.0001**
Threonine	110±7.3^{a}	75.3±5.4^{b}	97.9±2.5 ^{a}	98.7±4.7 ^{a}	**0.0104**	121±7.4	93.3±9.5	108±14	110±13	0.4546
Tyrosine	18.5±1.1^{a}	5.24±0.5^{c}	11.8±2.0^{b}	21.3±0.2^{a}	**<0.0001**	147±14^{ab}	96.2±10^{b}	166±12^{a}	116±15^{ab}	**0.0226**
Tryptophane	99.2±4.2	106±3.0	104±4.1	104±8.9	0.8656	298±10^{b}	304±3.7^{ab}	341±1.4^{a}	274±13^{b}	**0.0038**
Valine	91.2±0.9^{a}	60.9±3.4^{b}	83.3±4.7^{a}	82.4±5.1^{a}	**0.0032**	155±14^{a}	98.6±4.6^{b}	142±4.7^{ab}	106±13.7^{b}	**0.0111**

Data presented are mean ± SEM (n = 3). Different letters indicate significant differences according to the Tukey's HSD test (p=0.05)

4. Discussion

To the best of our knowledge, this is the first study that compared the anatomical traits, mineral accumulation and partitioning, and amino acids concentration of plants subjected to single N deficit or W deficit with the response of those plants subjected to the combination of both deficits at the same time.

Drought and N deficiency are major environmental factors affecting plant growth and productivity (Fernandes *et al.*, 2022). Various anatomical changes such as reduction in the size of the phloem, and xylem, reduced thickness of cortical cell layer as well as surface structures, including epidermal cell length, have been observed as a consequence of drought stress in plants (Shafqat *et al.*, 2021). Here, we observed a decreased cross-sectional hypocotyl area, as well as reduced xylem and phloem area in plants grown under single N or W deficit, and in those plants that were exposed to N+W deficit. The response in stem diameter under water deficit has been studied frequently, and the data shows that under drought stress, the stem may shrink (Lovisolo and Schubert, 1998). However, stem diameter exhibits diurnal variation, and it also depends on plant evaporative demand (Fernández and Cuevas, 2010). Size reduction in these anatomical traits is the direct response to stress conditions and plays a functional role in acclimation to drought. For instance, plants with decreased xylem vessel diameter showed a higher survival rate in comparison to plants with enlarged xylem vessel diameter under drought conditions (Shafqat *et al.*, 2021; Qaderi *et al.*, 2019). Furthermore, xylem thickness and xylem cell layers are reduced under low N supply (Song *et al.*, 2019). Moreover, low N supply together with drought stress was shown to cause even more decrease in xylem thickness and number of xylem cell layers of *Populus alba*, when compared to control conditions or drought treatment with adequate N supply (Song *et al.*, 2019). However, in our study, the anatomical traits were affected to the same extent by the three different deficits.

Several studies demonstrated that water and/or N deficit severely affect tomato performance leading to a growth reduction (recently reviewed by Machado et al, 2022). In our study, both deficits, either individually or in combination, decreased the LDW and SDW, with the combination of both resulting in a larger reduction (Figure 3b and c). RDW was also decreased in N and N+W deficit, but not in W deficit (Figure 3a). Plants subjected to W deficit increased root-to-shoot ratio, reflecting a higher partitioning towards the roots (Figure 3d). Indeed, due to the stronger effect of water deficit on shoots than on root growth, the root-to-shoot ratio tends to increase with drought (Du *et al.*, 2018; Moles *et al.*, 2018). This is a common mechanism used by plants to cope with water shortage and might have contributed to a better performance of these plants compared to those under N+W deficit, as demonstrated by higher chlorophyll content and photosynthetic capacity (Figure 3a, 4a, b). In

contrast, plants under N+W did not manage to develop their root system to reach the water available in deeper substrate layers and probably were subjected to a more severe water deficit, closing the stomata as a mechanism to decrease transpiration rate (Figure 3a, 4b, c, d). Indeed, stomatal closure is one of the first reactions to drought stress in most plants, being used as a mechanism to prevent water loss from transpirational pathways (Pirasteh-Anosheh *et al.*, 2016). On the other hand, N-deficient plants reduce their photosynthetic capacity due to the N partitioning dependence of the photosynthetic machinery (Bassi *et al.*, 2018). In our study, plants subjected to N deficit showed the lowest photosynthetic rate, probably caused by N remobilization, a common process occurring under N deficiency (Sakuraba, 2022), which resulted in lower chlorophyll content (Figure 4a and b). However, plants under N+W deficit had a different N remobilization strategy as in this case the decrease in the chlorophyll content was smaller which consequently led to a higher photosynthetic rate compared to single N deficit (Figure 4a, b).

In general, plants grown under all types of deficit had lower macronutrient accumulation compared to CTR conditions, with this decrease being more pronounced in plants subjected to N deficiency (single and combined) (Figure 5). This probably occurred due to the underdevelopment of the plant's root system compared to plants grown under CTR or W deficit (Figure 3a). Roots have an important role in water and nutrient uptake, with both N or water deficits being primarily perceived in this structure (Tajima, 2021). This also explains why plants subjected to W deficit managed to maintain N accumulation compared to CTR plants (Figure 3a and 3d and Figure 5a). This higher accumulation of N by plants under the W deficit may have facilitated coping with this deficit by producing defense molecules such as osmolytes. In fact, the NO_3^- molecule itself is known to have an osmoregulatory function as demonstrated by Handa *et al.* (1983) who attributed 4.5% of the total osmotic potential to this molecule in tomato plants. On the other hand, NO_3^- uptake has been shown to stimulate K^+ uptake, suggesting that the NO_3^- ion might be used as an accompanying anion during K^+ uptake and/or transport (Zhang *et al.*, 2010). This hypothesis is in accordance with our results as in plants under NO_3^- deficiency (N and N+W deficit) K accumulation was greatly decreased (Figure 5b). However, plants grown under N+W deficit altered K partitioning towards the shoots (Figure 6b). K has a known effect on osmoregulation being responsive for photosynthesis regulation through osmotic balances and changes in guard cell turgor, affecting stomatal and mesophyll conductance (Tränkner *et al.*, 2018). This agrees with the decrease in stomatal conductance found in plants grown under the N+W deficit (Figure 4c). On the other hand, P has poor mobility in soil not being easily accessible by plant roots (Holford, 1997). This lower accessibility is further aggravated by a smaller root system and lower soil moisture which impairs nutrient uptake (Hu *et al.*, 2007; Wang *et al.*, 2006). P

accumulation was lower in all deficits, especially under the N+W deficit where plants showed the lower RDW (Figure 3a and 5c). Ca acts as a secondary messenger in response to stress signals and its transport to the shoot depends on high transpiration rates (Gilliham *et al.*, 2011; Kumar *et al.*, 2014). In this study, plants grown under N deficiency may have activated that defense mechanism, however, when N deficit was applied in combination with W deficit, plants were unable to translocate Ca to the shoots due to a lower transpiration rate (Figure 4d and 6d). Mg is pointed out as having a role in increasing root growth and consequently root uptake of nutrients and water (Li *et al.*, 2020). Therefore, the lower partitioning of Mg to shoots in plants subjected to W deficit may be the cause of the improved root system of these plants, which might have improved the water uptake and consequently decreased the negative effects of water deficit (i.e. maintenance of chlorophyll content, photosynthesis, stomatal conductance, and transpiration rate) (Figure 3a, 2 and 6e). Interestingly, Mg partitioning to shoots is increased in N deficit (Figure 6e). N and Mg might act antagonistically because it was already found that Mg is enhanced by N deficiency and reduced by N supply in citrus plants (Heo and Park, 2022).

Understanding how plants accumulate N is important to comprehend how they respond to a specific stress. For example, in our study plants grown under N deficiency prioritized N towards growth (higher NUtE), while plants subjected to W deficit (single and combined) seem to have utilized N to produce drought-related compounds (Figure 7). Amino acids are the first stable products of inorganic N assimilation consequently decreasing under N deficiency (Nunes-Nesi *et al.*, 2010). This tendency was also observed in our study, as plants under N deficit generally showed a lower free amino acids concentration, especially in the roots (Table 1). When the N deficit was applied in combination with the W deficit, this trend was only observed in the leaves. On the other hand, free amino acids are known to act as osmolytes helping plants to increase their osmotic potential under drought stress (Ozturk *et al.*, 2021). One of the most abundant amino acids is glutamate which functions as an important precursor for other amino acids such as proline, one of the most conspicuous osmolytes (Alfosea-Simón *et al.*, 2020; Kumar *et al.*, 2017). Additionally, besides its function as an osmoprotectant, proline is also known for acting on the scavenging of drought-induced reactive oxygen species (Sharma *et al.*, 2011). In this study, proline synthesis was higher upon W deficit (single and combined) and seemed to mainly have occurred in the roots. This increase was accompanied by a lower glutamate concentration when plants were subjected to either one of the single deficits but not to the combination of both (Table 1). Additionally, both W and N+W deficits increased glutamine synthesis, contrarily to single N deficit where a decrease was found. Furthermore, arginine was specifically increased in the roots of plants grown under the N+W deficit (Table 1). Arginine is the amino acid with the highest N/C ratio with glutamate being an

intermediate product in its biosynthetic pathway (The *et al.*, 2020; Winter *et al.*, 2015). Arginine metabolism plays a key role in N distribution and recycling in plants but also acts as a precursor of the biosynthesis of polyamines and nitric oxide, which are important messengers involved in almost all physiological and biochemical processes related to plant acclimation to stress (Winter *et al.*, 2015). Although plants grown under single W deficit did a higher investment in glutamine and proline biosynthesis to increase their osmotic potential, plants grown under N+W deficit used glutamate to produce both amino acids and arginine, possibly as a strategy to increase their osmotic potential but also to improve N distribution and recycling (Table 1).

Another important amino acid for N transport and storage is asparagine which was reported to increase under drought conditions (Lea *et al.*, 2007). In our study, asparagine was also significantly increased in the roots of plants subjected to W deficit (single or combined) (Table 1). In most plant species, N partitioning within the plant occurs in the form of amino acids with root-synthesized amino acids being transported in the xylem to mature source leaves, where they can be used as an N source for the building of the photosynthetic apparatus (Perchlik and Tegeder, 2018). Asparagine has been pointed out as being the major N transport compound in the xylem from the root to the leaves (Lea *et al.*, 2007). Therefore, the observed increase in root-synthesized arginine and asparagine for plants subjected to N+W may explain why these plants were able to mitigate the detrimental effects of N deficiency on the photosynthetic machinery (Figure 4a and b).

Levels of alanine, tyrosine, and phenylalanine were also specifically higher in the roots of plants grown under the N+W deficit. These amino acids, together with arginine were previously reported as acting against osmotic stress in tolerant and sensitive varieties of rice (Matsunami *et al.*, 2020). Muscolo *et al.* (2015) also reported a large increase in phenylalanine and tyrosine in two drought-sensitive accessions of lentils which the authors related to the initiation of lignification, a mechanism used to alleviate damage from drought stress at the root level. Many secondary metabolites are synthesized as a mechanism to protect the cells from oxidative damage, with most of them being derived from the aromatic amino acids phenylalanine and tyrosine through the phenylalanine ammonia-lyase (PAL) pathway. These results suggest that plants under N+W deficit specifically changed their amino acid pool to cope with both abiotic stresses.

5. Conclusions

This study suggests that the mechanisms involved in tomato plants' response to combined N+W deficit are different and cannot be predicted from the single deficits. Plants grown under combined N+W deficit responded mainly through an increased root synthesis of

several amino acids. Arginine and asparagine increase at the root level contributed to lower N remobilization from the photosynthetic machinery as demonstrated by the lower reduction in chlorophyll content and photosynthetic rate in comparison with plants under single N deficit. Additionally, plants grown under combined N+W deficit increased the reallocation and synthesis of osmolytes such as K^+ and proline, respectively, which contributed to stomatal closure and a lower transpiration rate compared to plants under single deficit. Other amino acids related to osmoregulation such as alanine, tyrosine, and phenylalanine were also specifically higher in the roots of plants grown under N+W deficit. On the other hand, under single W deficit plants invested relatively more in the root system stimulating the uptake of water and nutrients compared to plants grown under combined N+W deficit, being able to maintain physiological processes such as photosynthesis.

CHAPTER 3

Exploring silicon and genotypic variation as strategies to cope with combined nitrogen and water deficit

Sub-chapter 3.1

The effect of silicon on the antioxidant system of tomato seedlings exposed to individual and combined nitrogen and water deficit

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The effect of silicon on the antioxidant system of tomato seedlings exposed to individual and combined nitrogen and water deficit

Abstract

Exploring sustainable strategies for improving crop water and nitrogen use efficiency is essential. Silicon (Si) has been reported as a beneficial metalloid for plants since it alleviates several abiotic stresses (including drought) by triggering the plants´ antioxidant system. However, its role in mitigating the negative impact of nitrogen (N) deficit alone or when combined with water (W) deficit is not well studied. This study applied 0 or 2 mM of Na_2SiO to three-week-old tomato cv. Micro-Tom seedlings that were grown under the following conditions: control (CTR; 100%N+100% Field Capacity), N deficit (N; 50% N + 100% Field Capacity), water deficit (W; 100% N + 50% Field Capacity) or combined stress (N+W; 50% N + 50% Field Capacity. The Si effect on tomato plant growth depended on the type of stress. Si could only alleviate stress caused by the N+W deficit resulting in a higher root dry weight (by 28%), total dry weight (by 23%), and root length (by 37%). Alongside this, there was an increase in the antioxidant (AOX) system activity with the root activity of the studied AOX enzymes APX and CAT being enhanced by 48% and by 263%, respectively. Si application also enhanced AOX enzyme activity when tomato plants were subjected to individual deficits but to a lesser extent. In conclusion, Si-treated tomato plants could efficiently modulate their AOX networks in a situation of combined N and water limitation, thus mitigating some of the adverse effects of this combined stress.

1. Introduction

Considering the increasing demand for food and the need for more environmentally friendly agriculture (Jhariya *et al.*, 2019), finding alternative and sustainable solutions to ensure the efficient use of water and nitrogen (N) is a great priority, as these two resources have a huge impact on crop yield (Fernandes *et al.*, 2022). Besides the direct consequences of climate change on water availability, this also brings an additional threat to food security as several studies have demonstrated that the continuous increase of the atmospheric CO2 concentration negatively impacts the N status of most C3 plants, leading to lower yield and nutritional crop value (Gojon, 2017).

Numerous morphological, physiological, and metabolic changes are common in plants subjected to N or drought stress (reviewed by Machado *et al.*, 2022). Commercial tomato

cultivars (*Solanum lycopersicum*) are quite sensitive to these deficits at all stages of plant development (Du *et al.*, 2018; Heuvelink *et al.*, 2020) meaning that large amounts of fertilizers and irrigation must be used during their production cycle (Sandhu *et al.*, 2021). Moreover, considering the increased consumption of fresh tomato and tomato-derived products and the economic importance of this crop worldwide (Costa and Heuvelink, 2018), it is of high importance to study its responses to these abiotic stresses.

A sustainable method that is receiving great attention in recent years, due to its role on alleviating the negative effects of several abiotic stresses, is the application of silicon (Si) (Bokor *et al.*, 2021; Nunes da Silva *et al.*, 2022). For instance, supplementation with Si has been adopted as an effective strategy for relieving drought stress and improving the drought resistance of several crops (recently reviewed by Bokor *et al.*, 2021; Malik *et al.*, 2021; Thorne *et al.*, 2020; Verma *et al.*, 2022; Wang *et al.*, 2021). In contrast, knowledge about the role of Si in alleviating nutrient deficiencies is far scarcer, as pointed out by Ali *et al.* (2020) and Pavlovic *et al.* (2021). In recent studies it was shown that the application of Si had a positive impact on N uptake, assimilation and remobilization, which in turn can contribute to improving N utilization within plant tissues (Minden *et al.*, 2021; Pavlovic *et al.*, 2021). Nevertheless, most of this research was conducted on Si accumulating species such as rice and wheat. Concerning Si non-accumulating species (Si excluders), such as tomato, which exhibit passive absorption and much less accumulation of Si (Hoffmann *et al.*, 2020; Liang *et al.*, 2007), the literature is limited.

One of the key mechanisms proposed by Liang *et al.* (2007) for the alleviation of abiotic stress in higher plants using Si has to do with the stimulation of the antioxidant system (AOX). Previous reports demonstrated that exogenously applied Si can enhance the AOX system under drought stress, including in tomato plants, where a Si-mediated significant decrease of reactive oxygen species (ROS) was observed (Shi *et al.*, 2016). In the same study, the authors reported an increase in the activity of the AOX enzymes superoxide dismutase (SOD) and catalase (CAT) as well as in the concentration of the non-enzymatic AOX molecules ascorbic acid and glutathione. For wheat and maize, under drought stress, it has also been shown that Si decreases oxidative damage, ROS, phenolics concentration, and ascorbate peroxidase (APX) activity, but increases SOD and CAT (Gong *et al.*, 2005; Parveen *et al.*, 2019; Sattar *et al.*, 2020; Xu *et al.*, 2017). Peroxidase (POD) showed some variability in responses: while some authors found no significant differences (Gong *et al.*, 2005), others noticed a decrease (Xu *et al.*, 2017) or reported an increase in the activity of this enzyme (Parveen *et al.*, 2019; Sattar *et al.*, 2020). Concerning the effects of Si on crops grown at N deficit, most studies focus on the role of Si on N uptake and transport directly and less on the AOX system, which needs further investigation (Ali *et al.*, 2020). Additionally, to the best of our knowledge, so far

there are no studies on the effects of Si on combined N and water deficit, which is surprising given that often plants do not face only one individual abiotic stress, but rather a combination of them. This requires that more research explores the complex acclimation mechanisms, which cannot be predicted from the responses obtained when stressors are applied individually (García-Martí *et al.*, 2019; Hussain *et al.*, 2018; Pandey *et al.*, 2015;). Thus, we hypothesize that tomato responses to Si supplementation under combined N and W deficit cannot be directly predicted from the responses obtained for the single stresses and that the AOX system plays an important role in the defence mechanism employed by tomato.

This study aimed to: (i) evaluate if Si supplementation can be used as a strategy to help tomato plants to cope with N deficit alone and/or when combined with water deficit; (ii) understand the impact of Si on the AOX responses (AOX activity, secondary metabolism responses and enzymatic activity) in plants subjected to single or combined N and W deficit.

2. Material and Methods

2.1. Plant material and growth conditions

The experiment was carried out in a growth chamber under controlled environmental conditions (temperature: 25 ºC day/ 23 ºC; night; relative humidity: 70%; photosynthetic photon flux density (PPFD): 150 µmol m^{-2} s^{-1}; photoperiod: 16 h light).

At the stage of third leaf appearance (approximately three weeks after sowing) seedlings from tomato plants cv. Micro-Tom were selected for uniformity and transplanted to single-plant pots (10 cm high, 10 cm diameter) filled with 0.1 to 1.5 mm-grade vermiculite (50 g). At this stage plants were divided into eight experimental groups, resulting from the combination of: four levels of deficit conditions [control (CTR; 10.5 mM N; 100% field capacity), N deficit (N; 5.3 mM N), water deficit (W; 50% field capacity) and combined deficit (N+W; 5.3 mM N; 50% field capacity] and two levels of Si supply [0mM or 2 mM of sodium silicate (Na_2SiO_3) solution (27% SiO_2 (w/v) dissolved in 14% NaOH (w/v); Sigma).

Since we aimed at having a progressive water deficit, as seedling transplanting is a critical phase of the cultivation period, just prior to transplanting, all pots were irrigated to field capacity (FC), determined using the soil gravimetric water content method (Joshi et al., 2021), by adding ~230 mL of the nutrient solution supplied with 0 or 2 mM of Na_2SiO_3, depending on the treatment. Two types of nutrient solutions were used. A full nutrient solution was applied to the CTR and W deficit plants [10 mM NO_3^-; 0.5 mM NH_4^+; 1.9 mM $H_2PO_4^-$; 6.1 mM K+; 3.6 mM Ca^{2+}; 1.6 mM SO_4^{2-}; 2.5 mM Mg^{2+}; 2.6 mM Cl^-; 0.5 mM HCO_3^- (pH 5.8 and electrical conductivity – EC – of 2.0 dSm^{-1})]. Plants subjected to single N deficit or combined N+W deficit were irrigated with a nutrient solution containing half the amount of N [5 mM NO_3^-; 0.3 mM NH_4^+; 1.9 mM $H_2PO_4^-$; 6.1 mM K+; 3.6 mM Ca^{2+}; 4.8 mM $SO_4$2-; 2.8 mM Mg^{2+}; 5.5 mM Cl^-; 0.5

mM HCO_3^- (pH 5.8 and EC of 2.1 dSm^{-1})]. Both nutrient solutions had the same composition in terms of micronutrients (35 µM Fe-EDDHA; 10 µM Mn^{2+}; 20.1 µM B^{3+}; 0.9 µM Cu^{2+}; 5.0 µM Zn^{2+}; 0.5 µM MoO_4^{2-}). Following seedling transplanting all the pots' surface was covered with black plastic to prevent evaporation.

During the experimental period each pot from the CTR and N treatment (with or without Si supplementation), was weighted and re-watered (with distilled water) on a daily basis, to maintain water content at 100% FC. In contrast, for plants under single or combined water deficit (W and N+W) with 0 or 2 mM of Na_2SiO_3, no additional irrigation was supplied, resulting in a progressive decrease of water content to 50% of FC (day 10) which was then kept at this point (with distilled water for an additional 17 days). At day 27 post-transplantation, all the plants were harvested and used for the analysis of morphological and physiological parameters (n=6). For the biochemical analysis, roots were carefully washed, and plants were quickly separated into roots and leaves being immediately frozen in liquid nitrogen and stored at -80 ºC until analyses. For the analyses (n = 3), plant organs were ground and homogenized in liquid nitrogen using a mortar and pestle. Each biological replicate resulted from a pool of three plants that were ground together.

2.2. Biometric traits

At the end of the experimental period, plant height, as well as the maximum root length, were recorded. The total leaf number was obtained by counting leaves larger than 1cm and total leaf area (LA) was measured using a LI-3100C area meter (LI-COR, Lincoln, Nebraska, USA). The dry weight (DW, 48 h at 105°C in a ventilated oven) of leaves (LDW), stems (SDW) and roots (RDW) were determined. Shoot-to-root ratio (S/R, ratio of the shoot to root DW; g g^{-1} DW) was calculated.

2.3. Total antioxidant capacity and non-enzymatic components

2.3.1. Preparation of the extracts

For the analysis of the total AOX activity (ABTS [2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)] free radical scavenging assay) and non-enzymatic components (total soluble polyphenols and flavonoids), 300 or 250 mg of frozen samples of roots and leaves, respectively, were extracted with 1.2 or 1.5 mL of 80 % methanol, respectively, under cold conditions. Samples were centrifuged at 15 000 x*g* for 15 min and the resulting supernatant was used for all the analyses.

2.3.2. ABTS Free Radical Scavenging Assay

ABTS [2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)] assay was performed following the protocol described by Gonçalves *et al.* (2009), with slight modifications for 96-well microplate. Briefly, ABTS•+ solution was generated through a chemical oxidation reaction with potassium persulfate and its concentration adjusted with methanol to an initial absorbance of 0.700 (± 0.020) at 734 nm (Multiskan GO Microplate Spectrophotometer, Thermo FisherScientific Inc., Waltham, MA, USA). To 20 µL of the sample or standard solution, 180 µL of the adjusted ABTS•+ solution was added. The mixture was then incubated for 5 min at 30 °C, the absorbance was measured at 734 nm and the results were obtained by calculating the reduction of percentage in sample absorbance (ASPL) with respect to the control (ACTL) using Equation 1. Trolox was used as a standard to prepare a calibration curve and the results were expressed as mmol Trolox equivalents/g DW. All reactions were performed in triplicate, and the radical stock solution was freshly prepared and filtered with a 0.45 µm syringe filter.

$$\text{ABTS scavenging } (\%) = (A_{CTL} - A_{SPL}) * \frac{100}{A_{CTL}} \quad (1)$$

2.3.3. Total phenolics and flavonoids

Determination of total phenolic contents was performed using the Folin-Ciocalteu reagent following Ramos *et al.* (2019), with slight modifications. Briefly, 20 µL of extract or standard was mixed with 80 µL of Folin-Ciocalteu reagent previously diluted 1:10, and 100 µL of 7.5 % (w/v) sodium carbonate was added to the mixture in a 96-well plate. After 60 min at room temperature and dark conditions, absorbance was measured at 750 nm. Results were expressed as mg gallic acid equivalents/g of fresh weight (mg GAE/g FW). Each reaction was performed in triplicate.

Total flavonoids were quantified through the aluminum chloride method adapted from Zafar *et al.* (2016). Briefly, an aliquot of each extract (40 µL) was mixed with 20 µL of 10% (w/v) aluminium chloride and 20 µL of 1 M potassium acetate solutions. Thereafter, distilled water was added to obtain a final volume of 200 µL in a 96-well plate. After 30 min of incubation in dark conditions and room temperature, the absorbance at 415 nm was measured by using a microplate reader. The calibration curve was drawn by using quercetin as standard and the flavonoid concentration was established in µg quercetin equivalents/g FW (µg QE/g FW).

2.4. Enzymatic assay

2.4.1. Enzyme extraction and activity quantification: SOD, CAT, and APX

The extraction of the AOX enzymes was performed under cold conditions according to Fidalgo *et al.* (2011), using frozen samples of around 250 mg. After centrifugation, the supernatants were collected and used for both protein and enzyme quantification following the protocols described by Soares *et al.* (2019b). Briefly, protein quantification was performed as described by Bradford (1976), using bovine serum albumin as standard. Superoxide dismutase (SOD, EC.1.15.1.1) activity was quantified with a spectrophotometric assay based on the inhibition of photochemical reduction of nitroblue tetrazolium (Donahue *et al.*, 1997). Absorbance was read at 560 nm and results are expressed as units of SOD mg^{-1} protein, in which one unit of SOD corresponds to the amount of enzyme needed to inhibit the photochemical reduction of NBT by 50% (Spormann *et al.*, 2022). Catalase (CAT, EC.1.11.1.6) activity was determined spectrophotometrically by measuring CAT-mediated H_2O_2 degradation ($\varepsilon_{240\ nm}$ = 39.4 mM^{-1} cm^{-1}) (Aebi, 1984). Absorbance measurements were recorded every 5 s for 60 s, and results were expressed as mmol H_2O_2 min^{-1} mg of protein. Ascorbate peroxidase (APX, EC.1.11.1.11) activity was determined by monitoring the oxidation of ascorbic acid (AsA) for 1 min, with readings every 5 s ($\varepsilon_{290\ nm}$ = 0.49 M^{-1} cm^{-1}) (Nakano and Asada, 1981); results were expressed as mmol dehydroascorbate (DHA) min/mg of protein. The protocols for quantifying CAT and APX activity were adapted to UV-microplates following the adaptation from Soares *et al.* (2019b).

2.5. Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). The effects of deficit level and silicon supply on plant growth parameters and AOX system were analysed with a two-way ANOVA, using deficit level, silicon supply and their interaction as fixed factors. Mean separation was based on Fisher's protected least significant difference (LSD) test at p = 0.05 using IBM SPSS Statistics 26 and GraphPad Prim 8.

3. Results

3.1. Plant Growth

The deficit level significantly (p < 0.001) affected all plant growth parameters (Table 1, Figures 1 and 2), but Si supplementation significantly (p < 0.001) increased root length at all deficit levels (21% CTR, 18% N, 30%W, 37% N+W). (Table SM1, Figure 2b). Deficit level and Si supplementation showed a significant interaction for RDW (p = 0.002) and TDW (p = 0.021). When Si was added to plants grown under W deficit a significant reduction of 15% occurred

in both RDW and TDW. On the other hand, plants grown under N+W deficit and supplemented with Si significantly increased their RDW by 29% and their TDW by 23%.

Table 1 – Effects of deficit level: control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen or water deficit (N+W; 5.3 mM N + 50% W)) and silicon supplementation (two levels: 0 mM Na_2SiO_3 or 2mM Na_2SiO_3) on the growth traits of tomato plants cv. 'Micro-Tom' grown for 27 days. F ratios and associated *p* values are shown. Significant *p* values (<0.05) are indicated in bold.

Growth Traits	Deficit Level		Silicon Supplementation		Interaction	
	$F_{(3,40)}$	Sig.	$F_{(1,40)}$	Sig.	$F_{(3,40)}$	Sig.
RDW	25.48	**<0.001**	0.001	0.979	5.830	**0.002**
SDW	72.28	**<0.001**	0.000	1.000	0.000	1.000
LDW	25.67	**<0.001**	0.127	0.723	2.146	0.110
TDW	44.56	**<0.001**	0.102	0.751	3.637	**0.021**
Shoot/Root Ratio	11.51	**<0.001**	0.186	0.668	1.410	0.254
LA	29.37	**<0.001**	1.004	0.322	1.399	0.257
Height	47.20	**<0.001**	0.014	0.906	0.681	0.569
Root Lenght	15.26	**0.001**	22.34	**0.001**	0.223	0.880

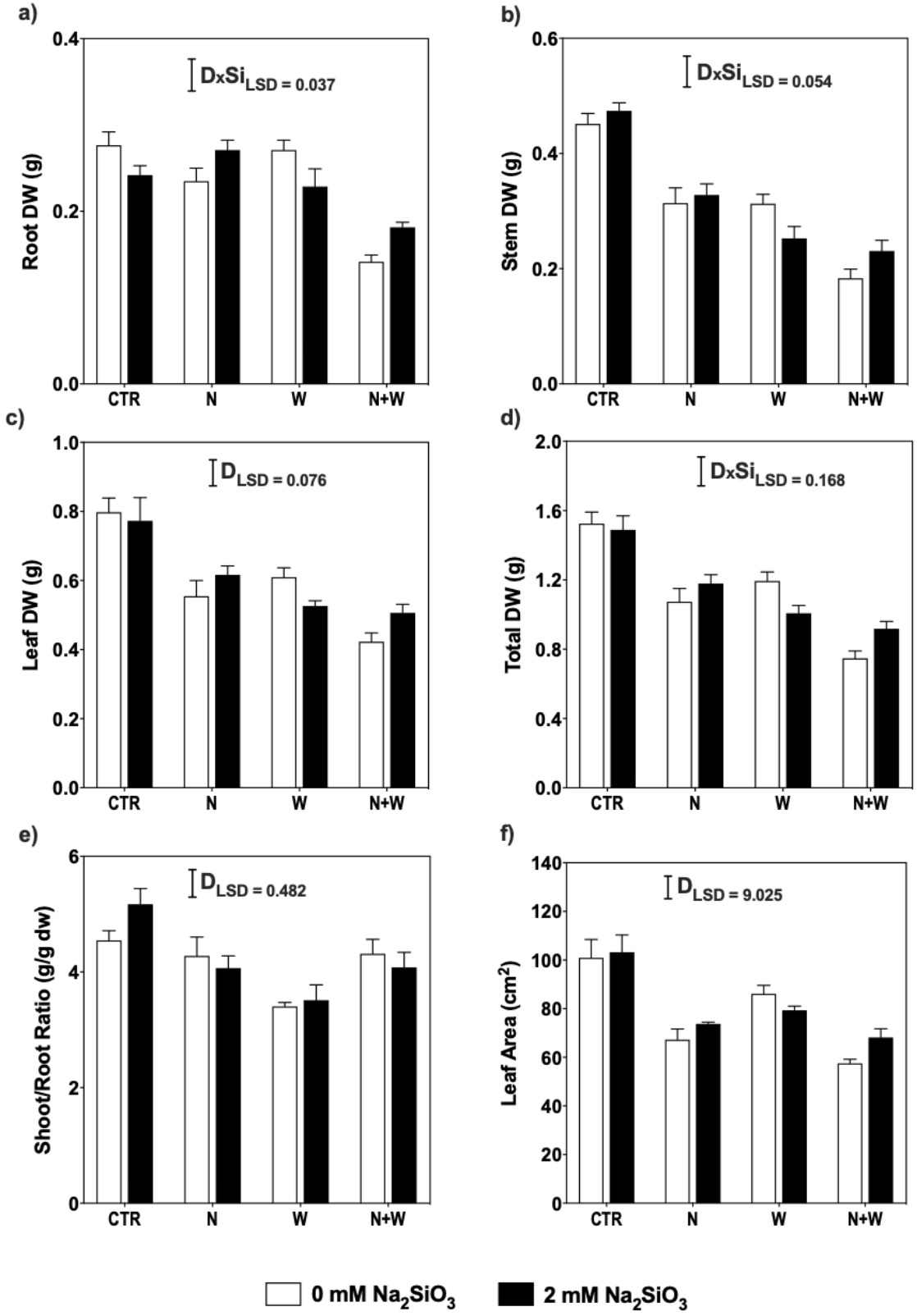


Figure 1 – Root dry weight **(a)**, stem dry weight **(b)**, leaf dry weight **(c)**, total dry weight **(d)**, shoot-to-root ratio **(e)**, and leaf area **(f)** of tomato plants cv. 'Micro-Tom' grown for 27 days under four levels of deficit [control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N+W; 5.3 mM N + 50% W)] without (0 mM Na_2SiO_3) or with (2mM Na_2SiO_3) of silicon (Si) supplementation. Data presented are mean ± SEM (n =

6). Bars represent Fisher's LSD ($p = 0.05$) for the significant interaction deficit level × silicon supplementation and for the independent effects (D, deficit level; Si, silicon supplementation) when the interaction was not statistically significant.

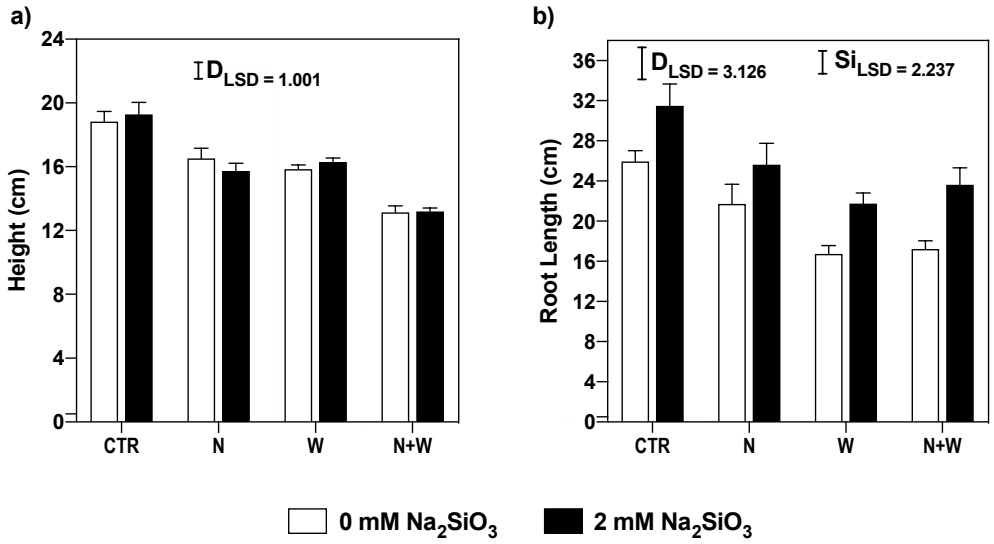


Figure 2 - Plant height **(a)**, and root length **(b)** of tomato plants cv. 'Micro-Tom' grown for 27 days under four levels of deficit [control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N+W; 5.3 mM N + 50% W)] without (0 mM Na_2SiO_3) or with (2mM Na_2SiO_3) of silicon (Si) supplementation. Data presented are mean ± SEM (n = 6). Bars represent Fisher's LSD ($p = 0.05$) for the significant interaction deficit levels × silicon supplementation and for the independent effects (D, deficit level; Si, silicon supplementation) when the interaction was not statistically significant.

3.2. Total antioxidant activity (ABTS assay), non-enzymatic components: total phenolics and flavonoids concentration

Neither the deficit level, the silicon supplementation, nor their interaction significantly affected the roots or leaves total antioxidant activity (ABTS assay) ($p > 0.05$, Table 2 and Figure 3a and b). However, phenolics concentration in roots was significantly affected either by the Si supplementation ($p = 0.024$) or the interaction between the deficit level and the Si supply ($p = 0.003$) (Table 2). When Si was added to CTR plants a significant increase of 83% was found (Figure 3c). A significant ($p = 0.030$) interaction between the deficit level and the Si supplementation was also found for the flavonoid concentration in the leaves, which was decreased in plants grown in CTR conditions and supplemented with Si by 31% (Table 2 and Figure 3f).

Table 2 - Effects of deficit level: control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen or water deficit (N+W; 5.3 mM N + 50% W)) and silicon supplementation (two levels: 0 mM Na_2SiO_3 or 2mM Na_2SiO_3) on the antioxidant system (root and leaves) of tomato plants cv. 'Micro-Tom' grown for 27 days. F ratios and associated *p* values are shown. Significant *p* values (<0.05) are indicated in bold.

AOX System	Deficit Level		Silicon Supplementation		Interaction	
	$F_{(3,16)}$	Sig.	$F_{(1,16)}$	Sig.	$F_{(3,16)}$	Sig.
ABTS (root)	1.345	0.295	1.109	0.308	0.504	0.685
ABTS (leaves)	2.180	0.130	2.153	0.162	0.175	0.912
Phenolics (root)	2.096	0.141	6.188	**0.024**	7.030	**0.003**
Phenolics (leaves)	0.486	0.697	2.972	0.104	2.143	0.135
Flavonoids (root)	0.819	0.502	0.000	0.991	1.640	0.222
Flavonoids (leaves)	0.080	0.970	1.621	0.221	3.872	**0.030**
APX (roots)	16.94	**<0.001**	0.930	0.349	22.23	**<0.001**
APX (leaves)	21.20	**<0.001**	12.53	**0.003**	7.147	**0.003**
CAT (roots)	40.82	**<0.001**	145.9	**<0.001**	26.49	**<0.001**
CAT (leaves)	103.0	**<0.001**	0.050	0.827	2.383	0.108
SOD (roots)	16.83	**<0.001**	1.529	0.234	2.533	0.094
SOD (leaves)	18.95	**<0.001**	60.09	**<0.001**	87.95	**<0.001**

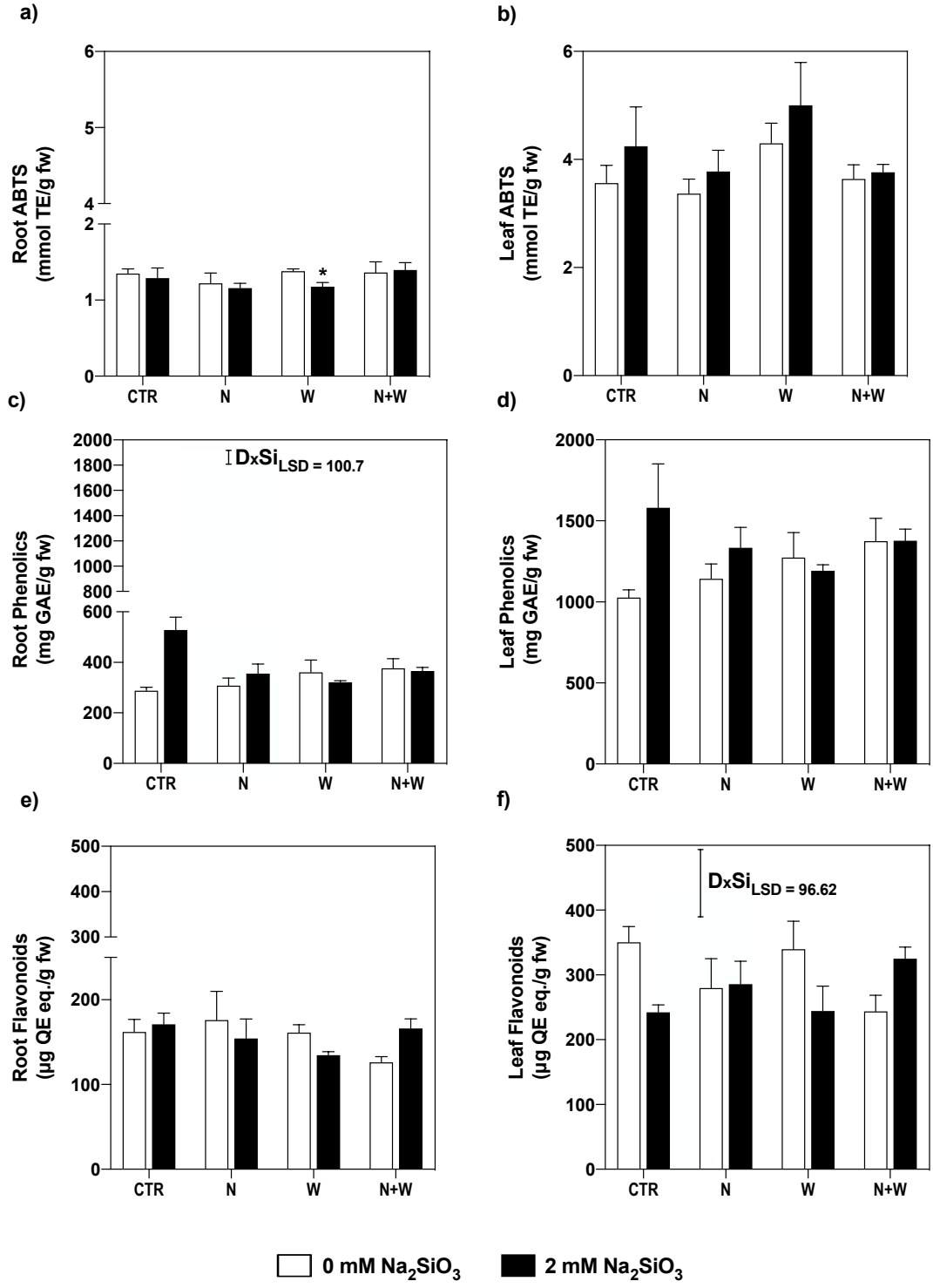


Figure 3 - Antioxidant capacity measured by ABTS in roots **(a)** and leaves (b), total phenolics in roots (c) and leaves (d), and total flavonoids in roots (e) and leaves (f) of tomato plants cv. 'Micro-Tom' grown for 27 days under four levels of deficit [control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N+W; 5.3 mM N + 50% W)] without (0 mM Na_2SiO_3) or with (2mM Na_2SiO_3) of silicon (Si) supplementation. Data presented are mean ± SEM (n = 3). Bars represent Fisher's LSD (p = 0.05) for the significant interaction deficit levels × silicon supplementation and for the independent effects (D, deficit level; Si, silicon supplementation) when the interaction was not statistically significant

3.3. Enzymatic components: SOD, CAT, APX

All enzyme's activity, both in roots and leaves, was significantly ($p < 0.001$) affected by the deficit level. Si supplementation alone only significantly affected CAT root activity ($p < 0.001$) and APX and SOD activity in leaves ($p = 0.003$ and $p < 0.001$, respectively) (Table 2).

Deficit level and Si supplementation showed a significant interaction for APX, both in roots and leaves ($p < 0.001$ and $p = 0.003$, respectively; Table 2). While in CTR plants Si addition led to a 52% significantly lower activity, in plants grown under N or N+W deficit and supplemented with Si APX activity was actually increased by 98% or by 48%, respectively (Figure 4a). An interaction between both factors also occurred for the APX activity in the leaves ($p = 0.003$; Table 2), being significantly higher (by 103%) in CTR plants grown with Si supplementation (Figure 4b). The activity of CAT in roots was also significantly increased by the interaction between deficit level and Si supplementation ($p < 0,0001$; Table 2). The N+W plants showed the highest increase (by 263 %), followed by N (by 176 %) and W (by 24 %) deficits (Figure 4c). Concerning SOD activity, only in leaves an interaction between both factors was found ($p < 0,0001$; Table 2). An increase in CTR (by 18%) and in W deficit (by 14%) plants occurred in SOD activity when Si was supplied in the nutrient solution (Figure 4f)

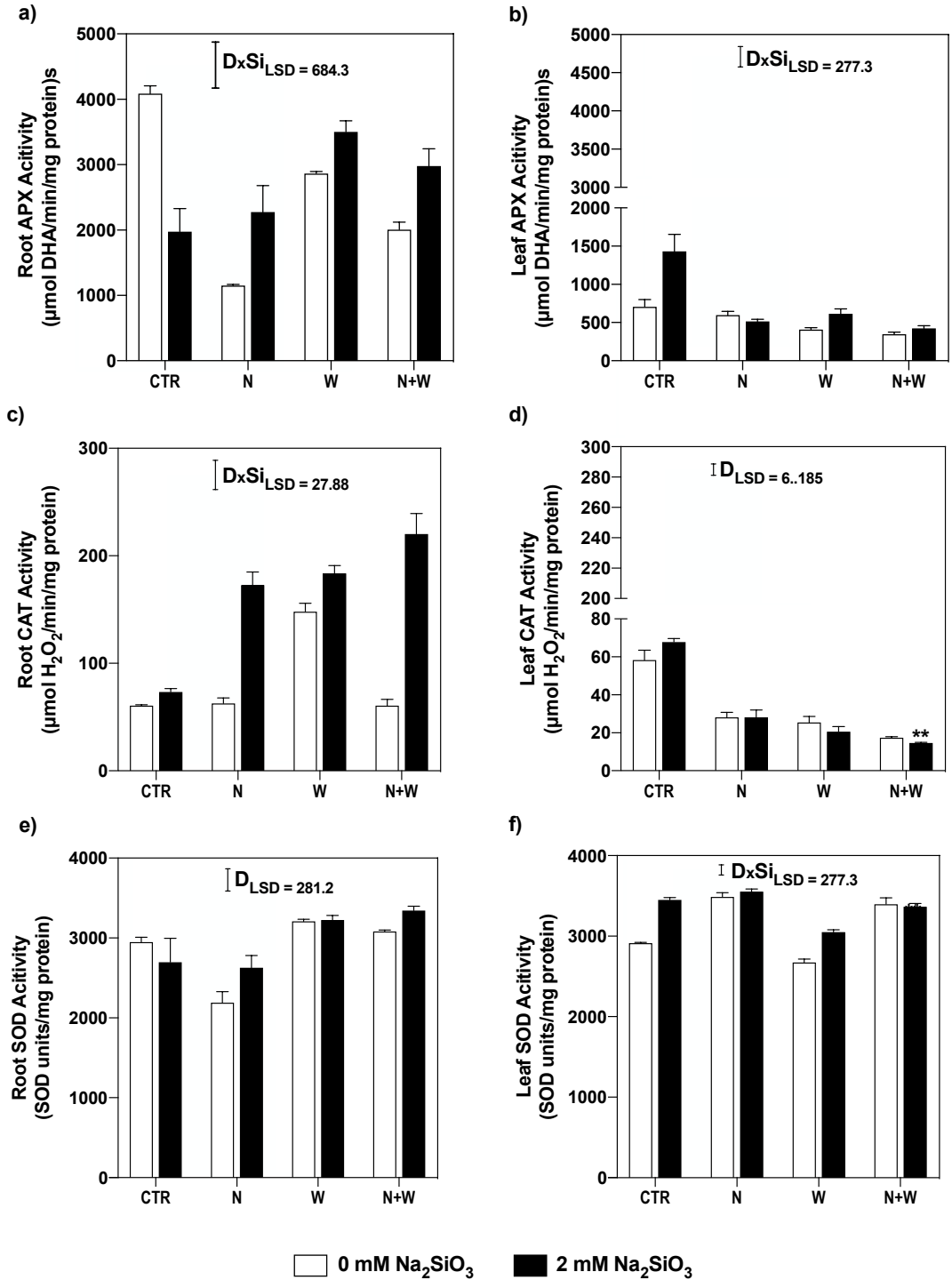


Figure 4 - APX activity in roots **(a)** and leaves **(b)**, CAT activity in roots **(c)** and leaves **(d)**, and SOD activity in roots **(e)** and leaves **(f)** of tomato plants cv. 'Micro-Tom' grown for 27 days under four levels of deficit [control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N+W; 5.3 mM N + 50% W)] without (0 mM Na_2SiO_3) or with (2mM Na_2SiO_3) of silicon (Si) supplementation. Data presented are mean ± SEM (n = 3). Bars represent Fisher's LSD ($p = 0.05$) for the significant interaction deficit levels × silicon supplementation and for the independent effects (D, deficit level; Si, silicon supplementation) when the interaction was not statistically significant

4. Discussion

To the best of our knowledge, this is the first study that compares the benefits of Si application on the AOX response of young tomato plants subjected to single N or W deficit or combined N+W deficit. By studying a set of plant growth traits and AOX biochemical parameters, we showed that single and combined N+W deficit hampered plant growth, particularly when no Si was added to the nutrient solution (Table 1; Figure 1 and 2) and that tomato plants grown under N+W deficit were able to modulate their AOX network in the presence of Si (Table 2; Figure 4).

It has been reported that Si plays an important role in plant functioning under stress conditions, yet under optimal conditions, its role is often reported as being minimal or non-existent (reviewed by Coskun *et al.*, 2019). Accordingly, concerning plant growth, in our study, the addition of Si did not cause significant effects in CTR plants, except for root length which increased (Table 1 and Figure 1 and 2). The positive effects of Si on root morphological traits, including root length was already observed for several crops and was recently reviewed by Tripathi *et al.* (2021). Oppositely, Si addition positively affected growth and biomass production in several plant species under environmental stress conditions (Bokor *et al.*, 2021; Coskun *et al.*, 2019). However, the literature is very scarce on the effects of Si supplementation in Si-excluder plants, such as tomato, subjected to N deficiency. In our study, Si addition to N-stressed plants did not significantly benefit any of the analyzed growth traits (Table 1 and Figures 1 and 2). The same was previously reported by Haddad *et al.* (2018), who also found that for the Si-excluder *Brassica napus* under N starvation, the Si treatment did not affect plant biomass. Furthermore, Leal *et al.* (2021) came to the same conclusion for cotton plants subjected to N deficiency and supplemented with Si. On the other hand, the positive effects of Si in mitigating the W deficit are widely reported (recently reviewed by Bokor *et al.*, 2021; Malik *et al.*, 2021; Verma *et al.*, 2022; Thorne *et al.*, 2020; Wang *et al.*, 2021;). However, in our work, Si addition to plants under W deficit alone did not show noticeable effects, except for RDW and TDW where a significant reduction was found, and for root length which was increased (Table 1 and Figure 1 and 2). This difference in the results could be due to different experimental design conditions such as the form and concentration of silicon given to the plants, the type of substrate used, or even using different tomato genotypes. For example, Zhang *et al.* (2018) and Shi *et al.* (2016) used potassium silicate instead of sodium silicate to conduct their experiment while Shi *et al.* (2014) utilized sodium silicate but in a lower concentration of 0.5 mM. Furthermore, a study by Ali *et al.* (2018) demonstrated that tomato genotypes subjected to drought stress responded differently to the application of sodium silicate in a concentration of 1.5 mM, with the tolerant genotype having no beneficial effects on growth upon Si application. Finally, in most of the

studies (Ali *et al.*, 2018; Shi *et al.*, 2016; Shi *et al.*, 2014; Zhang *et al.*, 2018), drought stress was imposed to tomato plants using polyethylene glycol and not through the decrease of watering *per se* which may also influence the results. Interestingly, although under single deficit (N or W deficit) Si addition to the nutrient solution did not result in significant benefits, under combined N+W deficit it led to an improvement in growth traits such as increased RDW, TDW, and root length (Table 1 and Figure 1 and 2). This, in turn may be linked to the stimulation of the AOX system (Table 2 and Figure 3 and 4) as already observed for other crops under abiotic stress (Bu *et al.*, 2016; Geng *et al.*, 2018; Kim *et al.*, 2017; Shi *et al.*, 2014).

It is known that under abiotic stress, plants have a complex AOX system for scavenging toxic ROS, which can be classified as non-enzymatic and enzymatic AOX systems, that work *in tandem* to counteract the overproduction of these by-products (Laxa *et al.*, 2019; Soares *et al.*, 2019a). Si application has been reported as a sustainable way of increasing the tolerance of plants under several abiotic stresses by boosting plant defence responses, such as those of the AOX system (reviewed by Kim *et al.*, 2017). In our work, the total AOX capacity (ABTS) and the non-enzymatic system (phenolics and flavonoids) did not significantly change after Si addition, except for phenolics content in the roots, which was increased, and for the flavonoid content in the leaves, which was decreased in CTR plants (Figure 3). Increasing evidence shows that Si influences the concentration of phenolic compounds (Hajiboland *et al.*, 2017) which is supported by our findings in CTR plants. Such increase has also been reported for unstressed strawberry plants (Hajiboland *et al.*, 2018).

However, the enzymatic system responded both to the imposed deficit as well as to the Si supplementation, demonstrating a higher importance of the enzymatic part of the AOX system on the deficits studied (Table 2 and Figure 4). In CTR plants supplemented with Si, APX was decreased in roots but increased in leaves, while CAT did not significantly differ in any plant organ, and SOD only increased in leaves (Figure 4). This suggests that Si impacted the tomato AOX response even in CTR conditions, as previously reported by Vega *et al.* (2019) after observing that Si application stimulated the AOX system of two healthy barley cultivars. Interestingly, although after Si addition, APX and CAT were increased in the roots of plants subjected to all the imposed deficits, it was in the N+W deficit where the Si application showed a higher impact (Figure 4a and 4c). Shi *et al.* (2016) observed that Si improved the growth and development of tomato plants under drought stress by improvement of root hydraulic conductance due to an increase of the AOX system response. In this study, the general improvement of the AOX system on the roots of combined N+W tomato plants may therefore be the reason why this group of plants responded better to the imposed stress. On the other hand, Si addition increased the activity of SOD in leaves of plants subjected to

W deficit, but not in the other stress treatments, where no significant changes were observed (Figure 4c and f). Although Si was already shown to increase the activities of SOD, CAT, and APX in crops such as wheat, tomato, chickpea, rapeseed, sugarcane and sunflower under drought conditions (Gunes *et al.*, 2007; Gunes *et al.*, 2008; Shi *et al.*, 2016; Verma *et al.*, 2020; Xu *et al.*, 2017;), we could not find literature reports about the effect of Si on the AOX system of plants subjected to singles N or combined N+W deficit. Our results suggest that Si differentially affects the response of the enzymatic system in plants under single stresses or in a combination of them, the latter occurring most often. While for single W deficit, Si seems to have stimulated the enzymatic machinery partly in roots and partly in the leaves, for combined N+W deficit, and to a certain extent for N deficit, it seems to have acted mostly at the root level, where a high increase was seen in the activity of APX and CAT enzymes. This greater impact of Si on the roots of plants grown under combined N+W deficit may be due to the fact that both the N deficit and the W deficit are primarily perceived by roots (Bengough *et al.*, 2011; Sun *et al.*, 2020a). On the other hand, since Si was also applied via the root, it may have led to a greater activation here, as a first line of defence, which could only later be extended to the leaf. Indeed, the significant role of Si on root development during stress has been previously noted (Hattori *et al.*, 2005) and was also observed in our study (Figure 2b). The root growth stimulation by Si application may be the result of root elongation by increasing the extensibility of cell walls (Hattori *et al.*, 2003).

5. Conclusions

Root Si application on tomato seedlings can partly alleviate the stress caused by combined N+W deficit as demonstrated by the increase in growth traits such as RDW and root length which ultimately contributed to their higher TDW. The better performance of plants in combined N+W deficit supplemented with Si seems to be related, at least in part, to the AOX system of the roots (where the stresses were first experienced), shown as increased activity of CAT and APX enzymes. Plants' response to combined stresses cannot be extrapolated from the responses observed for the single deficits, because for plants subjected to either N or W deficit the impact of Si was not so evident. These results are highly relevant if we consider that: 1) plants often do not face only one single abiotic stress, but rather a combination of them and 2) combined N+W deficit was the treatment where the highest decreases in growth traits were found, and therefore the treatment where the application of Si would bring more benefit. Comprehension of the full mechanisms involved in the complex interactions between these two abiotic stresses and Si application is still in its infancy. It is also not clear yet to what extent the present findings can be extrapolated to other crops and stress levels.

Sub-chapter 3.2

Exploring tomato phenotypic variability under combined nitrogen and water deficit

The content of this sub-chapter is published as:

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Exploring tomato phenotypic variability under combined nitrogen and water deficit

Abstract

Despite drought and nitrogen (N) deficit being two of the most important crops' growth limiting factors, only few studies have explored tomato phenotypic variability in response to both abiotic stresses. In this study, we aimed to perform a phenotypic evaluation and an analysis of the growth traits of 40 tomato genotypes (mostly focusing on old cultivars, but also including modern hybrids and wild tomato relatives' accessions as anchors) under combined N and water deficit. Each genotype was divided into two groups: control (100% N, 100% field capacity) and combined deficit (20% N, 50% field capacity). A total of 14 morpho-physiological traits were evaluated and further analyzed using multivariate statistical methods. The Principal Component Analyses revealed considerable phenotypical diversity among tomato genotypes, with four principal components explaining 82% of the variability. Data integration on a cluster analysis separated the studied genotypes into three distinct clusters based on their ability to handle the combined deficit. Tolerance was associated with traits such as lower specific leaf area, lower leaf area ratio and higher water use efficiency, comparing to the sensitive genotypes. This study shows that tomato tolerance to combined N and water deficit largely varies between genotypes and that old cultivars represent a valuable gene pool towards more sustainable food production systems.

1. Introduction

Population growth, especially in light of global climate change, demands a more rational use of agricultural resources (e.g. water, fertilizers and land), which poses several challenges to guarantee global security while promoting sustainable crop production (Fernandes *et al.*, 2022; Kyalo Willy *et al.*, 2019). The agricultural sector uses about 80% of the total available freshwater (which is becoming increasingly scarce) as well as high amounts of N fertilizers that often exceeds crop N requirements (Elbehri, 2015; Esteban *et al.*, 2016a; Liang *et al.*, 2019). Excessive N fertilization causes environmental problems, such as leaching of nitrate, being worldwide recognized as a serious issue of public and economic concern, due to its potential of increasing the eutrophication of freshwater ecosystems, the acidification of agricultural soils as well as its impact in greenhouse gas emissions (Esteban *et al.*, 2016a; Fernandes *et al.*, 2022; Liang *et al.*, 2018). To respond to these and other challenges, the recent European Green Deal (2020), which strives to make Europe the first climate-neutral continent, has established the need of reducing nutrient losses by at least 50% and fertilizer

application by at least 20% by 2030 (Schebesta and Candel, 2020). Therefore, it is imperative to increase agricultural production in a sustainable way where the available resources are used more efficiently.

Water and nitrogen (N) are two of the most important resources affecting crop growth and yield (Machado *et al.*, 2022). Several studies demonstrated that drought or N deficit have an impact on a large number of morphological traits (e.g. leaf area, specific leaf area, leaf weight ratio, root growth, root hydraulic conductivity and long-lasting root anatomical changes), as well as dry matter partitioning (promoting root development) (e.g. Du *et al.*, 2018; Machado *et al.*, 2022; Moles *et al.*, 2018; Wu and Cosgrove, 2000). Furthermore, both abiotic stresses are known to have a severe impact on the metabolic pathways, including water relations and photosynthesis impairment, either due to stomatal closure and/or metabolic damage (Ding *et al.*, 2018; Gonzalez-Dugo *et al.*, 2010). Moreover, it is clear that water and N deficits are certainly highly interdependent (Ding *et al.*, 2018; Gonzalez-Dugo *et al.*, 2010; Plett *et al.*, 2020). Despite this, exploring the genotypic variation and understanding how crops respond to combined N and water deficit is largely unknown.

Tomato plants (*Solanum lycopersicum*) are among the most cultivated vegetable crops worldwide (FAO, 2020b; Heuvelink *et al.*, 2020). Intensive tomato cultivation relies on high fertilizer inputs and regular irrigation for maximum yield (Sandhu et al., 2021). These inputs come at a significant economic cost (Engindeniz, 2006) and carbon footprint (Pereira *et al.*, 2021). Thus, considering the increased consumption of fresh tomato and tomato-derived products (Costa and Heuvelink, 2018), the development of strategies focused on a rational use of water and fertilizers is required.

Plant breeding can be a powerful tool to enhance crop resilience to these abiotic stresses (Monforte, 2020). Nonetheless, for several decades of high-pressure of genotype selection and crop breeding for high yields led to the so-called genetic erosion, and the tomato cultivars had often a narrow genetic basis, with the vast majority of them being sensitive to drought and N deficiency (Esteban *et al.*, 2016; Machado *et al.*, 2022; Shirasawa *et al.*, 2010). To introduce genotypic variation tomato breeding programs, for fruit quality and environmental adaptation, started to rely on wild tomato relatives (such as *Solanum pimpinellifolium*) (Conesa *et al.*, 2019). More recently, it has been highlighted that another valuable gene pool for tomato improvement are the old accessions (e.g. landraces and heirlooms) held in germplasm collections. Nonetheless, so far, they represent an untapped genetic resource (Conesa *et al.*, 2019; Roohanitaziani *et al.*, 2020). Therefore, it is important to explore the variation of this genetic resource that might offer opportunities for improving resource use efficiency, thereby increasing the environmental sustainability of production systems (Monforte, 2020).

Up to date, the number of studies that have screened a high number of tomato genotypes in response to drought and N deficiency is limited. In this study, we aimed to perform a phenotypical evaluation and analysis of the growth traits of 40 genotypes focusing on old tomato accessions (but also including, as anchors, four modern hybrids and wild tomato relatives' accessions) when subjected to combined N and water deficit. To that end, 14 morpho-physiological traits were evaluated and the contribution of the different growth components to the genotypic variation in growth response of all accessions was used to determine their potential relationships (based on a cluster analysis).

2. Materials and Methods

2.1. Plant material and growth conditions

This study included a total of 40 tomato genotypes and wild relatives (34 old accessions, four modern inbred lines/hybrids and two wild relatives: Table 1) from an existing collection at Wageningen University (EU–SOL tomato core collection) and two Dutch seed companies (Nunhems and Rijk Zwaan). The old accessions were selected taking into account their interesting phenotypical diversity for plant growth and fruit quality-related traits being considered as a relevant gene pool for plant breeding (Aflitos *et al.*, 2014; Roohanitaziani *et al.*, 2020; Roohanitaziani *et al.*, 2022). However, these have never been screened in response to N and water deficit, which is the focus of our study. The modern inbred lines/hybrids genotypes and wild tomato relatives' accessions were included in this study as anchors.

Table 1 - List of the tomato genotypes and wild relatives analyzed from a core germoplasm collection existing at Wageningen University. For simplification genotypes were further numbered from low to high percentage decrease in TDW in response to combined N and water deficit.

No.	Specie	Accession name	Category	Accession ID[1]
1	*S. lycopersicum*	Micro-Tom	Old accession	LA3911
2	*S. lycopersicum*	Cal J Tm VF	Old accession	EA02054/CGN20815
3	*S. lycopersicum*	Bolivar	Old accession	EA03222/LYC3155/LYC2513/T828
4	*S. lycopersicum*	Nunhems-FM001	Modern Inbred Line	Nunhems
5	*S. lycopersicum*	Tiffen Mennonite	Old accession	EA01088
6	*S. lycopersicum*	981136	Old accession	EA06932

7	*S. lycopersicum*	n/a	Old accession	T825/LYC3153/EA03221
8	*S. lycopersicum*	Ailsa Craig	Old accession	LA2838A/EA01101/EA00240
9	*S. lycopersicum*	Morne a L'Eau	Old accession	EA05979/ PI372385
10	*S. lycopersicum*	Winter Tipe (NOR)	Old accession	PC11029
11	*S. lycopersicum*	Foundation	Modern Hybrid	Nunhems
12	*S. lycopersicum*	Moneymaker	Old accession	LA2706/EA00840/EA02936
13	*S. lycopersicum*	Grosse Cotelee	Old accession	EA01042
14	*S. lycopersicum*	Nagcarlan	Old accession	EA05732/PI324065
15	*S. lycopersicum*	Black Cherry	Old accession	EA00027/LA4451
16	*S. lycopersicum*	OH88119	Old accession	EA06902
17	*S. lycopersicum*	Kecskemeti Koria Bibor	Old accession	EA03075
18	*S. lycopersicum*	Katinka Cherry	Old accession	EA00375
19	*S. lycopersicum*	Capriccia	Modern Hybrid	Rijk Zwaan
20	*S. lycopersicum*	Watermelon beefsteak	Old accession	EA01640
21	*S. lycopersicum*	DL/67/248	Old accession	EA05721/ PI320468
22	*S. lycopersicum*	Anto	Old accession	EA01835/V710029
23	*S. lycopersicum*	All Round	Old accession	LA2463/LYC1365/EA02617
24	*S. lycopersicum*	Momotaro	Old accession	TR00003
25	*S. pimpinellifolium*	N481	Wild tomato relative	LYC2798/EA02994
26	*S. lycopersicum*	Chang Li	Old accession	PI93302/EA04243
27	*S. lycopersicum*	Giant Belgium	Old accession	EA01037
28	*S. lycopersicum*	Sonora	Old accession	CGN15882/EA01985
29	*S. lycopersicum*	Chih Mu Tao Se	Old accession	PI158760/EA04828
30	*S. lycopersicum*	ABC Potato Leaf	Old accession	EA00915
31	*S. lycopersicum*	The Dutchman	Old accession	PI303721
32	*S. lycopersicum*	Belmonte	Old accession	EA00892/SG16
33	*S. lycopersicum*	Blondokee	Old accession	EA00282

34	*S. lycopersicum*	Rote Beere	Old accession	LYC11/EA01965/CGN15464
35	*S. lycopersicum*	Callanzo	Modern Hybrid	Rijk Zwaan
36	*S. lycopersicum*	*var. cerasiforme*	Old accession	PI365925/LA1324/EA05891
37	*S. lycopersicum*	Rutgers	Old accession	LA1090/EA00465
38	*S. lycopersicum*	*var. cerasiforme*	Old accession	LA1421/PI 365930/TR00027
39	*S. lycopersicum*	n/a	Old accession	PI129097/EA04710
40	*S. pimpinellifolium*	T495	Wild tomato relative	LYC2910/EA03058/T115

[1] Identification starting with "EA", "LA", "LYC", "PI", and "TR" are genotypes registered by "EU-SOL tomato core collection database" (Aflitos et al., 2014), while other are provided by the corresponding company. n/a: genotype without a specific accession name.

Seeds were sown in potting soil and approximately two weeks later (corresponding to the third leaf appearance) seedlings from each genotype were selected based on uniformity. These were individually transplanted to pots (10.5 width x 10.5 length x 12 cm height) filled with 110 g of vermiculite (0.1-1.5 mm grade) and were divided into two groups: control plants (100% N requirement; 100% field capacity) and plants subjected to combined deficit (20% N requirement; 50% field capacity). In both cases, just prior to seedling transplanting, each pot was irrigated to 100% field capacity (FC), determined using the soil gravimetric water content method (Joshi *et al.*, 2021). This led to 550 mL of the respective nutrient solution applied to each pot [Control (100% N): 10 mM NO_3^-; 1.9 mM $H_2PO_4^-$; 6.1 mM K^+; 3.6 mM Ca^{2+}; 1.9 mM SO_4^{2-}; 2.5 mM Mg^{2+}; 2.6 mM Cl^-; Combined deficit (20% N): 2 mM NO_3^-; 1.9 mM $H_2PO_4^-$; 6.2 mM K^+; 3.6 mM Ca^{2+}; 4.8 mM SO_4^{2-}; 2.8 mM Mg; 5.5 mM Cl^-], both having the same pH (5.5) and E.C. (2 dSm^{-1}) as well as the same amount of micronutrients: 35 µM Fe^{3+}; 8 µM Mn^{2+}; 20.1 µM B; 0.5 µM Cu^{2+}; 5.0 µM Zn^{2+}; 0.5 µM MoO_4^{2-}. The 10 mM NO_3^- concentration used, in the control nutrient solution, is a common level for guaranteeing an adequate plant growth in several species (Macduff *et al.*, 1993; Lacrampe *et al.*, 2020). Moreover, based on previous studies from our research team (under comparable environmental conditions and using cv. 'Moneymaker' as reference cultivar, during vegetative growth stage) it was possible to estimate tomato N needs for the overall experimental period. Briefly, considering the expected dry weight (2.54 g per plant) and an average N concentration in the plant tissues of 3% (Fiebig and Dodd, 2016; Sonneveld and Voogt, 2009), 76.2 mg N per plant were added in the 550 mL irrigation solution supplied to the control plants. Thus, for simplification, we referred to the N concentration supplied in the combined deficit (2 mM) as representing 20% of this reference optimal concentration (20% N).

Following seedling transplanting all pots were covered to prevent evaporation and no more nutrient solution was added until the end of the experiment. Concerning further irrigation, each pot from control plants was weighted and re-watered (with distilled water on a daily basis) to maintain FC at 100% during the experimental period, whereas in combined deficit no additional irrigation was supplied, resulting in a progressive decrease of substrate FC. Three plants from each genotype and growth condition were randomly collected during the cultivation period. The average fresh weight of these plants (per treatment) was subtracted from the total pot weight (per treatment) to calculate the actual weight of the water/nutrient solution present in the pot (Machado *et al.*, 2023b). The experiment ended after 21 to 28 days, depending on genotype, when the latter group of plants reached 53 ± 3 % FC. The experiment was carried out during the winter period (Dec/Jan), in a multispan Venlo-type glasshouse compartment (52°N, 6°E; Wageningen University, the Netherlands) set at 25 ºC day/ 23 ºC night and 60% RH. Supplementary light was provided with high sodium pressure lamps (HSP) to extend the natural photoperiod to 16h. HPS lamps (150 µmol s^{-1} m^{-2} of photosynthetic photon flux density measured at plant level, HPS, Philips SON-T Agro) were switched on when global radiation was below 300Wm^{-2} and switched off when it was above 400Wm^{-2}. Plants were distributed among eight tables (each representing a block) and each table was split in two halves: in one half the control plants of the 40 genotypes were randomly allocated and in the other half the plants under stress treatment were positioned in a mirrored arrangement. In each table an outer row of plants was placed around the experimental plants to prevent border effects.

2.2. Data collection: morphological and physiological measurements

At the end of the experimental period, the stomatal conductance (g_s; mmol m^{-2} s^{-1}) was measured between 08:00 (2h after the beginning of the light period) and 12:00, being carried out randomly among different plants. Measurements were performed on the adaxial side of the youngest fully developed and well-exposed leaf to assure fully active plants (n=8). The porometer (SC-1 porometer, Decagon Devices Inc., Pullman, Washington, USA) was calibrated and the readings were set at a fixed time-period (30 seconds) following the manufacturer's instructions, to ensure that all measurements were comparable and also to avoid the interference of leaving the sensor head on the leaf surface for a long period. Photosynthetic efficiency (F_v/F_m) was measured in 30 minutes dark-adapted leaves using the same leaf to estimate the photosynthetic capacity (n=8) (portable minipam, Model PAM 2000. Walz, Effeltrich, Germany). Thereafter several morphological parameters were determined (n=8): plant height (PH; cm), total leaf number (LN; > 1cm) and total leaf area (LA; cm^2; leaf area meter, Model LI-3100C, LI-COR, Lincoln, Nebraska, USA). Dry weight (DW, 48 h at 105°C in a ventilated oven; g) of leaves (LDW; g), stems (SDW; g), and roots (RDW; g), the

latter after being washed, was determined (n=8). Specific leaf area (SLA, ratio of leaf area to leaf dry weight; $cm^2\ g^{-1}$ dw), leaf weight ratio (LWR, ratio of leaf dry weight to total dry weight; $g\ g^{-1}$ dw), leaf area ratio (LAR, ratio of leaf area to total dry weight; $cm^2\ g^{-1}$ dw) and shoot-root ratio (S/R, ratio of shoot to root dry weight; $g\ g^{-1}$ dw) were calculated according to the ''classical approach'' described by Hunt (2012) (n=8). The water use efficiency (WUE; $g\ mL^{-1}$) was calculated as the ratio between total dry weight (TDW; g) and water consumption per plant (mL) (Supplementary Data, Figure S1) (n=8).

2.3. Data analysis

For comparing genotypes responsiveness to the combined deficit, all the data was transformed (following Zeng et al., 2002) into percentages of decrease, which has been further used as our studied variables. For this, within each table, control plants from a given genotype were paired with their mirrow plant on the opposite side of the table (corresponding to the same genotype but under combined N and water deficit). The difference between the value of the control and the value of the stressed plant was divided by the value of the control plant for each parameter (n=8). To simplify further analysis and interpretation, the genotypes were then ordered and numbered from low to high percentage of decrease in TDW.

Pearson's correlation coefficient was used to test associations between the percentage of decrease obtained for each of the 14 morpho-physiological parameters (F_v/F_m_D, g_s_D, LN_D, PH_D, LA_D, LDW_D, SDW_D, RDW_D, S/R_D, SLA_D, LWR_D, LAR_D, WUE_D, TDW_D). A Principal Component Analysis (PCA) with *Varimax* rotation was used to establish the relationship among these quantitative variables with the data set including the percentages of decrease of all traits, as active variables. However, TDW_D was excluded form PCA as this would be a redundant variable since it results from the sum of the different plant organs that are already taken into account in the analysis. Additionally, TDW of the control plants (TDW) was included as supplementary variable (active observation). After the PCA, a Clustering Analysis was applied to group the genotypes based in the individual coordinates of their projections into the coordinate space, following the *k-means* clustering with a Wilks' Lambda criterion (Everitt et al., 2001). Differences in traits among clusters were tested by the non-parametric Kruskal–Wallis test. All statistical analyses were performed using XL-Stat (Addinsoft, 2021).

3. Results

3.1. Screening for genotypic variation

All genotypes showed visual deficiency symptoms when grown under combined N+W deficit. However, some genotypes were more severely affected than others as shown in

Supplementary Information (Figure S2). Actually, already after the second week of the imposed deficit, it was visually observed that plants started to present phenotypic differences with regards to the severity of the combined N+W deficit symptoms, with the leaves becoming lighter green/ yellow (particularly the older ones) and often falling at variable degrees amongst genotypes. However, at this timepoint, no leaf rolling and/or wilting were observed.

At harvest (when the stressed plants reached 50% FC), a more detailed analysis of plant responses to the combined N and water deficit, based on 14 morpho-physiological traits, corroborated the observed visual differences between genotypes, with all the studied traits being negatively affected by the applied stress but to a different degree (Figure 1, Table S1). For instance, this impact was more severe on LA with an average decrease of 77% over all genotypes under combined deficit, followed by the biomass production parameters (TDW, RDW, SDW, LDW) and the g_s (all of them with an average decrease larger than 50%*)* (Figure 1). On the other hand, F_v/F_m and LWR were the least impacted traits by the combined deficit (5.1 and 11.2% decrease, respectively). Interestingly, in spite of LA being the most sensitive parameter to the combined deficit, all genotypes responded in a similar magnitude resulting in the lowest coefficient of variance (4.5%) (Figure 1). Regarding LWR, SLA and WUE a high variability was found among genotypes (56-59 % CV) (Figure 1). For SLA and WUE it is important to notice that, in general, a gradient was observed following the genotypes sorting order, which was not observed for LWR (Figure 1).

At the end of the experiment the control plants of the different genotypes had significantly different TDW (p <0.0001, varying between 1.17 and 3.99 g; Figure 2). Therefore, we hypothesized that faster growing genotypes could be more prone to the combined deficit as they had access to the same amount of water and N during the experimental period when compared to the smaller genotypes. To clarify this, we determined the correlation between the TDW of the control plants and the percentage of TDW decrease observed under the combined deficit (Figure 2). This analysis demonstrated a moderate correlation between these variables (R^2= 0.435). Therefore, as plant biomass did not have *per se* a significant impact on the plant's response to the combined deficit, all the genotypes were considered for the following analyses.



	TDW_D	RDW_D	SDW_D	LDW_D	S/R_D	PH_D	LN_D	LWR_D	LA_D	LAR_D	SLA_D	Fv/Fm_D	gs_D	WUE_D
Min (%)	42	12	36.4	46	0	19	9	0	67	9	0	1	7	2
Max (%)	77	68	76.1	80	42	48	54	30	85	47	47	10	72	69
Mean±SD (%)	65±7	56±10	62±8	69±8	25±11	38±5	33±9	11±7	77±3	31±10	22±12	5± 2	52±14	22±12
CV(%)	11	18	13	11.2	42	13	26	59	5	32	56	39	27	56

Figure 1 - Effects of combined nitrogen and water deficit on the percentage of decrease (_D) of total dry weight (TDW), root dry weight (RDW), stem dry weight (SDW), leaf dry weight (LDW), shoot/root ratio (S/R), plant height (PH), leaf number (LN), leaf weight ratio (LWR), leaf area (LA), leaf area ratio (LAR), specific leaf area (SLA), photosynthetic efficiency (F_v/F_m), stomatal conductance (g_s) and water use efficiency (WUE) in each of the 40 screened tomato genotypes and wild relatives, and the variation observed across them (minimum and maximum value, mean and standard deviation and the coefficient of variance (%). Color scale represents the mean of the percentage of decrease (the difference between the mean value of the control plants and mean of the stressed plants divided by the mean value of the control plants, n = 8). Different colors used in the genotype number correspond to a different category: old accessions (blue); modern inbred lines and hybrids (light orange); wild tomato relatives (green). The genotypes were ordered from low to high percentage decrease in TDW and their identification is given on Table 1

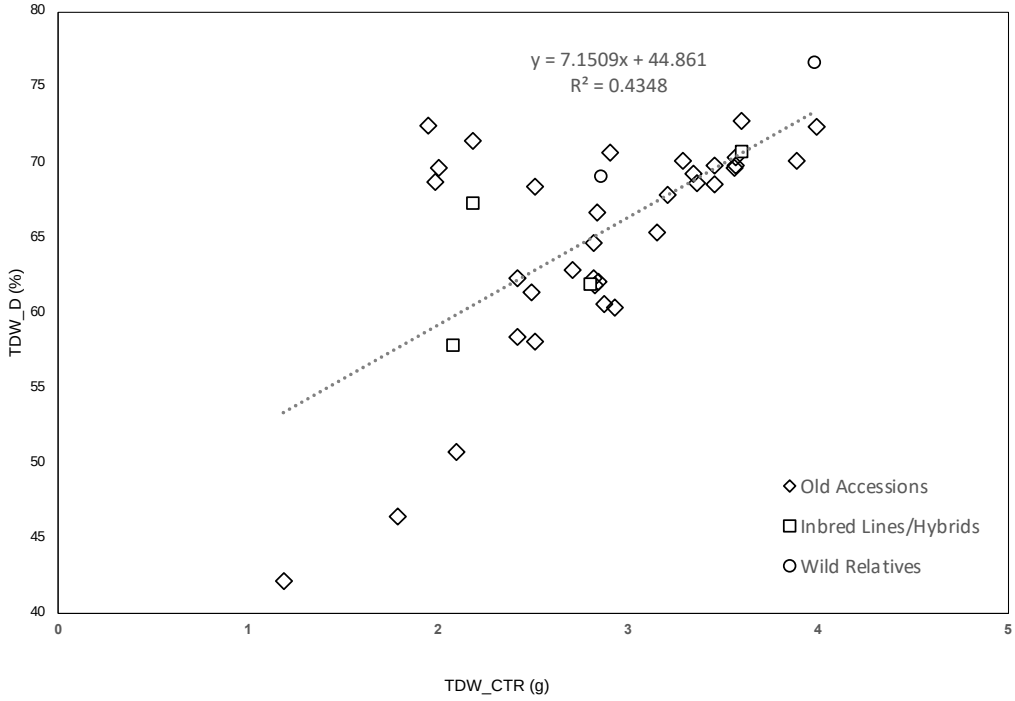


Figure 2 - Linear correlation between the percentage of decrease in total dry weight (TDW_D) after exposure to combined nitrogen and water deficit and the total dry weight of the control plants (TDW_CTR) for the 40 screened tomato genotypes and wild relatives. Different symbols used in the genotype number correspond to a different category: old cultivar (◊); modern inbred lines and hybrids (□); wild tomato relatives (○).

In order to analyze the relationship among the studied morpho-physiological traits, a Pearson's correlation coefficients were determined (Figure 3). Most traits showed a positive significant correlation among each other, with the exception of LAR and SLA which mostly showed a significant negative correlation with the other traits. For instance, the percentage of decrease in TDW was positively correlated with the percentage of decrease in RDW, SDW, LDW, PH, LN, LWR, LA, F_v/F_m and WUE but negatively correlated with the percentage of decrease in LAR and SLA (Figure 3). Additionally, regarding the physiological traits, the percentage of decrease of F_v/F_m was positively correlated with the percentage of decrease in TDW, LDW and PH whereas g_s decrease was not significantly correlated with any of the other analyzed traits (Figure 3). Finally, the percentage of decrease in WUE, one of the most important traits when particularly coping with water deficit, was positively correlated with the percentage of decrease in all evaluated parameters, with the exception of LAR and g_s, where no significative correlation was found (Figure 3).

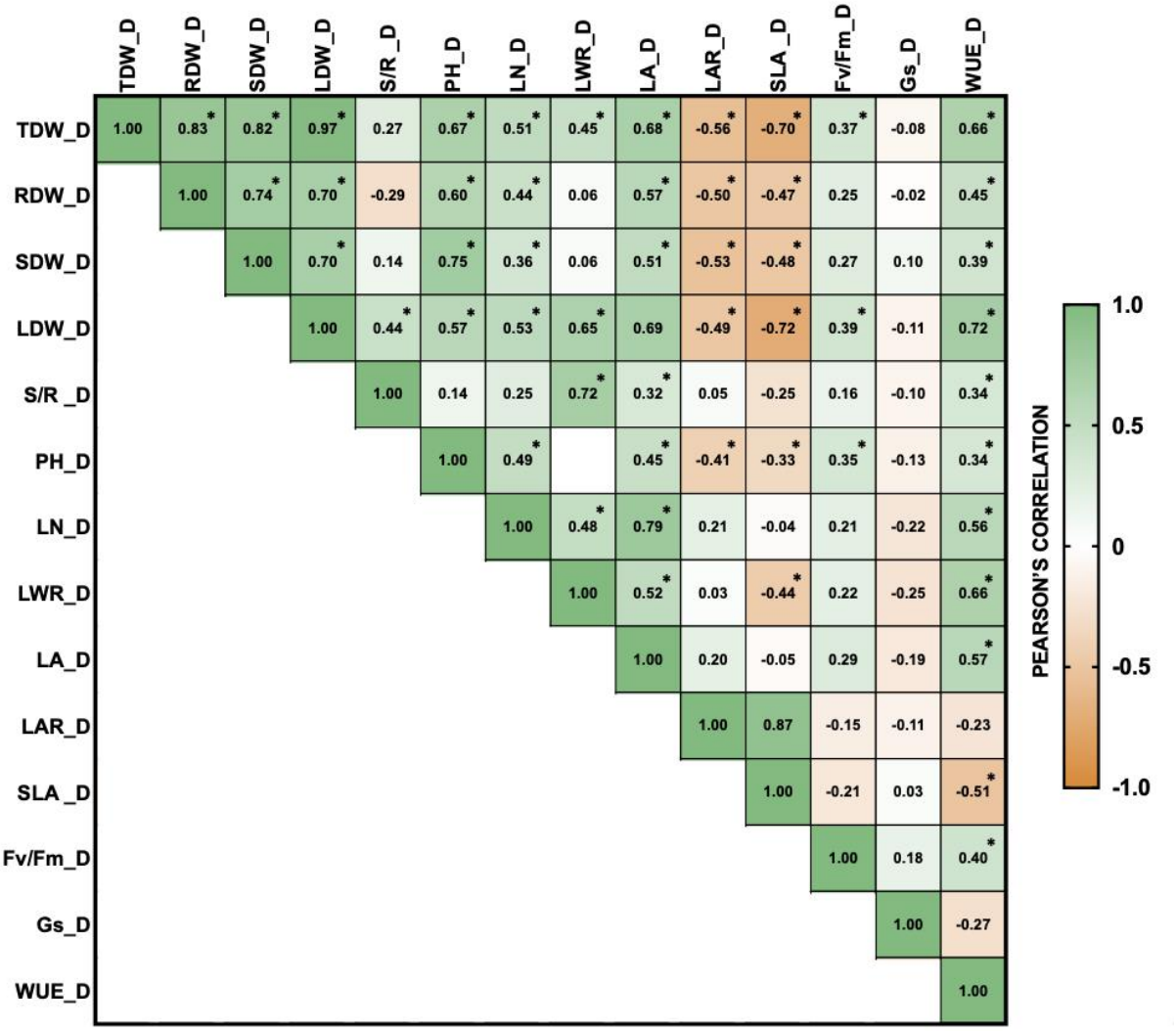


Figure 3 - Pearson's correlations coefficient for the percentage of decrease (_D) of each evaluated morpho-physiological parameters from the 40 screened tomato genotypes and wild relatives exposed to combined nitrogen and water deficit (* indicates significant correlation at p=0.05). Green and orange colours represent positive or negative correlations, respectively. Total dry weight = TDW; root dry weight = RDW, stem dry weight = SDW, leaf dry weight = LDW, shoot/root ratio = S/R, plant height = PH, leaf number = LN, leaf weight ratio = LWR, leaf area = LA, leaf area ratio = LAR, specific leaf area = SLA, photosynthetic efficiency = F_v/F_m, stomatal conductance = *gs*, Water use efficiency = WUE.

3.2. Multivariate statistical analyses of the genotypic variation

Results from the PCA yielded four main factors (those with an Eigenvalue >1), explaining over 82.4 % of the total variance (Table S2, Figure 4). This result reflects a close relationship between the evaluated traits and the genotype ability to handle with the combined deficit (Figure 4). Moreover, our study also revealed considerable phenotypical (and presumably genetic) diversity among the screened genotypes with the first two principal components explaining 60.7% of the variability (PC1 – 42.1% and PC2 – 18.6%), which demonstrates a different behavior when exposed to the combined N and water deficit (Figure 5). A further integration of these data in a cluster analysis separated the genotypes into three

distinct clusters based on their ability to cope with the combined deficit. Cluster 1 was composed by 15 genotypes (having 13 old cultivars, one modern inbred line and one modern hybrid), cluster 2 held 16 genotypes (13 old cultivars, two modern hybrids and one of the wild relatives) and cluster 3 comprised 9 genotypes (eight old cultivars and one wild relative) (Table 1). According to Figure 4, clusters 2 and 3 were both correlated with severe decreases in LDW, SDW and RDW when compared to cluster 1, pointing towards contrasting behavior among these groups. Genotypes from cluster 1, especially genotype 1, 2 and 3 (old cultivars), were the most tolerant genotypes in their responses to combined N and water deficit showing the lowest percentage decrease in TDW (Figure 1). In general, this cluster was characterized by a marked percentage of decrease in SLA, and LAR but also a higher percentage of decrease in g_s (although less pronounced). In all the other morphophysiological traits these genotypes had also a lower percentage of decrease. Cluster 2 mostly included genotypes with a higher TDW under control conditions and genotypes with a higher percentage of decrease in PH, SDW and RDW, as well as genotypes showing a lower percentage of decrease in LAR, SLA and g_s, all when grown under combined N and water deficit. Considering cluster 3, the parameters that were associated with their worst performance were the percentage of decrease in traits such as WUE, LWR, LA, S/R, LN and to a lower extent with the percentage of decrease in F_v/F_m.

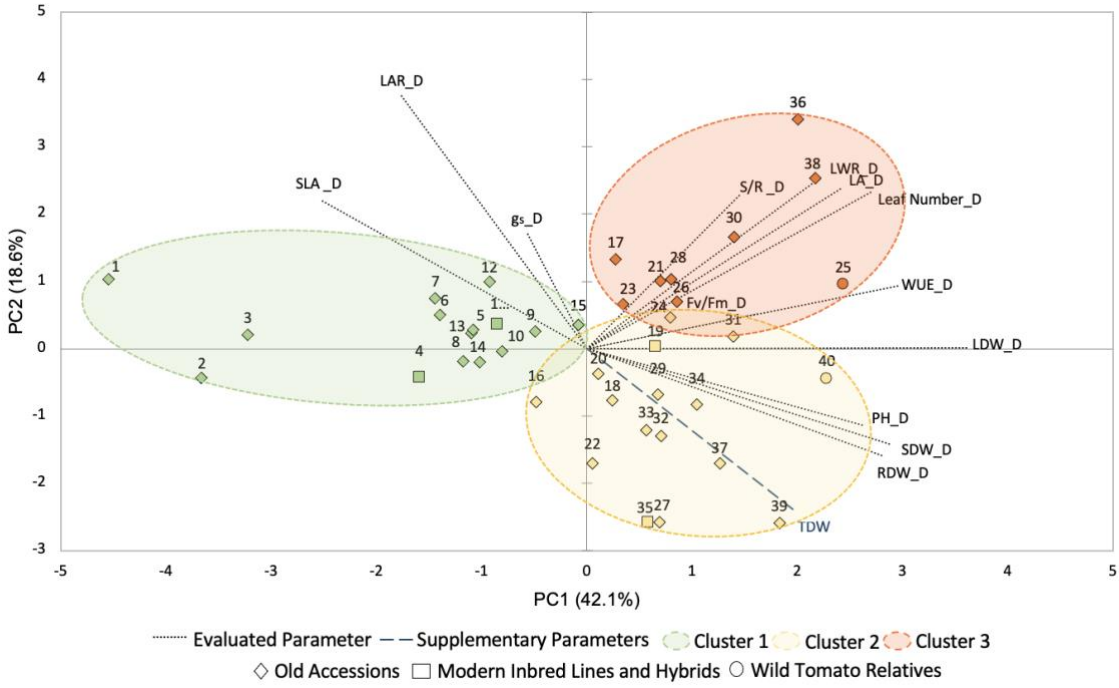


Figure 4 - Principal component analysis (PCA) of the percentage of decrease (_D) of 14 morpho-physiological traits evaluated in a total of 40 screened tomato genotypes and wild relatives subjected to combined nitrogen and water deficit. The two principal components (PC1, PC2) explained 60.7 % of the total variance. Different symbols used in the genotype number correspond to a different category: old accessions (◊); modern inbred lines and hybrids (□); wild tomato relatives (○). Total dry weight = TDW; root dry weight = RDW, stem dry weight = SDW, leaf dry weight = LDW, shoot/root ratio = S/R, plant height = PH, leaf number = LN, leaf weight ratio = LWR, leaf area = LA, leaf area ratio = LAR, specific leaf area = SLA, photosynthetic efficiency = F_v/F_m, stomatal conductance = g_s, Water use efficiency = WUE. The identification of the genotypes is given on Table 1

When characterizing the response of each cluster independently (Table S1), when subjected to the combined deficit, making use of all 14 morpho-physiological traits (Figure 5, 6 and 7) significantly differences were often found between them. For instance, the genotypes in cluster 1 had a percentage of decrease of TDW of around 58.0 ± 6.4 % whereas for the genotypes in clusters 2 and 3 this decrease was significantly higher reaching 69.8 ± 2.6 % and 69.3 ± 2.0 %, respectively (Figure 5a). This trend was also found for the biomass of the different plant organs (roots, stem, leaves) with cluster 1 always showing a significantly lower percentage of decrease on organ dry weight, when subjected to the combined deficit as compared to clusters 2 and 3. These results imply a differential response to the combined deficit conditions in the studied set of genotypes, with the ones from cluster 1 having lower losses of dry weight when exposed to the combined deficit, which suggests a more efficient behavior. All clusters responded with a decrease in S/R (Figure 5e), reflecting a higher partitioning towards the roots under combined deficit. However, this relative investment in the roots did not significantly vary among clusters.

Concerning the percentage of decrease in PH, no statistical differences were found among clusters (Figure 6a). However, the percentage of decrease in LN was significantly different, with cluster 1 and 2 having lower average values (29.8 ± 8.4 and 31.4 ± 4.4, respectively) than cluster 3 (42.8 ± 7.9) (Figure 6b). The same pattern was observed for the percentage of decrease in LWR (Figure 6c) and in LA (Figure 6d). However, for LAR cluster 2 showed the lowest decrease percentages (21.2 ± 7.1 %), being statistically different from cluster 1 (38.3 ± 4.3 %) and cluster 3 (38.3 ± 5.4 %) (Figure 6e). Finally concerning the clusters' response to combined N+W deficit in terms of SLA, cluster 1 showed a significantly higher percentage of decrease (33.2 ± 6.0 %) thus demonstrating a better ability to increase leaf thickness under this combined abiotic stress (Figure 6f).

Regarding the physiological traits, $F_v/F_{m,}$ only showed a significant difference among clusters 1 and 3, with the later having the higher percentage of decrease (6.5 ± 2.1 % in cluster 3 and 4.3 ± 2.1 % in cluster 1) (Figure 7a). For g_s significant differences were found among cluster 2 and 3, with the later having again the highest percentage of decrease (57.7 ± 14.0 % in cluster 3 and 44.8 ± 14.3 % in cluster 2 (Figure 7b). Finally, significant differences were also found for the percentage of decrease in WUE (Figure 7c). Cluster 1 had the most efficient genotypes in terms of water use, with the lowest percentages of decrease (11.9 ± 6.6), whereas cluster 2 and 3 were not significantly different among each other (resulting in an average value of 25.3 ± 5.8 and 33.7 ± 15.2, respectively) (Figure 7c).

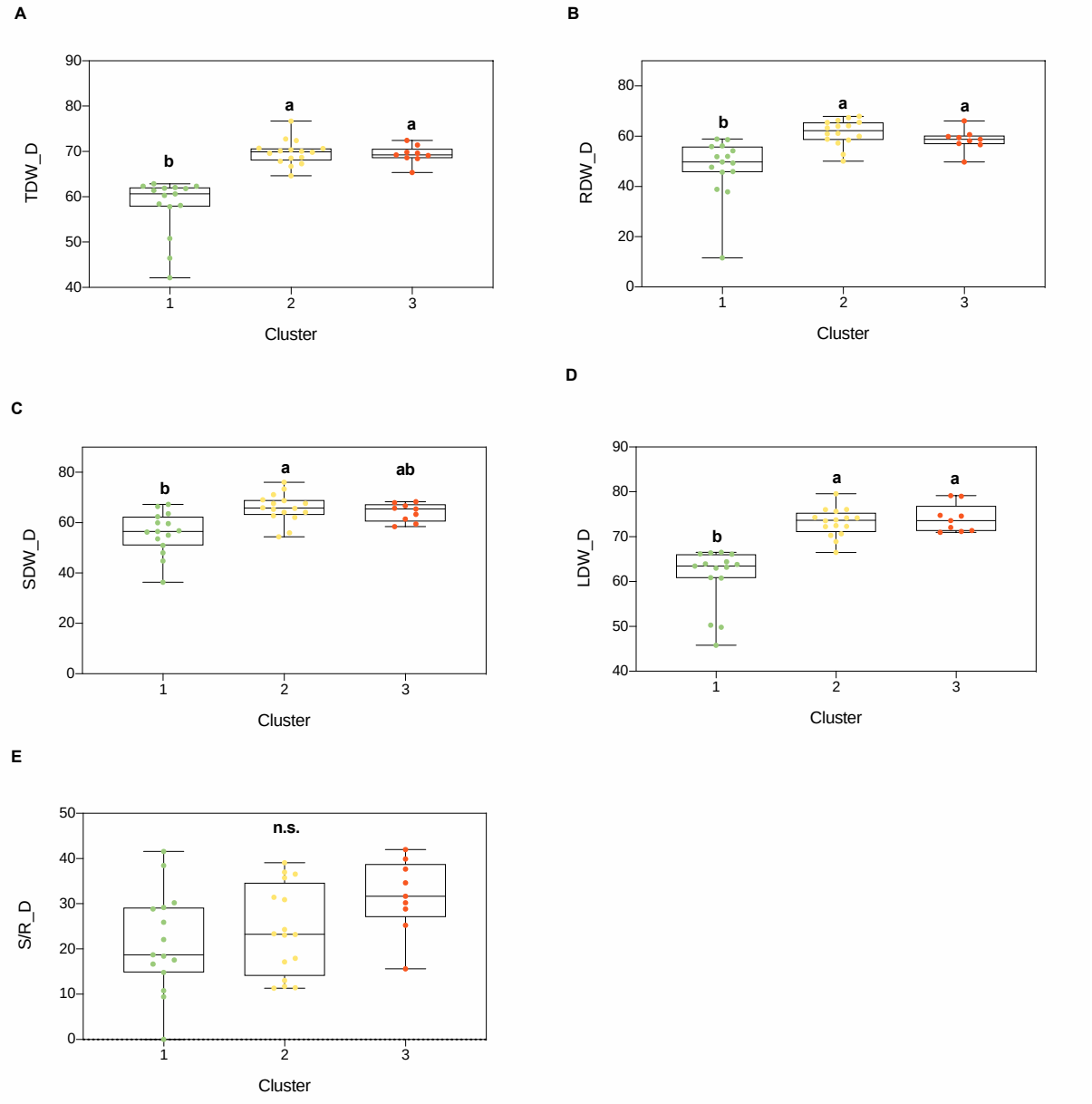


Figure 5 - Box and whisker plots of the different clusters concerning the percentage of decrease in total **(A)** root **(B)** stem **(C)** leaf **(D)** dry weight and shoot root ratio **(E)** in the 40 screened tomato genotypes and wild relatives subjected to combined nitrogen and water deficit. Boundaries of the boxes represent the 1st (lower limit) and the 3rd quartile (upper limit), while the black line inside the box marks the median. The whiskers, above and below the box, extend to the maximum and the minimum values, respectively. Different letters above box-plots indicate significant statistical differences and n.s. correspond to non-significant differences among clusters according to Kruskal–Wallis test at p=0.05. Colored points represent the average values for each genotype within the corresponding cluster. Total dry weight = TDW; root dry weight = RDW, stem dry weight = SDW, leaf dry weight = LDW, shoot/root ratio = S/R.

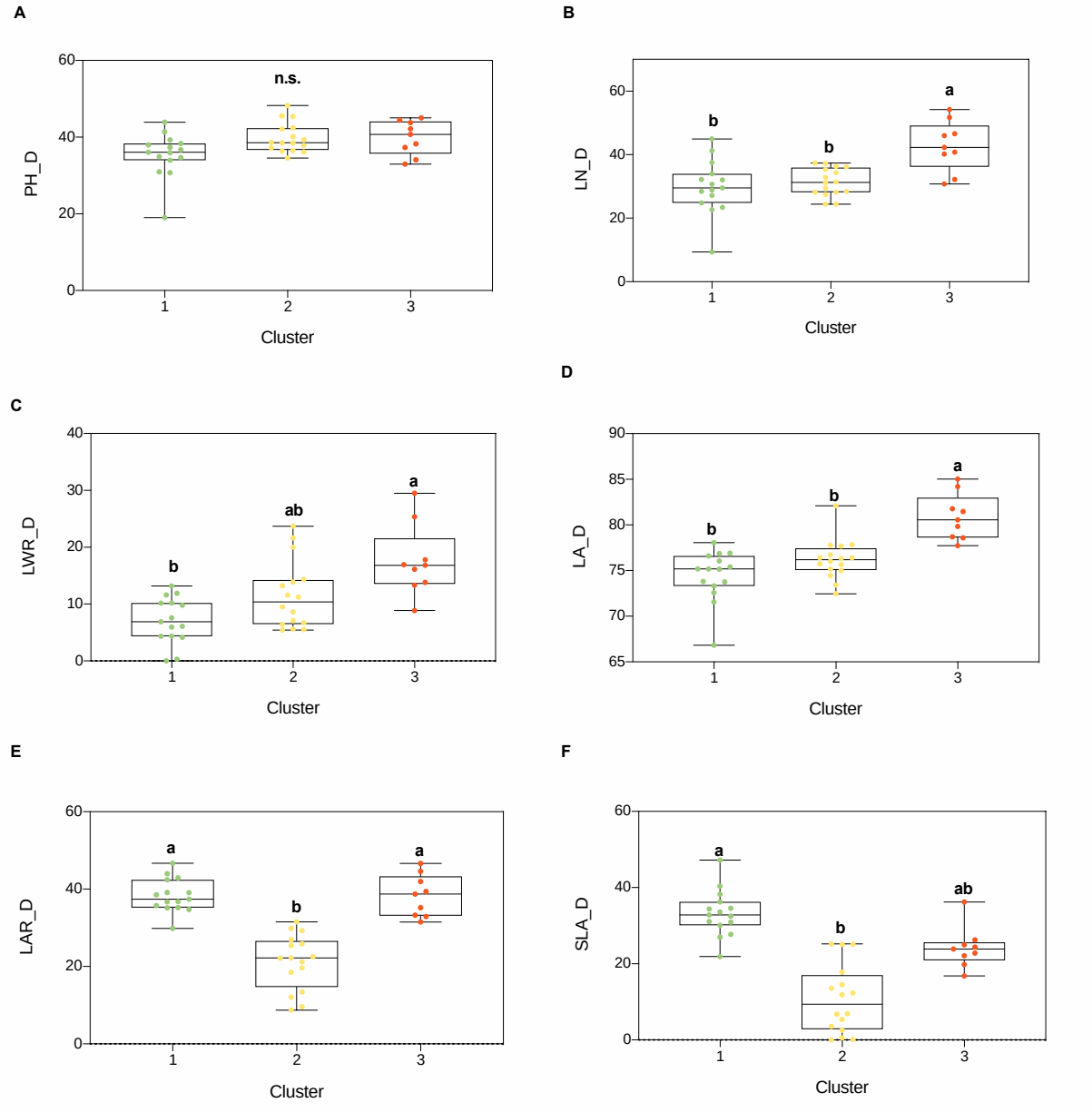


Figure 6 - Box and whisker plots of the different clusters concerning the percentage of decrease in plant height **(A)** leaf number **(B)** leaf weight ratio **(C)** leaf area **(D)** leaf area ratio **(E)** and specific leaf area **(F)** in the 40 screened tomato genotypes and wild relatives subjected to combined nitrogen and water deficit. Boundaries of the boxes represent the 1st (lower limit) and the 3rd quartile (upper limit), while the black line inside the box marks the median. The whiskers, above and below the box, extend to the maximum and the minimum values, respectively. Different letters above box-plots indicate significant statistical differences and n.s. correspond to non-significant differences among clusters according to a Kruskal–Wallis test at p=0.05. Colored points represent the average values for each genotype within the corresponding cluster. Plant height = PH, leaf number = LN, leaf weight ratio = LWR, leaf area = LA, leaf area ratio = LAR, specific leaf area = SLA.

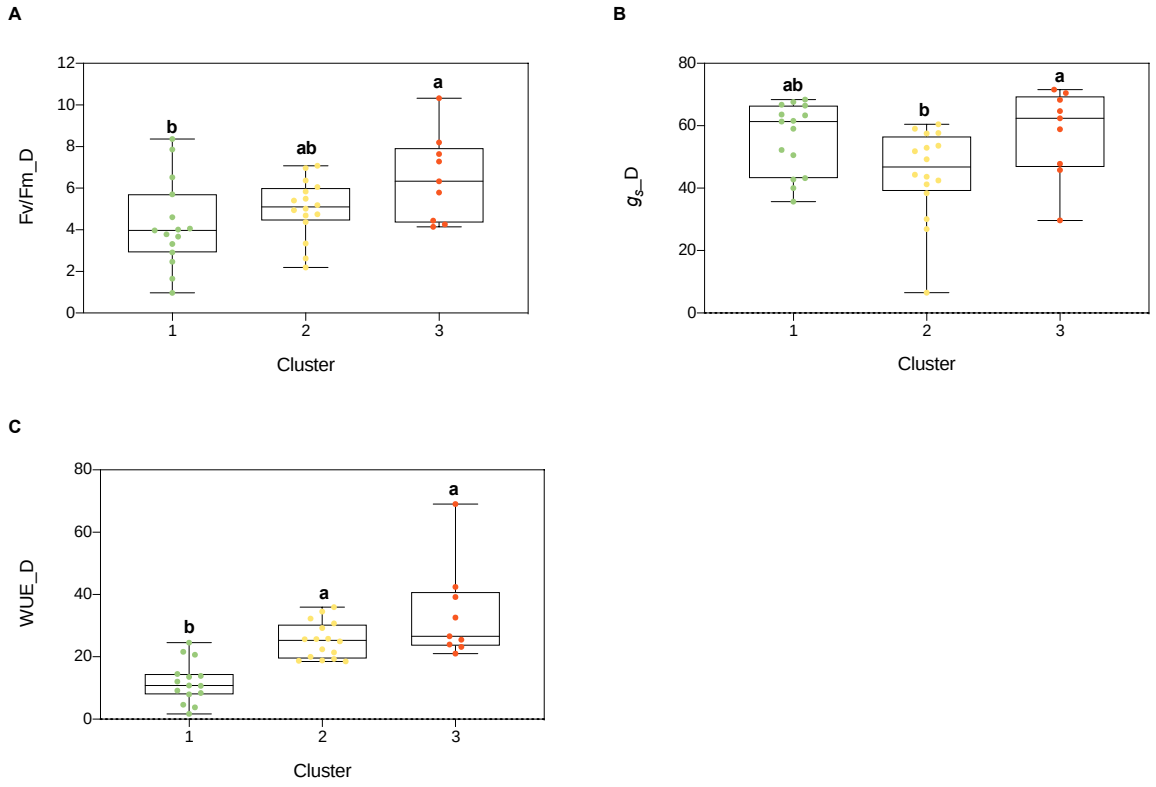


Figure 7 - Box and whisker plots of the different clusters concerning the percentage of decrease in the photosynthetic efficiency (F_v/F_m,) **(A)** stomatal conductance (g_s) **(B)** water use efficiency (WUE) **(C)** in the 40 screened tomato genotypes and wild relatives subjected to combined nitrogen and water deficit. Boundaries of the boxes represent the 1st (lower limit) and the 3rd quartile (upper limit), while the black line inside the box marks the median. The whiskers, above and below the box, extend to the maximum and the minimum values, respectively. Different letters above box-plots indicate significant statistical differences among cluster according to Kruskal–Wallis test at p=0.05. n.s. = not significant. Colored points represent the average values for each genotype within the corresponding cluster. Photosynthetic efficiency = F_v/F_m, stomatal conductance = g_s, water use efficiency = WUE.

4. Discussion

It is essential to enhance crop resilience to abiotic stresses towards sustainable food production systems (Carvalho and Vasconcelos, 2013). Nonetheless, this requires phenotypic variability being also of utmost importance to identify morpho-physiological traits associated with tolerance to these combined stresses. So far there is limited knowledge on tomato phenotypic variability to combined N and water deficit. Here we have screened the response of a large set of tomato genotypes to this combined deficit. In general, a vast majority of tomato cultivars are frequently referred has sensitive to these abiotic stresses (Machado *et al.*, 2022; Shirasawa *et al.*, 2010). Moreover, old accessions/cultivars (e.g. landraces and heirlooms) represent an underexploited wealth of genetic variation (Roohanitaziani *et al.*, 2020), that has been discarded due to undesired agronomic traits, but might encompass higher NUE and WUE than modern tomato cultivars. From our selection of 40 genotypes (including 34 old cultivars, four modern inbred lines/hybrids and two wild tomato relatives) it was found a large

variability in response to combined N and water deficit. The combined deficit severely affected all growth parameters, but to a different degree depending on the genotype and trait. Already after the second week of the imposed N and water deficit, it was visually observed that plants started to present phenotypic variability, with regard to the symptoms' severity. For instance, the older leaves become lighter green/ yellow (reflecting a common N deficiency symptom; (de Bang *et al.*, 2021)) and these have fallen at variable degrees amongst genotypes. Thereafter, at the end of the experimental period, the percentages of decrease in TDW ranged from 42 to 77 % (Figure 1 and 2). Previous studies also found some phenotypic variability in tomato, but they were limited to a much lower number of genotypes and were focused on the plant responses to independent stresses (Abenavoli *et al.*, 2016; Sánchez-Rodríguez *et al.*, 2010a).

PCA revealed considerable phenotypical diversity among the studied genotypes with four principal components explaining 82% of the variability (Figure 4). A further integration of our data, in a cluster analysis, was able to separate the screened genotypes into three distinct clusters based on their ability to handle the combined deficit (Figure 4). All genotypes responded to the combined deficit by changing the morpho-physiological traits, but genotypes from cluster 1 (composed only by 13 old accessions and two modern inbred lines/hybrids) showed higher tolerance under these conditions, pointing towards a more efficient behavior. In general, these tolerant genotypes showed a lower percentage of decrease in TDW as compared with more sensitive genotypes. Moreover, they were closely associated with traits such as a lower SLA, lower LAR and a higher WUE but also partly associated with lower g_s. Different authors have reported a negative effect on the dry weight of leaves, stems, and roots, and consequently on TDW when tomato plants were exposed to one of these abiotic stresses (Al Hassan *et al.*, 2015; Luna *et al.*, 2014; Khan *et al.*, 2015; Okunlola *et al.*, 2015: Zhou *et al.*, 2019). For instance, it has been found that water deficit at the early seedling stage might lead to higher RDW, longer roots and lower S/R, with all these traits being widely accepted as accurate indicators of the plant's ability to withstand drought stress (Makhadmeh *et al.*, 2022). Tripodi *et al.* (2022) also demonstrated that root architecture played an important role on tomato plant responses to N starvation or water deficit. In our study, the tomato genotypes from cluster 1 showed lower percentages of decrease in RDW (Figure 4 and 5b) but no significant differences were found concerning the S/R (Figure 5e).

Regarding SLA and LAR, the higher percentage of decrease in cluster 1 suggests that increasing leaf thickness (lower SLA) and investing less of their LDW in LA seems to be related to tomato ability to tolerate combined N and water deficit. Moreover, the reduction of SLA is believed to be a possible strategy to improve WUE. This is due to thicker leaves generally having a higher concentration of chlorophyll and proteins per leaf area unit leading to a greater

photosynthetic capacity and, consequently, a higher biomass production per unit leaf area than thinner leaves (Chatterjee and Solankey, 2015). Higher WUE can be defined as the amount of carbon assimilated as biomass produced per unit of water used (Cantero-Navarro *et al.*, 2016; Ullah *et al.*, 2019). Thus, an improvement in WUE has been related to a higher closing stomata capacity, and lower transpiration rate, with these traits being associated with higher tolerance to drought stress (Galmés *et al.*, 2013; Raja *et al.*, 2020). On the other hand, some studies have shown that a low nutrient availability may reduce WUE and plants' capacity to adapt to drought (Sardans *et al.*, 2013). Moreover, a screening of tomato landraces for drought tolerance, based on growth and chlorophyll fluorescence analyses, identified F_v/F_m as one of the most sensitive parameters for the detection of drought stress impact on tomato plants (Sousaraei *et al.*, 2021). Indeed, in our study, g_s and F_v/F_m were severely affected by the combined deficit, with cluster 3 having the higher decreases (Figure 7a and b). Although a wide phenotypic variation in the percentage of decrease of these traits was observed (Figure 1), our results suggest that, for this tomato collection, g_s and F_v/F_m were not the key parameters in tomato tolerance response to combined N and water deficit (Figure 4).

The two accessions of the studied wild tomato relatives *S. pimpinellifolium* were not part of the tolerant cluster, which was somehow unexpected as wild relatives have been reported as important genetic sources of abiotic tolerance traits (Conesa *et al.*, 2019; Martínez-Cuenca *et al.*, 2020). Recently, Tripodi *et al.* (2022) studied 42 tomato genotypes (including elite cultivars, heirlooms, landraces, and hybrids) grown in open field under single nitrogen deficit (no-N fertilization) or under single water deficit (30% of the amount given to the control plants). Among the studied genotypes, the two hybrids had the best performance in terms of yield, when grown under N starvation or water deficit, whereas in general the landraces and heirlooms exhibited a better fruit quality. In our study, two of the four screened modern inbred lines/hybrids were also part of the tolerant cluster, but interestingly most of the old accessions from this cluster even presented a better performance for the studied morphophysiological traits when grown under combined deficit (Figure 1 and 4).

Some recent studies have pondered that the physiological and molecular responses of crops to a combination of two stresses are unique, resulting in the deployment of stress-adaptation strategies which sometimes are different and contrasting to those seen under individual stresses, and other times are shared and similar (Hussain *et al.*, 2018; Pandey *et al.*, 2015). This study was a first step to address tomato response to combined N and water deficit and showed that old accessions represent a very interesting wealth of genetic diversity in terms of response to these abiotic stresses, with some of them (Cluster 1) having even a better performance than the two studied wild relative accessions from *S. pimpinellifolium* (Figure 4). In future works, the efficiency of these accessions to cope with the combined N

and water deficit, should be evaluated in other development stages, especially at the generative phase, as the plant structures have a variable degree of genotypic plasticity to adapt to stressful conditions. Indeed, the flowering and fruit development stages have been described as the most sensitive ones to water deficits in tomato plants (Khapte *et al.*, 2019; Machado *et al.*, 2022). Plus, the impact on yield and fruit quality at harvest would give additional relevant information. Moreover, it would also be important to validate our results (performed under controlled conditions) in field trials, preferably including different experimental sites with contrasting pedoclimatic conditions to address possible genotype and environment interactions (G × E). For instance, it would be relevant to design an experiment to investigate the role of soil type [e.g a sandy soil, with high leaching potential (Ayankojo *et al.*, 2020), compared a clay soil, with high water retention capacity (Tripodi *et al.*, 2022)] as well as contrasting climates (e.g. high versus low evaporative demanding conditions) to better analyze the G × E interactions. Indeed, Tripodi *et al.* (2022) highlighted the importance of exploring such interactions, although from their study the genotype was the main source of variation, with G × T (i.e. single water deficit or no-nitrogen supply) and E × T being less relevant.

Finally, further research on the underlying mechanisms behind tolerance (including single vs combined stresses) would also be of utmost importance to contribute to sustainable food production. Due to the amount of work involved in those type of analysis, a selection of contrasting genotypes (identified in this study) would be a good approach to explore in-depth these aspects.

5. Conclusion

This study represents one step forward in the current knowledge since it enabled to explore the tomato phenotypic variability focusing on old cultivars and contributed to a better view of plants' response to combined N and water deficit. The results obtained showed, for the first time, that tomato has a large phenotypic variability in its response to the combined deficit, and that old cultivars might represent a valuable gene pool in terms of tolerance to these abiotic stresses. Moreover, the data integration in a cluster analysis enabled to separate the 40 screened genotypes into three distinct clusters, based on their ability to handle the combined deficit. All genotypes responded to the combined deficit by adjusting several morpho-physiological traits, but genotypes from cluster 1 showed a more efficient behavior having a significantly lower percentage of decrease in their TDW, but also in the dry weight of each organ type. In general, these more tolerant genotypes were strongly associated with a lower SLA, a lower LAR and higher WUE as compared with less tolerant genotypes. Moreover, they were also associated, but to a lesser extent, with a lower g_s. It is concluded that tomato

tolerance to combined N and water deficit significantly varied between genotypes, and that old cultivars represent a valuable gene pool towards sustainable food production systems. Additionally, the identified contrasting genotypes might have the potential to be used in future studies, namely, to explore the biochemical and molecular mechanisms behind tolerance to these combined abiotic stresses, or to explore the potential of tolerant genotypes as rootstocks for improved NUE and WUE.

Supplementary Material

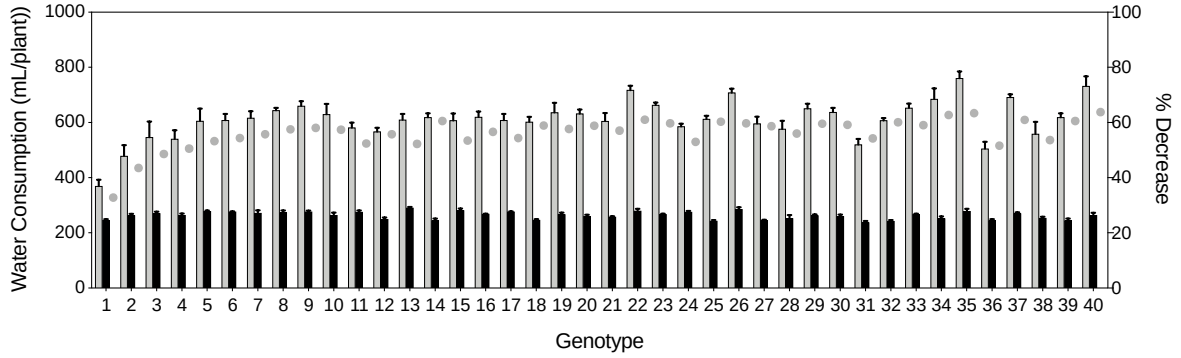


Figure S1 – Water consumption of the 40 screened tomato genotypes and wild relatives grown under control conditions (grey) and under combined nitrogen and water deficit. Circles represent the percentage of decrease on TDW in plants subjected to combined deficit. The genotypes were ordered from low to high percentage decrease in TDW and its identification is given on table 1. Data presented are mean ± SEM (n = 8).



Figure S2 – Example of the visual appearance of two tomato genotypes (3 (A) and 30 (B); Table 1). Plants on the left side of each photo were grown under control conditions, whereas plants on the right side were subjected to the combined nitrogen and water deficit. Genotype in panel A showed lower percentage decrease in several studied growth traits, behaving as a more tolerant genotype, than the one in panel B.

Table S1 - Effect of combined nitrogen and water deficit on different morpho-physiological traits found in the 40 screened tomato genotypes and wild relatives. Total dry weight = TDW; root dry weight = RDW, stem dry weight = SDW, leaf dry weight = LDW, shoot/root ratio = S/R, plant height = PH, leaf number = LN, leaf weight ratio = LWR, leaf area = LA, leaf area ratio = LAR, specific leaf area = SLA, photosynthetic efficiency = F_v/F_m, stomatal conductance = g_s, Water use efficiency = WUE. The identification of the genotypes is given on Table 1.

			Cluster	TDW (g)	RDW (g)	SDW (g)	LDW (g)	S/R	PH (cm)	LN	LWR	LA (cm^2)	LAR (cm^2g^{-1})	SLA (cm^2g^{-1})	F_v/F_m	g_s ($mmolm^{-2}s^{-1}$)	WUE ($mg\ mL^{-1}$)
GENOTYPE	1	CTR	1	1.19±0.02	0.16±0.01	0.27±0.01	0.75±0.02	6.7±0.6	22.9±0.7	9.0±0.4	0.63±0.01	219±2	184±3	291±7	0.83±0.00	497±14	3.3±0.1
		CMB		0.69±0.04	0.14±0.01	0.17±0.01	0.38±0.02	4.0±0.2	18.5±0.5	8.1±0.2	0.55±0.01	73±2	106±4	193±5	0.80±0.00	297±14	2.8±0.2
	2	CTR	1	1.79±0.16	0.41±0.05	0.32±0.02	1.06±0.09	3.5±0.3	35.5±1.0	9.6±0.2	0.59±0.00	403±33	225±2	381±4	0.83±0.00	660±38	3.7±0.1
		CMB		0.92±0.03	0.21±0.01	0.16±0.01	0.55±0.01	3.5±0.1	24.5±0.6	7.0±0.0	0.60±0.01	113±3	137±11	227±20	0.82±0.00	313±21	3.5±0. 1
	3	CTR	1	2.10±0.18	0.34±0.06	0,43±0.06	1.28±0.12	6.0±1.2	36.6±1.3	9.5±0.2	0.61±0.03	426±46	241±51	405±11	0.83±0.00	385±30	3.8±0. 1
		CMB		1.01±0.02	0.19±0.01	0.20±0.01	0.62±0.02	4.4±0.1	23.3±0.6	7.1±0.1	0.62±0.01	113±4	115±6	186±11	0.79±0.02	188±6	3.7±0.1
	4	CTR	1	2.08±0.16	0.29±0.03	0.58±0.06	1.21±0.07	6.3±0.3	53.9±1.6	7.9±0.3	0.58±0.01	252±12	124±5	211±5	0.82±0.00	614±29	3.8±0.1
		CMB		0.87±0.03	0.15±0.00	0.25±0.01	0.47±0.02	4.9±0.1	33.7±1.1	6.0±0.0	0.55±0.01	67±3	78±2	143±5	0.79±0.01	392±20	3.3±0.1
	5	CTR	1	2.51±0.26	0.41±0.05	0.61±0.07	1.50±0.16	5.4±0.4	42.2±1.2	11.0±0.2	0.60±0.00	430±11	181±24	303±39	0.83±0.01	569±16	4.1±0.2
		CMB		1.00±0.05	0.21±0.01	0.27±0.02	0.53±0.02	3.8±0.0	26.6±0.5	7.8±0.1	0.53±0.01	113±3	113±3	213±5	0.78±0.02	323±26	3.6±0.2
	6	CTR	1	2.42±0.20	0.43±0.05	0.39±0.02	1.60±0.14	4.8±0.2	35.7±0.8	9.6±0.2	0.66±0.00	450±9	191±17	290±27	0.84±0.01	622±52	4.0±0.2
		CMB		0.97±0.03	0.20±0.00	0.17±0.01	0.61±0.01	4.0±0.1	23.3±0.2	6.0±0.0	0.62±0.00	112±1	115±3	185±4	0.79±0.01	222±31	3.5±0.1
	7	CTR	1	2.93±0.03	0.47±0.02	0.73±0.05	1.82±0.08	5.4±0.1	48.9±1.6	10.4±0.1	0.62±0.02	462±13	158±2	255±7	0.83±0.00	693±49	4.9±0.1
		CMB		1.16±0.06	0.21±0.01	0.31±0.02	0.64±0.03	4.6±0.1	33.6±0.8	7.4±0.1	0.56±0.01	101±4	88±2	157±2	0.80±0.00	229±23	4.3±0.1
	8	CTR	1	2.87±0.09	0.54±0.04	0.54±0.02	1.87±0.07	4.6±0.3	39.5±0.5	11.0±0.2	0.65±0.01	491±11	172±3	265±7	0.83±0.00	638±45	4.6±0.2
		CMB		1.13±0.04	0.24±0.01	0.21±0.01	0.67±0.02	3.7±0.1	24.3±0.3	7.9±0.2	0.60±0.01	122±4	109±5	182±6	0.79±0.01	208±13	4.1±0.2

GENOTYPE																
	9	CTR	1	2.49±0.14	0.55±0.02	0.50±0.04	1.44±0.08	3.6 ±0.1	40.5±0.9	15.3±0.3	0.58±0.00	395±34	160±16	275±25	0.83±0.00	676±21	3.8±0.2
		CMB		0.95±0.05	0.26±0.01	0.16±0.01	0.53±0.02	2.7±0.1	22.7±0.4	8.4±0.1	0.55±0.00	96±2	101±6	183±11	0.79±001	219±3.7	3.4±0.1
	10	CTR	1	2.83±0.23	0.58±0.05	0.53±0.04	1.72±0.15	4.0±0.1	43.0±0.4	11.6±0.2	0.61±0.00	434±31	159±22	262±37	0.82±0.00	640±37	4.5±0.1
		CMB		1.05±0.03	0.23±0.00	0.21±0.01	0.61±0.01	3.6±0.1	26.7±0.4	7.9±0.1	0.58±0.00	98±4	94±6	161±10	0.75±0.02	231±10	4.0±0.1
	11	CTR	1	2.80±0.21	0.50±0.03	0.57±0.11	1.73±0.08	4.6±0.1	53.6±0.7	9.4±0.3	0.62±0.02	439±14	162±15	259±14	0.84±0.00	506±31	4.8±0.3
		CMB		1.04±0.04	0.20±0.01	0.26±0.02	0.58±0.02	4.1±0.1	34.3±1.1	5.5±0.4	0.56±0.00	105±4	100±3	180±5	0.81±0.00	289±45	3.8±0. 1
	12	CTR	1	2.84±0.19	0.47±0.04	0.48±0.02	1.89±0.13	5.2±0.1	38.3±0.8	9.4±0.2	0.67±0.00	494±17	180±9	270±15	0.83±0.00	599±59	5.0±0.2
		CMB		1.06±0.02	0.23±0.01	0.21±0.00	0.62±0.01	3.7±0.1	24.9±0.5	6.5±0.2	0.59±0.01	115±4	109±5	185±6	0.79±0.00	181±6	4.3±0.1
	13	CTR	1	2.82±0.11	0.49±0.02	0.49±0.01	1.85±0.08	4.9±0.1	41.1±1.1	11.0±0.2	0.65±0.00	554±13	199±10	305±17	0.83±0.00	592±29	4.7±0.1
		CMB		1.06±0.04	0.21±0.01	0.18±0.00	0.66±0.03	4.0±0.2	27.1±0.2	7.3±0.1	0.63±0.00	128±3	121±4	194±6	0.81±0.00	230±24	3.6±0.1
	14	CTR	1	2.42±0.16	0.50±0.05	0.48±0.03	1.44±0.09	3.9±0.2	41.2±0.5	12.6±0.3	0.60±0.01	458±12	195±14	326±20	0.84±0.00	554±15	3.9±0.2
		CMB		0.90±0.03	0.24±0.01	0.17±0.01	0.48±0.01	2.7±0.0	25.0±0.4	9.8±0.1	0.54±0.01	113±2	127±7	234±10	0.81±0.00	226±9	3.7±0. 3
	15	CTR	1	2.71±0.13	0.38±0.04	0.78±0.06	1.56±0.04	6.3±0.6	56.7±1.7	9.9±0.6	0.58±0.01	340±27	129±17	222±24	0.82±0.00	666±34	4.5±0. 1
		CMB		1.00±0.04	0.22±0.01	0.26±0.01	0.52±0.03	3.6±0.1	33.1±0.8	6.6±0.2	0.52±0.01	87.1±4	88.0±3	168±8	0.76±0.01	257±20	3.6±0. 2
	16	CTR	2	2.82±0.16	0.61±0.04	0.47±0.01	1.75±0.11	3.7±0.1	35.6±0.9	10.5±0.2	0.62±0.00	488±5	175±10	283±17	0.84±0.00	573±89	4.5±0. 1
		CMB		0.99±0.03	0.23±0.01	0.18±0.01	0.58±0.02	3.3±0.1	22.6±0.3	7.5±0.3	0.59±0.00	121±3	123±2	210±2	0.79±0.01	219±16	3.7±0.1
	17	CTR	3	3.16±0.21	0.44±0.04	0.71±0.05	2.01±0.13	6.5±0.3	45.7±0.8	9.6±0.5	0.64±0.01	565±17	182±13	287±20	0.82±0.00	521±24	5.2±0.2
		CMB		1.08±0.03	0.22±0.01	0.29±0.01	0.57±0.02	4.0±0.1	28.6±0.6	6.5±0.2	0.53±0.01	126±7	117±5	220±5	0.76±0.00	153±6	3.9±0.1
	18	CTR	2	2.84±0.05	0.46±0.02	0.62±0.04	1.77±0.02	5.3±0.3	39.1±1.1	14.5±0.2	0.62±0.01	527±13	186±4	298±6	0.84±0.00	354±15	4.7±0.1
		CMB		0.95±0.03	0.19±0.01	0.21±0.02	0.55±0.01	4.0±0.1	22.5±1.1	9.3±0.1	0.58±0.01	123±3	130±6	223±7	0.80±0.01	257±30	3.8±0.1

GENOTYPE																
	19	CTR	2	2.18±0.15	0.24±0.02	0.47±0.03	1.47±0.11	7.9±0.1	47.0±0.9	7.6±0.1	0.68±0.01	245±5	114±7	169±12	0.83±0.00	620±21	3.4±0.1
		CMB		0.71±0.02	0.12±0.00	0.21±0.01	0.38±0.02	5.0±0.1	28.9±0.6	5.1±0.2	0.54±0.01	62.7±3	89.0±2	167±2	0.78±0.01	285±16	2.7±0.1
	20	CTR	2	3.21±0.14	0.53±0.02	0.71±0.03	1.97±0.11	5.1±0.1	42.6±1.3	10.6±0.4	0.61±0.01	497±20	159±13	262±25	0.84±0.00	437±42	5.1±0. 2
		CMB		1.03±0.02	0.19±0.01	0.26±0.00	0.57±0.01	4.5±0.1	27.1±0.5	7.3±0.1	0.56±0.01	110±2	107±2	191±4	0.78±0.01	199±28	4.0±0. 1
	21	CTR	3	2.51±0.20	0.36±0.03	0.58±0.05	1.57±0.13	6.0±0.2	46.4±1.7	10.6±0.4	0.62±0.00	440±14	178±9	285±16	0.83±0.00	546±19	4.1±0.1
		CMB		0.78±0.02	0.15±0.01	0.18±0.01	0.45±0.02	4.2±0.1	25.7±0.5	6.3±0.3	0.57±0.01	80±5	103±4	179±8	0.79±0.00	295±8	3.0±0.1
	22	CTR	2	3.46±0.16	0.70±0.03	0.64±0.03	2.12±0.12	4.0±0.2	41.3±0.9	9.9±0.5	0.61±0.01	514±12	151±10	247±20	0.83±0.00	460±39	4.8±0. 2
		CMB		1.08±0.03	0.24±0.00	0.22±0.01	0.62±0.02	3.5±0.1	25.0±0.3	7.4±0.2	0.58±0.01	125±3	115±3	200±3	0.79±0.00	229±15	3.9±0.1
	23	CTR	3	3.36±0.11	0.44±0.01	0.67±0.02	2.17±0.07	6.8±0.2	44.0±1.2	10.1±0.2	0.65±0.02	510±9	153±7	237±9	0.82±0.00	614±43	5.0±0.1
		CMB		1.05±0.01	0.19±0.00	0.26±0.00	0.61±0.01	4.6±0.0	27.1±0.1	7.0±0.0	0.58±0.00	108±9	103±9	180±16	0.76±0.00	211±18	3.9±0.1
	24	CTR	2	1.99±0.06	0.26±0.01	0.45±0.01	1.24±0.04	6.6±0.2	47.9±0.5	8.3±0.4	0.62±0.02	196±6	99±3	159±8	0.84±0.01	476±49	3.3±0.1
		CMB		0.62±0.03	0.12±0.00	0.20±0.02	0.30±0.01	4.1±0.2	31.3±1.3	5.1±0.1	0.49±0.01	46±2	75±1	154±2	0.82±0.01	261±13	2.3±0.1
	25	CTR	3	2.86±0.07	0.69±0.02	0.54±0.01	1.63±0.04	3.1±0.1	42.1±1.2	17.5±0.7	0.57±0.00	441±8	157±8	275±14	0.86±0.00	355±22	8.2±2.1
		CMB		0.88±0.00	0.27±0.00	0.20±0.01	0.41±0.01	2.3±0.1	23.1±0.7	9.4±0.3	0.47±0.01	95±3	107±3	228±8	0.77±0.00	186±20	2.5±0. 6
	26	CTR	3	3.34±0.17	0.55±0.02	0.88±0.06	1.92±0.09	5.1±0.1	53.2±1.8	13.0±0.0	0.57±0.01	423±8	128±8	222±11	0.83±0.00	715±31	4.7±0.2
		CMB		1.02±0.03	0.22±0.01	0.30±0.01	0.50±0.02	3.6±0.1	31.4±0.7	7.5±0.2	0.49±0.01	85±5	84.0±5	170±7	0.79±0.00	291±16	3.6±0.1
	27	CTR	2	3.56±0.20	0.71±0.05	0.93±0.05	1.92±0.12	4.0±0.1	50.5±1.2	11.8±0.1	0.54±0.01	505±11	145±6	268±14	0.85±0.00	472±20	6.0±0.2
		CMB		1.07±0.02	0.25±0.01	0.30±0.01	0.52±0.02	3.3±0.1	31.1±0.7	8.9±0.1	0.49±0.01	139±3	130±1	267±7	0.79±0.01	134±22	4.4±0.1
	28	CTR	3	2.01±0.14	0.48±0.04	0.38±0.03	1.15±0.13	3.3±0.4	31.9±2.3	12.1±0.3	0.57±0.03	281±33	140±12	246±13	0.84±0.00	365±23	3.5±0.2
		CMB		0.60±0.08	0.16±0.01	0.13±0.02	0.31±0.06	2.5±0.4	20.7±0.6	7.3±0.3	0.49±0.04	52±7	93±7	194±26	0.80±0.01	253±12	2.3±0.3

GENOTYPE																
	29	CTR	2	3.57±0.13	0.58±0.02	0.80±0.03	2.19±0.08	5.0±0.1	46.1±0.6	9.8±0.1	0. 61±0.00	559±9	159±5	259±8	0.84±0.00	285±34	5.5±0.1
		CMB		1.07±0.03	0.23±0.00	0.28±0.01	0.56±0.01	3.8±0.1	28.9±0.7	7.0±0.0	0.52±0.00	124±5	116±3	221±4	0.77±0.01	103±4	4.1±0.1
	30	CTR	3	3.46±0.14	0.73±0.03	0.65±0.02	2.05±0.08	3.7±0.0	43.2±0.5	15.3±0.3	0.59±0.00	621±17	181±6	304±10	0.82±0.00	338±26	5.4±0.1
		CMB		1.04±0.05	0.31±0.03	0.22±0.00	0.52±0.03	2.4±0.1	25.0±0.5	8.1±0.4	0.50±0.01	115±7	111±4	223±7	0.78±0.00	190±21	4.0±0.3
	31	CTR	2	3.30±0.25	0.51±0.05	0.87±0.07	1.92±0.13	5.6±0.2	53.1±1.0	10.6±0.2	0.58±0.00	521±18	161±7	275±11	0.83±0.00	440±48	6.3±0.3
		CMB		0.97±0.01	0.21±0.01	0.30±0.01	0.45±0.01	3.6±0.2	33.0±0.7	6.8±0.3	0.47±0.00	116±6	120±4	256±7	0.79±0.00	169±9	4.1±0.1
	32	CTR	2	3.89±0.09	0.67±0.02	0.73±0.03	2.49±0.07	4.9±0.1	44.2±1.2	9.8±0.1	0.64±0.00	569±10	147±4	229±7	0.82±0.00	261±9	6.4±0.1
		CMB		1.16±0.03	0.24±0.01	0.22±0.00	0.69±0.02	3.8±0.1	25.5±0.4	6.9±0.1	0.60±0.00	136±2	118±2	197±4	0.80±0.01	161±12	4.8±0.1
	33	CTR	2	3.57±0.12	0.60±0.03	0.70±0.04	2.17±0.06	4.8±0.2	41.2±1.1	10.1±0.4	0.61±0.02	538±21	152±10	248±9	0.84±0.00	575±41	5.3±0.0
		CMB		1.05±0.01	0.25±0.01	0.24±0.01	0.57±0.01	3.3±0.2	26.2±0.2	7.3±0.1	0.54±0.00	134±4	127±3	234±4	0.81±0.02	236±20	3.9±0.1
	34	CTR	2	2.91±0.19	0.56±0.05	0.88±0.05	1.44±0.13	4.2±0.2	58.0±3.5	12.3±0.5	0.49±0.02	408±30	142±10	287±14	0.86±0.01	496±87	4.3±0.4
		CMB		0.85±0.04	0.22±0.02	0.25±0.02	0.38±0.03	2.8±0.1	31.3±3.1	7.6±0.7	0.44±0.03	95±6	111±5	252±8	0.82±0.00	205±32	3.4±0.2
	35	CTR	2	3.60±0.15	0.54±0.04	0.93±0.03	2.13±0.08	5.8±0.3	56.3±2.0	8.8±0.3	0.59±0.00	403±23	114±11	191±18	0.83±0.00	583±28	4.7±0.1
		CMB		1.05±0.02	0.17±0.01	0.29±0.01	0.59±0.01	5.1±0.3	34.4±0.7	6.0±0.0	0.56±0.01	106±1	101±3	181±3	0.78±0.01	184±8	3.8±0.1
	36	CTR	3	2.18±0.19	0.55±0.06	0.50±0.05	1.13±0.08	2.9±0.2	40.3±1.3	17.1±0.8	0.52±0.01	306±14	142±7	272±9	0.83±0.00	412±41	4.3±0.2
		CMB		0.60±0.04	0.22±0.01	0.15±0.01	0.23±0.03	1.7±0.3	27.0±0.5	8.1±0.7	0.37±0.03	47±9	78.0±9	207±9	0.79±0.03	172±14	2.5±0.2
	37	CTR	2	3.99±0.15	0.65±0.03	0.92±0.05	2.42±0.09	5.2±0.2	47.1±0.7	11.4±0.1	0.61±0.01	548±6	138±6	228±8	0.83±0.01	271±26	5.8±0.2
		CMB		1.10±0.04	0.22±0.01	0.30±0.01	0.58±0.02	3.9±0.1	28.2±0.6	8.3±0.1	0.53±0.01	134±2	121±3	231±6	0.80±0.01	187±9	4.1±0.1
	38	CTR	3	1.95±0.12	0.44±0.04	0.27±0.04	1.24±0.06	3.5±0.2	31.8±0.7	14.8±0.5	0.64±0.02	322±15	168±13	263±23	0.83±0.01	364±54	3.5±0.2
		CMB		0.53±0.04	0.17±0.00	0.10±0.00	0.26±0.03	2.1±0.2	17.9±0.3	6.8±0.1	0.48±0.02	48±2	93±8	197±25	0.78±0.00	314±29	2.1±0.1

39	**CTR**	**2**	3.60±0.15	0.78±0.03	0.98±0.03	1.83±0.12	3.6±0.3	49.7±0.8	15.3±0.1	0.51±0.01	530±14	148±8	293±22	0.83±0.00	350±28	5.8±0.1
	CMB		0.97±0.04	0.25±0.01	0.26±0.01	0.46±0.03	2.9±0.1	25.7±1.4	9.9±0.6	0.47±0.01	124±9	127±5	268±7	0.80±0.01	193±9	4.0±0.3
40	**CTR**	**2**	3.98±0.28	0.66±0.06	0.80±0.06	2.52±0.16	5.1±0.2	46.1±0.7	12.4 ±0.2	0.63±0.00	558±11	143±14	226±20	0.84±0.00	617±37	5.4±0.1
	CMB		0.92±0.05	0.22±0.02	0.19±0.01	0.51±0.03	3.1±0.2	25.1±0.7	8.1±0.2	0.56±0.01	100±3	109±4	196±8	0.78±0.01	242±26	3.5±0.1

Table S2 - Factor loadings of the percentage of decrease (_D) of different morpho-physiological traits found in the 40 screened tomato genotypes and wild relatives after exposure to combined nitrogen and water deficit into the first four components (PC1, PC2, PC3, PC4) retained from the PCA, with *Varimax* rotation, applied to the full set of genotypes and explaining 82.4 % of the total variance. Total dry weight = TDW; root dry weight = RDW, stem dry weight = SDW, leaf dry weight = LDW, shoot/root ratio = S/R, plant height = PH, leaf number = LN, leaf weight ratio = LWR, leaf area = LA, leaf area ratio = LAR, specific leaf area = SLA, photosynthetic efficiency = F_v/F_m, stomatal conductance = g_s, Water use efficiency = WUE.

Morpho-physiological traits	**PC1**	**PC2**	**PC3**	**PC4**
LDW_D	**0.963**	0.004	0.129	0.035
SDW_D	**0.768**	-0.309	-0.306	0.066
RDW_D	**0.742**	-0.342	-0.447	-0.056
SLA_D	**-0.669**	0.475	-0.499	-0.147
PH_D	**0.699**	-0.246	-0.354	-0.140
WUE_D	**0.786**	0.203	0.204	-0.065
LWR	**0.584**	**0.545**	**0.543**	0.035
LAR	-0.466	**0.813**	-0.274	-0.154
LA_D	**0.721**	**0.507**	-0.340	-0.165
LN_D	**0.642**	**0.516**	-0.333	-0.225
S/R_D	0.383	0.493	**0.571**	0.054
g_s_D	-0.151	0.371	-0.361	**0.698**
F_v/F_m_D	0.431	0.114	-0.147	**0.612**
Variance explained (%)	*42.1*	*18.6*	*13.8*	*7.9*
Eigenvalue	*5.47*	*2.42*	*1.80*	*1.02*

CHAPTER 4

General Discussion

Environmental stresses trigger various plant responses, ranging from altered gene expression and cellular metabolism to changes in growth rate and crop productivity. Previous studies demonstrated that N and water deficit severely affect plant performance leading to a growth reduction in several crops, including tomato (Farooq *et al.*, 2009; Abenavoli *et al.*, 2016; Moles *et al.*, 2018; Kapoor *et al.*, 2020). However, plants do not often face single abiotic and/or biotic stresses, but rather a combination of several adverse growth conditions, especially under the current context of climate change and environmental instability (Pascual *et al.*, 2022; Sánchez-Bermúdez *et al.*, 2022). The combination of abiotic stressors induces a unique plethora of responses that cannot be extrapolated from those observed under single exposure (García-Martí *et al.*, 2019; Hussain *et al.*, 2018; Pandey *et al.*, 2015; Spormann *et al.*, 2022). These findings highlight the complexity and relevance of studying plants response to multiple stresses. Still, literature analysing the mechanisms involved in responses of young tomato plants subjected to single N or water deficit with the response of those plants subjected to the combination of both deficits, or focused on strategies targeted to improve tomato plants performance grown under such conditions is scarce. This knowledge gaps led to the two main goals of this thesis which are addressed and discussed below.

1. Unravelling the mechanisms involved in tomato responses to combined nitrogen and water deficit

Both N and water deficits severely impact metabolic pathways (including osmotic adjustment, nutrient uptake, and photosynthesis) due to stomatal closure and/or oxidative damage (Ding *et al.*, 2018; Gonzalez-Dugo *et al.*, 2010)). Proline is one of the most conspicuous osmolytes that accumulates at high levels in response to osmotic stress. It may also represent a substantial reserve of substrate for growth in the form of organic N that is easily remobilized in response to N deficiency (Hayat *et al.*, 2012; Szabados and Savouré, 2010;). Beyond that, several enzymes and genes involved in N metabolism are affected either by N or water deficit (Kavoosi *et al.*, 2014; Lemaître *et al.*, 2008; Nacry *et al.*, 2013; Plett *et al.*, 2020). Furthermore, one of the most critical consequences of abiotic stresses is the disturbance of the equilibrium between the generation of ROS and AOX defense systems which often results in a loss of cell viability and death (Laxa *et al.*, 2019; Soares *et al.*, 2019a). Indeed, the plant redox status is commonly affected by N or water deficit exposure (Mittler *et al.*, 2011). Taking this into account, in this thesis we explored several mechanisms involved in N remobilization and plants' osmoregulation, as well as in its AOX response to the combined N and water deficit showing, for the first time, that exposing tomato plants for 16 days to a gradual

and mild combined N+W deficit led to: 1) similarities between plants grown under single N or combined N+W deficit concerning the morphological traits; 2) molecular and biochemical plant' responses under combined N+W deficit that could not be deduced from single deficit responses and were organ (root versus shoot) dependent (**Chapters 2.1** and **2.2)**. Briefly, we could show that plants grown under single N or combined N+W deficit had lower chlorophyll content, SDW, RDW, TDW, LA and N concentration as well as higher NUE. Interestingly, concerning plant metabolism, we observed a dual type of response in both plant organs. Root characteristics of plants grown under combined N+W deficit were similar to those plants exposed to W deficit alone, showing higher proline and NO_3^- concentration, higher NR activity, and *GS1* and *NR* expression, and lower C concentration. Both groups of plants presented a higher activity of APX and CAT enzymes and *Cu,ZnSOD, APX* and *CAT2*, expression profiles. Still, at the root level plants grown under N deficit or combined N+W deficits presented similar responses concerning protein and N concentration and C/N ratio but a differential response regarding flavonoid concentration and total AOX activity (ABTS), which were respectively, lower, and higher than in plants exposed to N or W deficit alone. On the other hand, shoot characteristics of plants grown under combined N+W deficit were more like those of plants exposed to N deficit alone, showing higher C/N ratio, NR and GS activity, and *rcbS* and *rcbL* transcript levels, but lower proline, protein and N concentrations, as well as *GS2.2* transcript levels than plants grown under W deficit. Interestingly, concerning the AOX system responses at shoot level, plants subjected to N+W deficit were different from the single deficits mainly due to higher activation of the defence mechanisms (including the increased anthocyanin concentration, higher activity of SOD, APX and CAT enzymes, and *APX* and *CAT2* expression as well as intermediate levels of flavonoids) compared to plants exposed to N or W deficit alone. These results demonstrated that tomato plants could employ specific defense strategies to cope with combined N+W deficit that contributed to the maintenance of the redox homeostasis, with unchanged values of MDA (lipid peroxidation) and H_2O_2 compared to CTR plants.

In **Chapters 2.1** and **2.2**, the W deficit level imposed did not impact several plant growth related traits but when combined with N deficit it was enough to activate multiple osmoregulatory and AOX responses. This suggests that N deficit had a higher impact on these traits but raised the hypothesis that this W deficit level may have played a role in the acclimation of young tomato plants to the combined N+W deficit, possibly making the plants more fitted for handling additional abiotic stresses. A recent study in maize has shown beneficial stress memory responses induced by priming the seedlings with combined heat and drought stress which prompt their defence system to trigger more

effective clearance mechanisms against subsequent exposure to these combined stresses (Ru *et al.*, 2022). The same stress memory response may also have occurred in our tomato plants subjected to combined N+W deficit, suggesting that it could also be used as a priming strategy for preparing the plants to cope with future abiotic stresses. However, further studies are needed, and the level of N deficit will need to be thoroughly adjusted to avoid, as in this thesis, impairments in plant growth.

As aforementioned, several enzymes and genes involved in N metabolism were shown to be affected by combined N+W deficit (**Chapter 2.1)**. One of the affected enzymes was glutamine synthetase which is responsible for assimilating ammonium into amino acids (Bernard and Habash, 2009). On the other hand, some amino acids play an important role in N remobilization (e.g. arginine and asparagine) and osmoregulation (e.g. proline and glycine betaine) (Masclaux-Daubresse *et al.*, 2010; Winter *et al.*, 2015; Ghosh *et al.*, 2021; Ozturk *et al.*, 2021). Several studies, including some using tomato plants, demonstrated that N deficiency often leads to lower total amino acids content, since N is a central nutrient for their formation (Ruan *et al.*, 2010; Yang *et al.*, 2020; Ková ik *et al.*, 2006; Sung *et al.*, 2015). Contrarily, under water deficit the content of free amino acids is increased in tomato plants (Živanović *et al.*, 2020). Therefore, we hypothesized that free amino acids content might be specifically adjusted after exposure to combined N+W deficits. On the other hand, water deficit has a severe effect on root nutrient uptake due to the reduction in soil moisture which causes a decreased rate of nutrient diffusion from the soil matrix to the absorbing root surface and translocation to the leaves (Rouphael *et al.*, 2012). Since N is a dominant macronutrient, its deficit also impacts other nutrients, with its ionic form controlling cation and anion uptake through the charge balance mechanism in ion uptake (Borgognone *et al.*, 2013). This impact might be worse in plants grown under combined N+W deficit. Furthermore, the xylem and phloem are responsible for macronutrient and amino acid transport, playing an essential role in shoot nutrition (White, 2012), suggesting that combined N+W deficit may impact anatomical characteristics of vascular tissues responsible for meeting plant water and nutrient demands. Therefore, in **Chapter 2.3** we decided to further explore the morphological and physiological responses to the combined N+W deficit, but also additional mechanisms involved in N remobilization and osmoregulatory responses. However, contrarily to **Chapter 2.1** and **Chapter 2.2** where we focused on tomato plants response to a gradual and mild single and combined N+W deficit (16 days), in **Chapter 2.3** we decided to explore plant responses to a more prolonged N and/or water deficit (27 days), as it is know that plants' response is often dependent on the level of N or water deficit to which the plants are subjected (Parkash and Singh, 2020). In this chapter we showed,

for the first time, that exposing tomato plants for 27 days to a combined N+W deficit resulted in specific plant' responses that could not be predicted from those observed under the single deficits. Contrarily to **Chapter 2.1** and **Chapter 2.2** (where, single N deficit had a higher negative impact on the tomato biometric traits than water scarcity, with the first group of plants sharing several similarities to those grown under combined N+W deficit), in **Chapter 2.3** both single deficits negatively impacted tomato biometric traits to the same extent, but the combined N+W deficit caused a higher reduction on tomato biometric traits. In contrast, hypocotyl, phloem and xylem area on the cross-section were negatively affected to the same extent in all types of deficit treatments. Furthermore, in **Chapter 2.1** and **Chapter 2.2,** different molecular and biochemical plant responses were shown to be differently employed by tomato plants subjected to the combined deficit, which were organ dependent. The same differential response was observed in **Chapter 2.3**. Plants grown under combined N+W deficit responded mainly with an increased root synthesis of arginine and asparagine compared to plants grown under the single deficits. This probably resulted in lower N remobilization from the photosynthetic machinery, as demonstrated by the lower reduction in chlorophyll content and photosynthetic rate compared to plants grown under single N deficit. Additionally, alanine, tyrosine and phenylalanine levels were specifically increased in the roots of plants grown under the combined N+W deficit. These three amino acids, together with arginine, were previously reported as acting against osmotic stress in rice (Matsunami *et al.*, 2020). Plants grown under combined N+W deficit also increased the reallocation and synthesis of osmolytes such as K^+ and proline, respectively, contributing to stomatal closure and a lower transpiration rate than plants under the single W deficit. On the other hand, plants grown under single W deficit invested relatively more in the root system, stimulating uptake of water and nutrients compared to plants grown under combined N+W deficit.

Taken together, the data from **Chapter 2** indicate that there is a differential behavior between combined N+W deficit and single deficits, as well as between plant organs (roots and shoots), which highlights that tomato plants grown under combined stresses employ specific response mechanisms that cannot be elucidated from the responses obtained when these stressors are applied individually. This holistic approach supports earlier reports that also show that the combination of others abiotic and/or biotic stresses generates specific plant responses (García-Martí et al., 2019; Hussain et al., 2018; Pandey et al., 2015).

2. Exploring sustainable mitigation strategies to enhance tomato plants' performance under combined nitrogen and water deficit

So far, research focused on agronomical strategies for stress mitigation mostly rely on studies targeted to single deficits. However, the current context of climate change and environmental instability, where combined stresses are expected to be prevalent, urges exploring strategies that can improve crop performance under these conditions (Fernandes et al., 2022). A sustainable method that is receiving much attention in recent years, due to its role in alleviating the adverse effects of several abiotic stresses, is the application of silicon (Si) (Bokor *et al.*, 2021; Nunes da Silva *et al.*, 2022). Supplementing agronomic crops with Si has been adopted as an effective strategy for alleviating drought stress (recently reviewed by Bokor *et al.*, 2021; Malik *et al.*, 2021; Thorne *et al.*, 2020; Verma *et al.*, 2022; Wang *et al.*, 2021). In contrast, the literature concerning Si potential in mitigating nutrient deficiencies is far scarcer (Ali *et al.*, 2020; Pavlovic *et al.*, 2021). Si positively impacts N uptake, assimilation, and remobilization, which in turn can contribute to improved N utilization within plant tissues (Minden *et al.*, 2021; Pavlovic *et al.*, 2021). However, the effects of Si supplementation in plants exhibiting passive absorption and low accumulation of Si (Si-excluder plants), such as tomato (Hoffmann *et al.*, 2020; Liang *et al.*, 2007), especially when subjected to combined N+W deficit, has been overlooked. One of the critical mechanisms proposed for alleviating abiotic stress after Si supplementation relates to stimulating the plants' AOX system (Liang *et al.*, 2007). In **Chapter 1.2** we summarize reports demonstrating that Si supplementation can enhance the AOX system of several crops subjected to drought stress (Bokor *et al.*, 2021). However, the potential impact of Si on plants' AOX system in crops grown under N deficit, needs further investigation (Ali *et al.*, 2020). In **Chapter 3.1** we analysed, for the first time, the effect of 2 mM Na_2SiO_3 supplementation in tomato plants exposed for 27 days to a prolonged single or combined N+W deficit, resulting in a significantly higher RDW and root length and consequently higher TDW compared to control plants. The better performance of Si-supplemented plants grown under combined N+W deficit may be related, at least partly, to the root's AOX system where an increase in all the studied AOX enzymes activities (APX, CAT and SOD) was found. This more pronounced impact of Si at root level may be because both the N deficit and the W deficit perception takes place in the roots (Bengough *et al.*, 2011; Sun *et al.*, 2020a), where the supplementation of Si also occurred. In **Chapter 3.1** we confirmed that the strategies used to mitigate the single deficit may activate different responses under a combined N+W deficit. For plants subjected to either N or W deficit, the impact of Si was not as evident when compared to combined N+W deficit plants. This difference in the results could be due to different

experimental design conditions such as the form and concentration of silicon given to the plants, the type of substrate used, or even using different tomato genotypes (previously discussed in Chapter 3.1). Nonetheless, our results are highly relevant since the combined deficit was the treatment with the largest negative impact on the growth traits and where Si application would bring the most benefits.

Another powerful tool that can enhance crop resilience to abiotic stresses is plant breeding (Monforte, 2020). Nonetheless, several decades of genotype selection and crop breeding for high yields led to so-called genetic erosion. Modern tomato cultivars have now very narrow genetic bases, most of which are sensitive to N deficit and drought, as this selection have been conducted under high N and water supplies (Esteban *et al.*, 2016; Machado *et al.*, 2022; Shirasawa *et al.*, 2010). Although the genetic variation within a species could be used as a valuable tool for screening and breeding for WUE and NUE, the genotypic variation in plants' response to combined N and water deficit is largely unknown (Ruggiero *et al.*, 2022). Previous studies reported genotypic variation in tomato in response to N or water deficiency, but they were limited to a much lower number of genotypes and most of them were focused on the plant responses to single stresses (Abenavoli *et al.*, 2016; Sánchez-Rodríguez *et al.*, 2010a). Furthermore, most tomato breeding programs, that introduce genotypic variability for fruit quality and environmental adaptation make use of wild tomato relatives (Conesa *et al.*, 2020). However, old accessions held in germplasm collections (e.g. landraces and heirlooms) might also represent a valuable gene pool for tomato improvement. Nonetheless, they represent an untapped genetic resource (Conesa *et al.*, 2020; Roohanitaziani *et al.*, 2020). Therefore, in **Chapter 3.2** we explored the potential of old accessions as a source of variation concerning NUE and WUE. We used 40 tomato genotypes which included 34 old accessions (four modern inbred lines/hybrids and two wild tomato relatives were also used as anchors) that were grown under combined N+W deficit. We showed a large genotypic variation in response to combined N+W deficit exists in old accessions. A Principal Component Analysis alongside with data integration in a cluster analysis separated the studied genotypes into three distinct clusters based on their ability to handle the combined N+W deficit. Though the combined deficit severely affected all growth parameters, this effect depended on the genotype and evaluated trait. This analysis allowed us to demonstrate that the tolerance of these genotypes was associated with traits such as lower SLA, lower LAR and higher WUE, compared to the sensitive genotypes. Moreover, they were also associated, but to a lesser extent, with a lower g_s. Although g_s and F_v/F_m have been widely used in plant stress-tolerance physiology (Liang *et al.*, 2020), our results suggest that these two parameters are not key in tomato

tolerance response to combined N+W deficit. Nevertheless, none of the studies that associate tolerance with these two physiological traits have focused on tomato plants' response to the combined stress. Interestingly, we observed that the two accessions of the studied wild tomato relative *S. pimpinellifolium* were not part of the tolerant cluster. This observation was somehow unexpected since wild relatives are essential genetic sources of abiotic tolerance traits (Conesa *et al.*, 2020; Martínez-Cuenca *et al.*, 2020). On the other hand, we showed that old accessions may represent a valuable gene pool. This result highlights that old accessions, such as landraces and heirlooms, may represent an underexploited wealth of genetic variation. Despite being discarded due to undesired agronomic traits, they might encompass high NUE and WUE, which can be of utmost importance for future plant breeding programs. In a very recent study, Ruggiero *et al.* (2022) also exposed ten tomato genotypes (including genotype M82 as reference and landraces from Southern Italy) to a combination of drought stress and low NO_3^-, identifying among the landraces, tomato genotypes with contrasting morphophysiological behaviors, which reinforces that these underutilized accessions may be promising tools to increase NUE and WUE.

Taken together, **Chapter 3.1** and **Chapter 3.2** represent one step further on the available knowledge, but the is still the need for further studies focused on strategies to support tomato plants in coping with low inputs of N and water thus improving their NUE and WUE.

Conclusions and Future Prospects

The overall goal of this thesis was to explore the underlying mechanisms (morphophysiological, biochemical and molecular) involved in tomato responses to combined N+W deficit and compare these with the response to single N or W deficit. Furthermore, this thesis analyzed sustainable mitigation strategies towards more resilient tomato plants to cope with these combined abiotic deficits.

We showed for the first time the differential response mechanisms of tomato plants exposed to combined N+W deficit, compared to the single deficit, at gene expression level and regarding metabolite accumulation and phenotype translation **(Chapter 2)**. The stress levels studied in this thesis activated relevant N remobilization and osmoregulation strategies. The AOX system also played an important role in tomato acclimation to the combined N+W deficit. We also demonstrated that the response pattern to combined deficit is quite distinct between root and shoot **(Chapter 2 and Chapter 3.1)**. Si application and genotypic variation were shown to be potential valuable tools to mitigate some of the adverse effects caused by combined N+W deficit **(Chapter 3)**. Applying 2 mM Na_2SiO_3 to tomato seedlings' roots could partly alleviate the stress caused by combined N+W deficit by improving plant growth and stimulating the AOX system **(Chapter 3.1)**. In this thesis it was also shown, for the first time, that tomato has a large genotypic variation in its response to the combined N+W deficit, and that old accessions represent a valuable gene pool in terms of tolerance to these combined abiotic stresses, offering good prospects for plant breeding programs targeted to promote sustainable food production. SLA, LAR and WUE were key parameters in the tolerance response. On the other hand, g_s and F_v/F_m were not distinctive traits in terms of tolerance **(Chapter 3.2)**.

Although in this work several mechanisms and potential sustainable mitigation strategies involved in tomato responses to combined N+W deficit were identified, further studies are needed. We identified several indicators of N remobilization and osmoregulation, but the complete pathways involved in these processes still need to be better explored. We showed that the AOX system is highly relevant for combined N+W deficit acclimation, but hormonal regulation might also play an important role, which needs elucidation. Indeed, understanding the complete set of mechanisms involved in the complex interactions between combined N+W deficit is still in its infancy. An untargeted and integrated approach involving high throughput methodologies could contribute to unravelling other relevant pathways, preferably exploring contrasting genotypes in their tolerance to these combined abiotic stresses. The responses

observed under tomato exposure to mild levels of combined N+W deficit also raised the hypothesis that low levels of this combined stress may activate AOX responses, which might act as a priming strategy for preparing the plants to cope with future situations of low N and water inputs. In future studies, it would be important to test this hypothesis and determine whether the current findings apply to other tomato cultivars and plant species. Si application to tomato seedlings showed promising results for combined N+W deficit mitigation. Future studies should test which application mode (fertigation vs foliar application) and frequency (single vs multiple application) provides best results. Finally, the efficiency of the old accessions identified as tolerant to N+W deficit, during the vegetative phase, should be evaluated in other development stages, especially in the fruit-bearing phase. Furthermore, understanding the impact of the combined N+W deficit on yield and fruit quality is essential as it impacts the economic revenue of the tomato crop. Exploring the underlying mechanisms behind the different tolerance levels found in the studied tomato genotypes (including single vs combined stresses) would also bring important information for future breeding programs.

Strengths and limitations

As strength of the current work, we highlight the novel findings concerning the identification of several mechanisms differentially used by plants grown under a combined N+W deficit compared to plants grown under single N or W deficit. Additionally, we showed that Si application and making use of old accessions in tomato breeding programs may be valuable approaches to mitigate some of the adverse effects caused by combined N+W deficit. The indications that mild combined N+W deficit during the seedling stage may be used to prime plants for future exposure to adverse conditions is also great of importance.

As limitation, we acknowledge that the higher level of N deficit compared to W deficit, imposed in **Chapter 2.1** and **2.2,** may have influenced some of the responses observed in plants grown under combined N+W deficit when compared to single deficit. Nonetheless, under environmental conditions, most of the times, plants are not exposed to the same level of stress, so we believe that the results presented here continue to make a valuable contribution to the current knowledge. Furthermore, we are aware that many of the plant responses here observed, can vary throughout the tomato life cycle. Considering that fruit production is the growers' target, we emphasize the need for more applied studies during the generative stage in order to evaluate the impact of the combined deficit on the yield and fruit quality.

With the large dataset collected in the present thesis, we hope to motivate future studies addressing the aforementioned limitations to further understand the complex interactive consequences of N starvation and water shortage on this crop. We also acknowledge that the screening performed in **Chapter 3.2** does not allow to conclude about the contribution of each individual deficit to the behavior observed under the combined N+W deficit treatment, but we strongly believe that the identified contrasting genotypes can be a useful basis for an in-depth analysis of the tolerance mechanisms to these combined abiotic stresses.

References

Abenavoli, M. R., Longo, C., Lupini, A., Miller, A. J., Araniti, F., Mercati, F., Princi, M. P. & Sunseri, F. (2016). Phenotyping two tomato genotypes with different nitrogen use efficiency. *Plant Physiology and Biochemistry* 107: 21-32.

AD, C. (2019).The fruit and vegetable sector in the EU - a statistical overview Eurostat2019.

Addinsoft. 2021. XLSTAT statistical and data analysis solution. New York, USA. https://www.xlstat.com.

Aebi, H. (1984).[13] Catalase in vitro. In *Methods in enzymology*, Vol. 105, 121-126: Elsevier.

Afzal, Z., Howton, T., Sun, Y. & Mukhtar, M. S. (2016). The roles of aquaporins in plant stress responses. *Journal of developmental biology* 4(1): 9.

Aghaie, P., Tafreshi, S. A. H., Ebrahimi, M. A. & Haerinasab, M. (2018). Tolerance evaluation and clustering of fourteen tomato cultivars grown under mild and severe drought conditions. *Scientia Horticulturae* 232: 1-12.

Ahmed, M., Fayyaz-ul-Hassan & Asif, M. (2014). Amelioration of drought in sorghum (*Sorghum bicolor* L.) by silicon. *Communications in soil science and plant analysis* 45(4): 470-486.

Akl, I. A., Savvas, D., Papadantonakis, N., Lydakis-Simantiris, N. & Kefalas, P. (2003). Influence of ammonium to total nitrogen supply ratio on growth, yield and fruit quality of tomato grown in a closed hydroponic system. *European Journal of Horticultural Science* 68(5): 204-211.

Al Hassan, M., Fuertes, M. M., Sánchez, F. J. R., Vicente, O. & Boscaiu, M. (2015). Effects of salt and water stress on plant growth and on accumulation of osmolytes and antioxidant compounds in cherry tomato. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca* 43(1): 1-11.

Al-Abdallat, A. M., Al-Debei, H. S., Ayad, J. Y. & Hasan, S. (2014). Over-expression of *SLSHN1* gene improves drought tolerance by increasing cuticular wax accumulation in tomato. *International Journal of Molecular Sciences* 15(11): 19499-19515.

Alaoui, S. M., Salghi, R., Abouatallah, A., Jaouhari, N. & Hammouti, B. (2014). Impact of drip irrigation scheduling on vegetative parameters in tomato (*Lycopersicon esculentum*) under unheated greenhouse. *International Journal of Engineering Research and Applications* 4(1): 71-76.

Albert, E., Segura, V., Gricourt, J., Bonnefoi, J., Derivot, L. & Causse, M. (2016). Association mapping reveals the genetic architecture of tomato response to water deficit: focus on major fruit quality traits. *Journal of Experimental Botany* 67(22): 6413-6430.

Alexieva, V., Sergiev, I., Mapelli, S. & Karanov, E. (2001). The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. *Plant, Cell & Environment* 24(12): 1337-1344.

Alfosea-Simón, M., Simón-Grao, S., Zavala-Gonzalez, E. A., Cámara-Zapata, J. M., Simón, I., Martínez-Nicolás, J. J., Lidón, V. & García-Sánchez, F. (2020). Physiological, Nutritional and Metabolomic Responses of Tomato Plants After the Foliar Application of Amino Acids Aspartic Acid, Glutamic Acid and Alanine. *Frontiers in Plant Science* 11: 581234.

Ali, N., Réthoré, E., Yvin, J.-C. & Hosseini, S. A. (2020). The Regulatory Role of Silicon in Mitigating Plant Nutritional Stresses. *Plants* 9(12): 1779.

Ali, N., Schwarzenberg, A., Yvin, J. C. & Hosseini, S. A. (2018). Regulatory Role of Silicon in Mediating Differential Stress Tolerance Responses in Two Contrasting Tomato Genotypes Under Osmotic Stress. *Front Plant Sci* 9: 1475.

Alscher, R. G., Erturk, N. & Heath, L. S. (2002). Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *Journal of Experimental Botany* 53(372): 1331-1341.

Amin, M., Ahmad, R., Ali, A., Hussain, I., Mahmood, R., Aslam, M. & Lee, D. J. (2018). Influence of silicon fertilization on maize performance under limited water supply. *Silicon* 10(2): 177-183.

Anjana, S. U. & Iqbal, M. (2007). Nitrate accumulation in plants, factors affecting the process, and human health implications. A review. *Agronomy for Sustainable Development* 27: 45-57.

Aoyama, S., Huarancca Reyes, T., Guglielminetti, L., Lu, Y., Morita, Y., Sato, T. & Yamaguchi, J. (2014). Ubiquitin ligase ATL31 functions in leaf senescence in response to the balance between atmospheric CO_2 and nitrogen availability in Arabidopsis. *Plant and Cell Physiology* 55(2): 293-305.

Ashraf, M. & Harris, P. J. (2013). Photosynthesis under stressful environments: an overview. *Photosynthetica* 51(2): 163-190.

Ashraf, M. & Sultana, R. (2000). Combination effect of NaCl salinity and nitrogen form on mineral composition of sunflower plants. *Biologia Plantarum* 43(4): 615-619.

Aulakh, M. S. & Malhi, S. S. (2005). Interactions of nitrogen with other nutrients and water: effect on crop yield and quality, nutrient use efficiency, carbon sequestration, and environmental pollution. Advances in Agronomy 86: 341-409.

Avin-Wittenberg, T., Baluška, F., Bozhkov, P. V., Elander, P. H., Fernie, A. R., Galili, G., Hassan, A., Hofius, D., Isono, E., Le Bars, R., Masclaux-Daubresse, C., Minina, E. A., Peled-Zehavi, H., Coll, N. S., Sandalio, L. M., Satiat-Jeunemaitre, B., Sirko, A., Testillano, P. S. & Batoko, H. (2018). Corrigendum: Autophagy-related approaches for improving nutrient use efficiency and crop yield protection. *Journal of Experimental Botany* 69(12): 31-73.

Ayankojo, I. T., Morgan, K. T., Kadyampakeni, D. M. & Liu, G. D. (2020). Tomato growth, yield, and root development, soil nitrogen and water distribution as affected by nitrogen and irrigation rates on a Florida sandy soil. *American Society for Horticultural Science* 55(11): 1744-1755.

Baquedano, F. & Castillo, F. (2006). Comparative ecophysiological effects of drought on seedlings of the Mediterranean water-saver Pinus halepensis and water-spenders *Quercus coccifera* and *Quercus ilex*. *Trees* 20(6): 689-700.

Barbagallo, R. N., Di Silvestro, I. & Patanè, C. (2012). 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. *Journal of the Science of Food and Agriculture* 93: 1449-1457.

Bassham, D.C. (2007). Plant autophagy - more than a starvation response. *Current Opinion in Plant Biology* 10: 587-593.

Bassi, D., Menossi, M. & Mattiello, L. (2018). Nitrogen supply influences photosynthesis establishment along the sugarcane leaf. *Scientific reports* 8(1): 1-13.

Bates, L. S., Waldren, R. P. & Teare, I. (1973). Rapid determination of free proline for water-stress studies. *Plant and Soil* 39(1): 205-207.

Bedu, M., Marmagne, A., Masclaux-Daubresse, C. & Chardon, F., (2020). Transcriptional plasticity of autophagy-related genes correlates with the genetic response to nitrate starvation in *Arabidopsis thaliana*. *Cells* 9(4): 10-21.

Beńard, C., Bourgaud, F. & Gautier, H. (2011). Impact of temporary nitrogen deprivation on tomato leaf phenolics. *International Journal of Molecular Sciences* 12(11), 7971-7981.

Bénard, C., Gautier, H., Bourgaud, F., Grasselly, D., Navez, B., Caris-Veyrat, C., Weiss, M. & Génard, M. (2009). Effects of low nitrogen supply on tomato (*Solanum lycopersicum*) fruit yield and quality with special emphasis on sugars, acids,

ascorbate, carotenoids, and phenolic compounds. *Journal of Agricultural and Food Chemistry* 57(10): 4112-4123.

Bengough, A. G., McKenzie, B. M., Hallett, P. D. & Valentine, T. A. (2011). Root elongation, water stress, and mechanical impedance: a review of limiting stresses and beneficial root tip traits. *Journal of Experimental Botany* 62(1): 59-68.

Bernard, S. M. & Habash, D. Z. (2009). The importance of cytosolic glutamine synthetase in nitrogen assimilation and recycling. *New Phytologistl* 182(3): 608-620.

Bhatt, R. M. & Srinivasa Rao, N. K. (1987). Seed germination and seedling growth responses of tomato cultivars to imposed water stress. *Journal of Horticultural Science* 62(2): 221-225.

Biju, S., Fuentes, S. & Gupta, D. (2017). Silicon improves seed germination and alleviates drought stress in lentil crops by regulating osmolytes, hydrolytic enzymes and antioxidant defense system. *Plant Physiology and Biochemistry* 119: 250-264.

Bista, D. R., Heckathorn, S. A., Jayawardena, D. M., Mishra, S. & Boldt, J. K. (2018). Effects of Drought on Nutrient Uptake and the Levels of Nutrient-Uptake Proteins in Roots of Drought-Sensitive and -Tolerant Grasses. *Plants (Basel)* 7(2).

Blum, A. (2017). Osmotic adjustment is a prime drought stress adaptive engine in support of plant production. *Plant, Cell & Environment* 40(1): 4-10.

Bokor, B., Santos, C. S., Kostoláni, D., Machado, J., da Silva, M. N., Carvalho, S. M. P., Vaculík, M. & Vasconcelos, M. W. (2021). Mitigation of climate change and environmental hazards in plants: Potential role of the beneficial metalloid silicon. *Journal of Hazardous Materials* 416: 126-193.

Bolger, A., Scossa, F., Bolger, M. E., Lanz, C., Maumus, F., Tohge, *et al.* (2014). The genome of the stress-tolerant wild tomato species *Solanum pennellii*. *Nature Genetics* 46: 1034-1038.

Borgognone, D., Colla, G., Rouphael, Y., Cardarelli, M., Rea, E. & Schwarz, D. (2013). Effect of nitrogen form and nutrient solution pH on growth and mineral composition of self-grafted and grafted tomatoes. *Scientia horticulturae* 149: 61-69.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry* 72(1-2): 248-254.

Bu, R., Xie, J., Yu, J., Liao, W., Xiao, X., Lv, J., Wang, C., Ye, J. & Calderón-Urrea, A. (2016). Autotoxicity in cucumber (Cucumis sativus L.) seedlings is alleviated by silicon through an increase in the activity of antioxidant enzymes and by mitigating lipid peroxidation. *Journal of Plant Biology* 59(3): 247-259.

Calero Hurtado, A., Chiconato, D. A., Prado, R. d. M., Sousa Junior, G. d. S., Olivera Viciedo, D. & Piccolo, M. d. C. (2020). Silicon application induces changes C:N:P stoichiometry and enhances stoichiometric homeostasis of sorghum and sunflower plants under salt stress. *Saudi Journal of Biological Sciences* 27(12): 3711-3719.

Candar-Cakir, B., Arican, E. & Zhang, B. (2016). Small RNA and degradome deep sequencing reveals drought-and tissue-specific micrornas and their important roles in drought-sensitive and drought-tolerant tomato genotypes. *Plant Biotechnology* Journal 14(8): 1727-1746.

Cannella, D., Möllers, K., Frigaard, N.-U., Jensen, P., Bjerrum, M., Johansen, K. & Felby, C. (2016). Light-driven oxidation of polysaccharides by photosynthetic pigments and a metalloenzyme. *Nature communications* 7(1): 1-8.

Cantero-Navarro, E., Romero-Aranda, R., Fernández-Muñoz, R., Martínez-Andújar, C., Pérez-Alfocea, F. & Albacete, A. (2016). Improving agronomic water use efficiency in tomato by rootstock-mediated hormonal regulation of leaf biomass. *Plant Science* 251: 90-100.

Cao, B.-l., Ma, Q., Zhao, Q., Wang, L. & Xu, K. (2015). Effects of silicon on absorbed light allocation, antioxidant enzymes and ultrastructure of chloroplasts in tomato leaves under simulated drought stress. *Scientia Horticulturae* 194: 53-62.

Cao, B.-l., Wang, L., Gao, S., Xia, J. & Xu, K. (2017). Silicon-mediated changes in radial hydraulic conductivity and cell wall stability are involved in silicon-induced drought resistance in tomato. *Protoplasma* 254(6): 2295-2304.

Cao, X., Zhong, C., Zhu, C., Zhu, L., Zhang, J., Wu, L. & Jin, Q. (2018). Ammonium uptake and metabolism alleviate PEG-induced water stress in rice seedlings. *Plant Physiology and Biochemistry* 132: 128-137.

Carvalho, D. R. A., Vasconcelos, M. W., Lee, S., Vreugdenhil, D., Heuvelink, E. & Carvalho, S. M. P. (2017). Moderate salinity improves stomatal functioning in rose plants grown at high relative air humidity. *Environmental and Experimental Botany* 143: 1-9.

Carvalho, S. M. P. & Vasconcelos, M. W. (2013). Producing more with less: Strategies and novel technologies for plant-based food biofortification. *Food Research International* 54(1): 961-971.

Chapin, F. S., Walter, C. H. S. & Clarkson, D. T. (1988). Growth response of barley and tomato to nitrogen stress and its control by abscisic acid, water relations and photosynthesis. *Planta* 173: 352-366.

Chatterjee, A. & Solankey, S. (2015). Functional physiology in drought tolerance of vegetable crops: an approach to mitigate climate change impact. *Climate dynamics in horticultural science* 1: 149-171.

Chaves, M. M., Flexas, J. & Pinheiro, C. (2009). Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. *Annals of Botany* 103(4): 551-560.

Chaves, M. M., Maroco, J. P. & Pereira, J. S. (2003). Understanding plant responses to drought from genes to the whole plant. *Functional Plant Biology* 30 (3): 239-264.

Chen, D., Cao, B., Qi, L., Yin, L., Wang, S. & Deng, X. (2016a). Silicon-moderated K-deficiency-induced leaf chlorosis by decreasing putrescine accumulation in sorghum. *Annals of botany* 118(2): 305-315.

Chen, D., Cao, B., Wang, S., Liu, P., Deng, X., Yin, L. & Zhang, S. (2016b). Silicon moderated the K deficiency by improving the plant-water status in sorghum. *Scientific reports* 6(1): 1-14.

Chen, D., Wang, S., Xiong, B., Cao, B. & Deng, X. (2015). Carbon/nitrogen imbalance associated with drought-induced leaf senescence in Sorghum bicolor. *PloS one* 10(8): e0137026.

Chen, H. & Jiang, J.-G. (2010). Osmotic adjustment and plant adaptation to environmental changes related to drought and salinity. *Environmental Reviews* 18: 309-319.

Chen, J., Kang, S., Du, T., Qiu, R., Guo, P. & Chen, R. (2013). Quantitative response of greenhouse tomato yield and quality to water deficit at different growth stages. *Agricultural Water Management* 129: 152-162.

Chen, W., Yao, X., Cai, K. & Chen, J. (2011). Silicon alleviates drought stress of rice plants by improving plant water status, photosynthesis and mineral nutrient absorption. *Biological trace element research* 142(1): 67-76.

Cohen, I., Halpern, M., Yermiyahu, U., Bar-Tal, A., Gendler, T. & Rachmilevitch, S. (2019). CO_2 and nitrogen interaction alters root anatomy, morphology, nitrogen partitioning and photosynthetic acclimation of tomato plants. Planta 250(5): 1423-1432.

Conesa, M., Fullana-Pericàs, M., Granell, A. & Galmés, J. (2019). Mediterranean Long Shelf-Life Landraces: An Untapped Genetic Resource for Tomato Improvement. *Frontiers in Plant Sciences* 10: 1651.

Consortium, T. T. G. S., Aflitos, S., Schijlen, E., de Jong, H., de Ridder, D., Smit, S., Finkers, R., Wang, J., Zhang, G., Li, N., Mao, L., Bakker, F., Dirks, R., Breit, T., Gravendeel, B., Huits, H., Struss, D., Swanson-Wagner, R., van Leeuwen, H., van Ham, R. C. H. J., Fito, L., Guignier, L., Sevilla, M., Ellul, P., Ganko, E., Kapur, A., Reclus, E., de Geus, B., van de Geest, H., te Lintel Hekkert, B., van Haarst, J., Smits, L., Koops, A., Sanchez-Perez, G., van Heusden, A. W., Visser, R., Quan, Z., Min, J., Liao, L., Wang, X., Wang, G., Yue, Z., Yang, X., Xu, N., Schranz, E., Smets, E., Vos, R., Rauwerda, J., Ursem, R., Schuit, C., Kerns, M., van den Berg, J., Vriezen, W., Janssen, A., Datema, E., Jahrman, T., Moquet, F., Bonnet, J. & Peters, S. (2014). Exploring genetic variation in the tomato (*Solanum Lycopersicon*) clade by whole-genome sequencing. *The Plant Journal* 80(1): 136-148.

Coruzzi, G. M. & Zhou, L. (2001). Carbon and nitrogen sensing and signaling in plants: emerging 'matrix effects'. *Current opinion in plant biology* 4(3): 247-253.

Ćosić, M., Djurović, N. T., Todorović, M., Maleti ć, R., Zečević, B. &Stričević, R. (2015). Effect of irrigation regime and application of kaolin on yield, quality and water use efficiency of sweet pepper. *Agricultural Water Management* 159: 139147.

Coskun, D., Britto, D. T., Shi, W. & Kronzucker, H. J. (2017a). How plant root exudates shape the nitrogen cycle. *Trends in Plant Science* 22(8): 661-673.

Coskun, D., Britto, D. T., Shi, W. & Kronzucker, H. J. (2017b). Nitrogen transformations in modern agriculture and the role of biological nitrification inhibition. *Nature Plants* 3(6): 1-10.

Coskun, D., Deshmukh, R., Sonah, H., Menzies, J. G., Reynolds, O., Ma, J. F., Kronzucker, H. J. & Bélanger, R. R. (2019). The controversies of silicon's role in plant biology. *New Phytologist* 221(1): 67-85.

Costa, J. M. & Heuvelink, E. (2018).The global tomato industry. In *Tomatoes*, Vol. 27, 1-26: CABI Boston, USA.

Crusciol, C. A., Pulz, A. L., Lemos, L. B., Soratto, R. P. & Lima, G. P. (2009). Effects of silicon and drought stress on tuber yield and leaf biochemical characteristics in potato. *Crop Science* 49(3): 949-954.

da Silva, D. L., de Mello Prado, R., Tenesaca, L. F. L., da Silva, J. L. F. & Mattiuz, B.-H. (2021). Silicon attenuates calcium deficiency by increasing ascorbic acid content, growth and quality of cabbage leaves. *Scientific Reports* 11(1): 1-9.

de Bang, T. C., Husted, S., Laursen, K. H., Persson, D. P. & Schjoerring, J. K. (2021). The molecular–physiological functions of mineral macronutrients and their consequences for deficiency symptoms in plants. *New Phytologist* 229(5): 2446-2469.

de Carvalho, J. S., Frazão, J. J., de Mello Prado, R., de Souza Júnior, J. P. & Costa, M. G. (2022). Silicon modifies C:N:P stoichiometry and improves the physiological efficiency and dry matter mass production of sorghum grown under nutritional sufficiency. *Scientific Reports* 12(1): 16082.

De Groot, C. C., Boogaard, R. V. D., Marcelis, L. F., Harbinson, J. & Lambers, H. (2003). Contrasting effects of N and P deprivation on the regulation of photosynthesis in tomato plants in relation to feedback limitation. *Journal of Experimental Botany* 54(389): 1957-1967.

de Oliveira Filho, A. S. B., de Mello Prado, R., Teixeira, G. C. M., de Cássia Piccolo, M. & Rocha, A. M. S. (2021). Water deficit modifies C:N:P stoichiometry affecting sugarcane and energy cane yield and its relationships with silicon supply. *Scientific Reports* 11(1): 20916.

de Souza Júnior, J. P., de Oliveira, T. L., de Mello Prado, R., de Oliveira, K. R. & Soares, M. B. (2022). Analyzing the Role of Silicon in Leaf C:N:P Stoichiometry and Its Effects on Nutritional Efficiency and Dry Weight Production in Two Sugarcane Cultivars. *Journal of Soil Science and Plant Nutrition* 22(2): 2687-2694.

Deak, K. I. & Malamy, J. (2005). Osmotic regulation of root system architecture. *The Plant Journal* 43(1): 17-28.

Deus, A. C. F., de Mello Prado, R., de Cássia Félix Alvarez, R., de Oliveira, R. L. L. & Felisberto, G. (2020). Role of silicon and salicylic acid in the mitigation of nitrogen deficiency stress in rice plants. *Silicon* 12(5): 997-1005.

Diaz, C., Lemaître, T., Christ, A., Azzopardi, M., Kato, Y., Sato, F., Morot-Gaudry, J.-F., Le Dily, F. & Masclaux-Daubresse, C. (2008). Nitrogen recycling and remobilization are differentially controlled by leaf senescence and development stage in Arabidopsis under low nitrogen nutrition. *Plant Physiology* 147(3): 1437-1449.

Ding, L., Lu, Z., Gao, L., Guo, S. & Shen, Q. (2018). Is nitrogen a key determinant of water transport and photosynthesis in higher plants upon drought stress? *Frontiers in Plant Science* 9: 1143.

Djangsou, H., Enrico, F., Domenico, R. & Matteo, B. (2019). Blossom end-rot in tomato (*Solanum lycopersicum* L.): a multi-disciplinary overview of inducing factors and control strategies. *Scientia Horticulturae* 249: 49-58.

Djurović, N., Ćosić, M., Stričević, R., Savić, S. & Domazet, M. (2016). Effect of irrigation regime and application of kaolin on yield, quality and water use efficiency of tomato. *Scientia Horticulturae* 201: 271-278.

Domínguez-Figueroa, J., Carrillo, L., Renau-Morata, B., Yang, L., Molina, R. V., Marino, D., Canales, J., Weih, M., Vicente-Carbajosa, J., Nebauer, S. G. & Medina, J. (2020). The arabidopsis transcription factor cdf3 is involved in nitrogen responses and improves nitrogen use efficiency in tomato. *Frontiers in Plant Science* 11: 601558.

Donahue, J. L., Okpodu, C. M., Cramer, C. L., Grabau, E. A. & Alscher, R. G. (1997). Responses of antioxidants to paraquat in pea leaves (relationships to resistance). *Plant Physiology* 113(1): 249-257.

Dong, Y., Zhai, J., Yan, J., Li, K. & Xu, H. (2022). Physiological and transcriptomic responses of antioxidant system and nitrogen metabolism in tomato seedlings treated with nitrogen starvation and re-supply. *The Journal of Horticultural Science and Biotechnology*: 1-15.

dos Santos Sarah, M. M., de Mello Prado, R., Teixeira, G. C. M., de Souza Júnior, J. P., de Medeiros, R. L. S. & Barreto, R. F. (2022). Silicon supplied via roots or leaves relieves potassium deficiency in maize plants. *Silicon* 14(3): 773-782.

Du, Y. & Scheres, B. (2018). Lateral root formation and the multiple roles of auxin. *Journal of Experimental Botany* 69(2): 155-167.

Du, Y.-D., Gu, X.-B., Wang, J.-W. & Niu, W.-Q. (2019). Yield and gas exchange of greenhouse tomato at different nitrogen levels under aerated irrigation. *Science of The Total Environment* 668: 1156-1164.

Du, Y.-D., Niu, W.-Q., Gu, X.-B., Zhang, Q. & Cui, B.-J. (2018). Water-and nitrogen-saving potentials in tomato production: A meta-analysis. *Agricultural Water Management* 210: 296-303.

Du, Y., Cao, H., Liu, S., Gu, X. & Cao, Y. (2017). Response of yield, quality, water and nitrogen use efficiency of tomato to different levels of water and nitrogen under drip irrigation in Northwestern China. *Journal of Integrative Agriculture* 16(5): 1153-1161.

Egea, I., Albaladejo, I., Meco, V., Morales, B., Sevilla, A., Bolarin, M.C., *et al.* (2018). The drought-tolerant *Solanum pennellii* regulates leaf water loss and induces genes involved in amino acid and ethylene/jasmonate metabolism under dehydration. *Scientific Reports* 8: 2791.

Elbehri, A. (2015). *Climate change and food systems: global assessments and implications for food security and trade.* Food and Agriculture Organization of the United Nations (FAO).

Elia, A. & Conversa, G. (2012). Agronomic and physiological responses of a tomato crop to nitrogen input. *European Journal of Agronomy* 40: 64-74.

Engindeniz, S. (2006). Economic analysis of pesticide use on processing tomato growing: A case study for Turkey. *Crop Protection* 25(6): 534-541.

Errebhi, M. & Wilcox, G. E. (1990). Tomato growth and nutrient uptake pattern as influenced by nitrogen form ratio. *Journal of Plant Nutrition* 13(8): 1031-1043.

Esan, V. I., Ayanbamiji, T. A., Adeyemo, J. O. & Oluwafemi, S. (2018). Effect of drought on seed germination and early seedling of tomato genotypes using polyethylene glycol 6000. *International Journal of Science* 2: 36-43.

Esteban, R., Ariz, I., Cruz, C. & Moran, J. (2016a).Review: mechanisms of ammonium toxicity and the quest for tolerance. *Plant Science* 248: 92-101.

Esteban, R., Ariz, I., Cruz, C. & Moran, J. F. (2016b). Mechanisms of ammonium toxicity and the quest for tolerance. *Plant Science* 248: 92-101.

Everitt, B. S., Landau, S. & Lesse M. (2001). Cluster Analyses (4th Edition). Arnold, London

Ewas, M., Gao, Y., Wang, S., Liu, X., Zhang, H., Nishawy, E. M. E., Ali, F., Shahzad, R., Ziaf, K., Subthain, H., Martin, C. & Luo, J. (2016). Manipulation of *SLMXL* for enhanced carotenoids accumulation and drought resistance in tomato. *Science Bulletin* 61: 1413-1418.

Fageria, N. K. & Baligar, V. C. (2005). Enhancing nitrogen use efficiency in crop plants. *Advances in Agronomy* 88: 97185.

Fageria, V. D. (2001). Nutrient interactions in crop plants. *Journal of Plant Nutrition* 24 (8): 1269-1290.

Fan, J.-B., Zhang, Y.-L., Turner, D., Duan, Y.-H., Wang, D.-S. & Shein, Q.-R. (2010). Root physiological and morphological characteristics of two rice cultivars with different nitrogen-use efficiency. P*edosphere* 20(4): 446-455.

FAO (2020a).The State of Food and Agriculture 2020. Overcoming water challenges in agriculture. FAO Rome, Italy.

FAO (2020b). Statistical Database: Production Statistics. Available online: Available from: http:// www.fao.org/faostat/en/#data/QC/visualize.

Farooq, M., Wahid, A., Kobayashi, N., Fujita, D. & Basra, S. (2009).Plant drought stress: effects, mechanisms and management. In *Sustainable agriculture*, 153-188: Springer.

Fátima, T., Teasdale, J. R., Bunce, J. & Mattoo, A. K. (2012). Tomato response to legume cover crop and nitrogen: differing enhancement patterns of fruit yield, photosynthesis and gene expression. *Functional Plant Biology* 39(3): 246.

Fernandes, A., Machado, J., Fernandes, T., Vasconcelos, M. & Carvalho, S. (2022). Water and nitrogen fertilization management in light of climate change: Impacts on food security and product quality. *Plant Nutrition and Food Security in the Era of Climate Change*: 147-178.

Fernández, J. E. & Cuevas, M. V. (2010). Irrigation scheduling from stem diameter variations: A review. *Agricultural and Forest Meteorology* 150(2): 135-151.

Ferrario-Méry, S., Valadier, M.-H. & Foyer, C. H. (1998). Overexpression of nitrate reductase in tobacco delays drought-induced decreases in nitrate reductase activity and mRNA. *Plant physiology* 117(1): 293-302.

Fidalgo, F., Freitas, R., Ferreira, R., Pessoa, A. M. & Teixeira, J. (2011). Solanum nigrum L. antioxidant defence system isozymes are regulated transcriptionally and posttranslationally in Cd-induced stress. *Environmental and experimental botany* 72(2): 312-319.

Fiebig, A. & Dodd, I. C. (2016). Inhibition of tomato shoot growth by over-irrigation is linked to nitrogen deficiency and ethylene. *Physiologia plantarum* 156(1): 70-83.

Flexas, J., Gallé, A., Galmés, J., Ribas-Carbo, M. & Medrano, H. (2012). The response of photosynthesis to soil water stress. In: Aroca, R. (Ed.), Plant Responses to Drought Stress. *Springer*, Berlin, Heidelberg, pp. 129-144.

Flexas, J., Ribas-Carbó, M., Bota, J., Galmés, J., Henkle, M., Martínez-Cañellas, S. & Medrano, H. (2006). Decreased Rubisco activity during water stress is not induced by decreased relative water content but related to conditions of low stomatal conductance and chloroplast CO_2 concentration. *New Phytologist* 172(1): 73-82.

Florencio-Ortiz, V., Sellés-Marchart, S., Zubcoff-Vallejo, J., Jander, G. & Casas, J. L. (2018). Changes in the free amino acid composition of *Capsicum annuum* (pepper) leaves in response to *Myzus persicae* (green peach aphid) infestation. A comparison with water stress. *PloS One* 13(6): e0198093.

Foolad, M. R. (2007). Current status of breeding tomatoes for salt and drought tolerance. In: Jenkes, M.A., Hasegawa, P.M., Jain, S.M. (Eds.), Advances in Molecular Breeding Toward Drought and Salt Tolerant Crops. *Springer*, Dordrecht, pp. 669-700.

Foolad, M. R., Zhang, L. P. & Subbiah, P. (2003). Genetics of drought tolerance during seed germination in tomato: inheritance and QTL mapping. *Genome* 46(4): 536-545.

Foyer, C. H., Valadier, M.-H., Migge, A. & Becker, T. W. (1998). Drought-induced effects on nitrate reductase activity and mRNA and on the coordination of nitrogen and carbon metabolism in maize leaves. *Plant Physiology* 117(1): 283-292.

Franco, J. A., Bañón, S., Vicente, M. J., Miralles, J. & Martínez-Sánchez, J. J. (2011). Review article: root development in horticultural plants grown under abiotic stress conditions - a review. *The Journal of Horticultural Science and Biotechnology*. 86(6): 543-556.

Franks, P. J. & Beerling, D. J. (2009). Maximum leaf conductance driven by CO_2 effects on stomatal size and density over geologic time. *Proceedings of the National Academy of Sciences* 106: 10343-10347.

Frazão, J. J., Prado, R. d. M., de Souza Júnior, J. P. & Rossatto, D. R. (2020). Silicon changes C:N:P stoichiometry of sugarcane and its consequences for photosynthesis, biomass partitioning and plant growth. *Scientific Reports* 10(1): 12492.

Fukushima, R. S. & Hatfield, R. D. (2001). Extraction and isolation of lignin for utilization as a standard to determine lignin concentration using the acetyl bromide spectrophotometric method. *Journal of Agricultural and Food Chemistry* 49(7): 3133-3139.

Fukutoku, Y. & Yamada, Y. (1984). Sources of proline-nitrogen in water-stressed soybean (*Glycine max*). II. Fate of 15N-labelled protein. *Physiologia Plantarum* 61(4): 622-628.

Galangau, F., Daniel-Vedele, F., Moureaux, T., Dorbe, M.-F., Leydecker, M.-T. & Caboche, M. (1988). Expression of leaf nitrate reductase genes from tomato and tobacco in relation to light-dark regimes and nitrate supply. *Plant Physiology* 88(2): 383-388.

Galangau, F., Daniel-Vedele, F., Moureaux, T., Dorbe, M.-F., Leydecker, M.-T. & Caboche, M. (1988). Expression of leaf nitrate reductase genes from tomato and tobacco in relation to light-dark regimes and nitrate supply. *Plant physiology* 88(2): 383-388.

Galdon-Armero, J., Fullana-Pericas, M., Mulet, P. A., Conesa, , M. À., Martin, C. & Galmés, J. (2018). The ratio of trichomes to stomata is associated with water use efficiency in *Solanum lycopersicum* (tomato). *The Plant Journal* 96(3): 607-619.

Galmés J, Conesa, M. À., Ochogavía, J. M., Perdomo, J. A., Francis, D. M., Ribas-Carbó, M., Savé, R., Flexas, J., Medrano, H. & Cifre, J. (2011). Physiological and

morphological adaptations in relation to water use efficiency in mediterranean accessions of *Solanum lycopersicum*. *Plant, Cell & Environment* 34(2): 245-260.

Galmés, J., Ochogavía, J. M., Gago, J., Roldán, E. J., Cifre, J. & Conesa, M. À. (2013). Leaf responses to drought stress in Mediterranean accessions of *Solanum lycopersicum*: anatomical adaptations in relation to gas exchange parameters. *Plant, Cell & Environment* 36(5): 920-935.

Gao, X., Zou, C., Wang, L. & Zhang, F. (2006). Silicon decreases transpiration rate and conductance from stomata of maize plants. *Journal of plant nutrition* 29(9): 1637-1647.

Gao, Y. F., Liu, J. K., Yang, F. M., Zhang, G. Y., Wang, D., Zhang, L., Ou, Y. B. & Yao, Y. A. (2020). The WRKY transcription factor WRKY8 promotes resistance to pathogen infection and mediates drought and salt stress tolerance in *Solanum lycopersicum*. *Physiologia Plantarum* 168(1): 98-117.

García-Martí, M., Piñero, M. C., García-Sanchez, F., Mestre, T. C., López-Delacalle, M., Martínez, V. & Rivero, R. M. (2019). Amelioration of the oxidative stress generated by simple or combined abiotic stress through the K^+ and Ca^{2+} supplementation in tomato plants. *Antioxidants* 8(4): 81.

Garnett, T., Conn, V. & Kaiser, B. N. (2009). Root based approaches to improving nitrogen use efficiency in plants. *Plant, Cell & Environment* 32(9): 1272-1283.

Geng, A., Wang, X., Wu, L., Wang, F., Wu, Z., Yang, H., Chen, Y., Wen, D. & Liu, X. (2018). Silicon improves growth and alleviates oxidative stress in rice seedlings (*Oryza sativa* L.) by strengthening antioxidant defense and enhancing protein metabolism under arsanilic acid exposure. *Ecotoxicology and Environmental Safety* 158: 266-273.

Gerendás, J. & Sattelmacher, B. (1990). Influence of nitrogen form and concentration on growth and ionic balance of tomato (*Lycopersicon esculentum*) and potato (*Solanum tuberosum*). In: van Beusichem, M.L. (Ed.), Plant Nutrition—Physiology and Applications. Developments in Plant and Soil Sciences, vol. 41. *Springer*, Dordrecht, pp. 33-37.

Gerendás, J., Zhu, Z., Bendixen, R., Ratcliffe, R. G. & Sattelmacher, B. (1997). Physiological and biochemical processes related to ammonium toxicity in higher plants. *Z. Pflanzenernaehr. Bodenk.* 160(2): 239-251.

Ghosh, U. K., Islam, M. N., Siddiqui, M. N. & Khan, M. A. R. (2021). Understanding the roles of osmolytes for acclimatizing plants to changing environment: a review of potential mechanism. *Plant Signal Behaviour* 16(8): 1913306.

Giardini, L., Giovanardi, R. & Borin, M. (1988). Water consumption and yield response of tomato in relation to water availability at different soil depth. *Acta Horticulturae* 228: 119-126.

Gilliham, M., Dayod, M., Hocking, B. J., Xu, B., Conn, S. J., Kaiser, B. N., Leigh, R. A. & Tyerman, S. D. (2011). Calcium delivery and storage in plant leaves: exploring the link with water flow. *Journal of Experimental Botany* 62(7): 2233-2250.

Gojon, A. (2017). Nitrogen nutrition in plants: rapid progress and new challenges. *Journal of Experimental Botany* 68(10): 2457-2462.

Gonçalves, B., Falco, V., Moutinho-Pereira, J., Bacelar, E., Peixoto, F. & Correia, C. (2009). Effects of elevated CO_2 on grapevine (*Vitis vinifera* L.): volatile composition, phenolic content, and in vitro antioxidant activity of red wine. *Journal of Agricultural and Food Chemistry* 57(1): 265-273.

Gong, H. j., Chen, K. m., Chen, G. c., Wang, S. m. & Zhang, C. l. (2003). Effects of silicon on growth of wheat under drought. *Journal of Plant Nutrition* 26(5): 1055-1063.

Gong, H., Zhu, X., Chen, K., Wang, S. & Zhang, C. (2005). Silicon alleviates oxidative damage of wheat plants in pots under drought. *Plant Science* 169(2): 313-321.

Gong, P., Zhang, J., Li, H., Yang, C., Zhang, C., Zhang, X., Khurram, Z., Zhang, Y., Wang, T., Fei, Z. & Ye, Z. (2010). Transcriptional profiles of drought-responsive

genes in modulating transcription signal transduction, and biochemical pathways in tomato. *Journal of Experimental Botany* 61(13): 3563-3575.

Gonzalez-Dugo, V., Durand, J.-L. & Gastal, F. (2010). Water deficit and nitrogen nutrition of crops. A review. *Agronomy for Sustainable Development* 30(3): 529-544.

Goss, M. J., Miller, M. H., Bailey, L. D. & Grant, C. A. (1993). Root growth and distribution in relation to nutrient availability and uptake. *European Journal of Agronomy* 2:(2): 57-67.

Groher, T., Schmittgen, S., Noga, G. & Hunsche, M. (2018). Limitation of mineral supply as tool for the induction of secondary metabolites accumulation in tomato leaves. *Plant Physiology and Biochemistry*: 130: 105-111.

Gruber, B. D., Giehl, R. F. H., Friedel, S. & von Wiren, N. (2013). Plasticity of the arabidopsis root system under nutrient deficiencies. *Plant Physiology* 163 (1): 161-179.

Guidi, L., Lorefice, G., Pardossi, A., Malorgio, F., Tognoni, F. & Soldatini, G. F. (1997). Growth and photosynthesis of *Lycopersicum esculentum* (L.) plants as affected by nitrogen deficiency. *Biologia Plantarum* 40(2): 235-244.

Gunes, A., Alpaslan, M. & Inal, A. (1998). Critical nutrient concentrations and antagonistic and synergistic relationships among the nutrients of NFT-grown young tomato plants. *Journal of Plant Nutrition* 21(10): 2035-2047.

Gunes, A., Pilbeam, D. J., Inal, A. & Coban, S. (2008). Influence of silicon on sunflower cultivars under drought stress, I: Growth, antioxidant mechanisms, and lipid peroxidation. *Communications in soil science and plant analysis* 39(13-14): 1885-1903.

Gunes, A., Pilbeam, D. J., Inal, A., Bagci, E. G. & Coban, S. (2007). Influence of silicon on antioxidant mechanisms and lipid peroxidation in chickpea (*Cicer arietinum* L.) cultivars under drought stress. *Journal of Plant Interactions* 2(2): 105-113.

Guo, N., Zhang, S., Gu, M. & Xu, G. (2021). Function, transport, and regulation of amino acids: What is missing in rice? *The Crop Journal* 9(3): 530-542.

Guo, S., Zhou, Y., Shen, Q. & Zhang, F. (2007). Effect of ammonium and nitrate nutrition on some physiological processes in higher plants growth, photosynthesis, photorespiration, and water relations. *Plant Biology* 9(1): 21-29.

Gur, A. & Zamir, D. (2004). Unused natural variation can lift yield barriers in plant breeding. *PLoS Biology* 2(10): e245.

Habibi, G. (2014). Silicon supplementation improves drought tolerance in canola plants. *Russian Journal of Plant Physiology* 61(6): 784-791.

Haddad, C., Arkoun, M., Jamois, F., Schwarzenberg, A., Yvin, J.-C., Etienne, P. & Laine, P. (2018). Silicon promotes growth of *Brassica napus* L. and delays leaf senescence induced by nitrogen starvation. *Frontiers in Plant Science* 9: 516.

Hajiboland, R., Bahrami-Rad, S. & Poschenrieder, C. (2017). Silicon modifies both a local response and a systemic response to mechanical stress in tobacco leaves. *Biologia Plantarum* 61(1): 187-191.

Hajiboland, R., Moradtalab, N., Eshaghi, Z. & Feizy, J. (2018). Effect of silicon supplementation on growth and metabolism of strawberry plants at three developmental stages. *New Zealand Journal of Crop and Horticultural Science* 46(2): 144-161.

Halpern, M., Bar-Tal, A., Lugassi, N., Egbaria, A. & Granot, D., Yermiyahu, U. (2018). The role of nitrogen in photosynthetic acclimation to elevated [CO_2] in tomatoes. Plant & Soil 434: 397-411.

Hao, S. X., Cao, H. X., Wang, H. B. & Pan, X. Y. (2019). Effects of water stress at different growth stages on comprehensive fruit quality and yield in different bunches of tomatoes in greenhouses. *International Journal of Agricultural and Biological Engineering* 12(3): 67-76.

Hasanuzzaman, M., Bhuyan, M. H. M. B., Zulfiqar, F., Raza, A., Mohsin, S. M., Mahmud, J. A., Fujita, M. & Fotopoulos, V. (2020). Reactive Oxygen Species and Antioxidant Defense in Plants under Abiotic Stress: Revisiting the Crucial Role of a Universal Defense Regulator. *Antioxidants* 9(8): 681.

Hasanuzzaman, M., Nahar, K., Anee, T. I., Khan, M. I. R. & Fujita, M. (2018). Silicon-mediated regulation of antioxidant defense and glyoxalase systems confers drought stress tolerance in Brassica napus L. *South African Journal of Botany* 115: 50-57.

Hattori, T., Inanaga, S., Araki, H., An, P., Morita, S., Luxová, M. & Lux, A. (2005). Application of silicon enhanced drought tolerance in Sorghum bicolor. *Physiologia plantarum* 123(4): 459-466.

Hattori, T., Inanaga, S., Tanimoto, E., Lux, A., Luxová, M. & Sugimoto, Y. (2003). Silicon-Induced Changes in Viscoelastic Properties of Sorghum Root Cell Walls. *Plant and Cell Physiology* 44(7): 743-749.

Hattori, T., Sonobe, K., Inanaga, S., An, P., Tsuji, W., Araki, H., Eneji, A. E. & Morita, S. (2007). Short term stomatal responses to light intensity changes and osmotic stress in sorghum seedlings raised with and without silicon. *Environmental and Experimental Botany* 60(2): 177-182.

Hayat, S., Hasan, S. A., Fariduddin, Q. & Ahmad, A. (2008). Growth of tomato (*Lycopersicon esculentum*) in response to salicylic acid under water stress. *Journal of Plant Interactions* 3(4): 297-304.

Hayat, S., Hayat, Q., Alyemeni, M. N., Wani, A. S., Pichtel, J. & Ahmad, A. (2012). Role of proline under changing environments: a review. *Plant Signaling & Behavior* 7(11): 1456-1466.

Haynes, R. J. & Goh, K. M. (1978). Ammonium and nitrate nutrition of plants. *Biological Reviews* 53: 465-510.

He, W., Yoo, G., Moonis, M., Kim, Y. & Chen, X. (2019). Impact assessment of high soil CO_2 on plant growth and soil environment: a greenhouse study. *PeerJ* 7: e6311.

Heath, R. L. & Packer, L. (1968). Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics* 125(1): 189-198.

Helaly, M. N., El-Hoseiny, H., El-Sheery, N. I., Rastogi, A. & Kalaji, H. M. (2017). Regulation and physiological role of silicon in alleviating drought stress of mango. *Plant Physiology and Biochemistry* 118: 31-44.

Heo, S. & Park, W.-P. (2022). Effects of nitrogen deficiency and resupply on the absorption of mineral nutrients by tangor cultivar 'Shiranuhi' (*Citrus unshiu* × *C. sinensis*) grown in a hydroponic system. *Plants* 11(18): 2351.

Hernandez-Espinoza, L. H. & Barrios-Masias, F. H. (2020). Physiological and anatomical changes in tomato roots in response to low water stress. *Scientia Horticulturae* 265: 109208.

Hernández, V., Hellín, P., Fenoll, J. & Flores, P. (2020). Impact of nitrogen supply limitation on tomato fruit composition. Scientia Horticulturae 264: 109173.

Heuvelink, E., Okello, R. C., Peet, M., Giovannoni, J. J. & Dorais, M. (2020). *Tomato.* CABI International.

Hildebrandt, Tatjana M., Nunes Nesi, A., Araújo, Wagner L. & Braun, H.-P. (2015). Amino Acid Catabolism in Plants. *Molecular Plant* 8(11): 1563-1579.

Hildebrandt, U., Schmelzer, E. & Bothe, H. (2002). Expression of nitrate transporter genes in tomato colonized by an arbuscular mycorrhizal fungus. *Physiologia Plantarum* 115(1): 125-136.

Hoffmann, C., Ladewig, E., Claassen, N. & Jungk, A. (1994). Phosphorus uptake of maize as affected by ammonium and nitrate nitrogen measurements and model calculations. *Journal of Plant Nutrition and Soil Science* 157(3), 225-232.

Hoffmann, J., Berni, R., Hausman, J.-F. & Guerriero, G. (2020). A Review on the Beneficial Role of Silicon against Salinity in Non-Accumulator Crops: Tomato as a Model. *Biomolecules* 10(9): 1284.

Holford, I. C. R. (1997). Soil phosphorus: its measurement, and its uptake by plants. *Soil Research* 35(2): 227-240.

Hooker, T. S. & Thorpe, T. A. (1997). Effects of water deficit stress on the developmental growth of excised tomato roots cultured in vitro. *In Vitro Cellular & Developmental Biology – Plant* 33(3): 245-251.

Hosseini, S. A., Maillard, A., Hajirezaei, M. R., Ali, N., Schwarzenberg, A., Jamois, F. & Yvin, J.-C. (2017). Induction of barley silicon transporter HvLsi1 and HvLsi2, increased silicon concentration in the shoot and regulated starch and ABA homeostasis under osmotic stress and concomitant potassium deficiency. *Frontiers in Plant Science* 8: 1359.

Hosseini, S. A., Naseri Rad, S., Ali, N. & Yvin, J.-C. (2019). The ameliorative effect of silicon on maize plants grown in Mg-deficient conditions. *International Journal of Molecular Sciences* 20(4): 969.

Hsiao, T. C. & Xu, L. K. (2000). Sensitivity of growth of roots versus leaves to water stress: bio-physical analysis and relation to water transport. *Journal of Experimental Botany* 51: 1595-1616.

Hsieh, P.-H., Kan, C.-C., Wu, H.-Y., Yang, H.-C. & Hsieh, M.-H. (2018). Early molecular events associated with nitrogen deficiency in rice seedling roots. *Scientific Reports* 8: 12207.

Hu, Y., Burucs, Z., von Tucher, S. & Schmidhalter, U. (2007). Short-term effects of drought and salinity on mineral nutrient distribution along growing leaves of maize seedlings. *Environmental and experimental botany* 60(2): 268-275.

Huang, N.-C. (1999). Cloning and functional characterization of an arabidopsis nitrate transporter gene that encodes a constitutive component of low-affinity uptake. *Plant Cell Online* 11 (8): 1381-1392.

Huett, D. O. & Dettmann, E. B. (1988). Effect of nitrogen on growth, fruit quality and nutrient uptake of tomatoes grown in sand culture. *Australian Journal of Experimental Agriculture* 28 (3): 391-399.

Hunt, R. (2012). *Basic growth analysis: plant growth analysis for beginners.* Springer Science & Business Media.

Hussain, H. A., Hussain, S., Khaliq, A., Ashraf, U., Anjum, S. A., Men, S. & Wang, L. (2018). Chilling and drought stresses in crop plants: implications, cross talk, and potential management opportunities. *Frontiers in Plant Science* 9: 393.

Iovieno, P., Punzo, P., Guida, G., Mistretta, C., Van Oosten, M. J., Nurcato, R., Bostan, H., Colantuono, C., Costa, A., Bagnaresi, P., Chiusano, M. L., Albrizio, R., Giorio, P., Batelli, G. & Grillo, S. (2016). Transcriptomic changes drive physiological responses to progressive drought stress and rehydration in tomato. *Frontiers in Plant Science* 7: 371.

Jaleel, C. A., Manivannan, P., Wahid, A., Farooq, M., Somasundaram, R. & Panneerselvam, R. (2009). Drought stress in plants: a review on morphological characteristics and pigments composition. *International Journal of Agriculture and Biology* 11: 100-105.

Jangam, A.P. & Raghuram, N. (2015). Nitrogen and stress. In: Pandey, G. (Ed.), Elucidation of abiotic stress signaling in plants. *Springer*, New York, NY, pp. 323339.

Jhariya, M. K., Banerjee, A., Meena, R. S. & Yadav, D. K. (2019).Agriculture, forestry and environmental sustainability: a way forward. in *sustainable agriculture, forest and environmental management*, 1-29 (Eds M. K. Jhariya, A. Banerjee, R. S. Meena and D. K. Yadav). Singapore: *Springer*, Singapore.

Jin, C. W., Du, S. T., Zhang, Y. S., Lin, X. Y. & Tang, C. X. (2009). Differential regulatory role of nitric oxide in mediating nitrate reductase activity in roots of tomato (*Solanum lycocarpum). Annals of Botany* 104(1): 9-17.

Jin, R., Wang, Y., Liu, R., Gou, J. & Chan, Z. (2015). Physiological and metabolic changes of purslane (*Portulaca oleracea* L.) in response to drought, heat, and combined stresses. *Frontiers in Plant Science* 6: 1123.

Jin, X., Yang, G., Tan, C. & Zhao, C. (2015). Effects of nitrogen stress on the photosynthetic CO_2 assimilation, chlorophyll fluorescence and sugar-nitrogen ratio in corn. *Scientific Reports* 5: 9311

Joshi, R., Wani, S. H., Singh, B., Bohra, A., Dar, Z. A., Lone, A. A., Pareek, A. & Singla-Pareek, S. L. (2016). Transcription factors and plants response to drought stress: current understanding and future directions. *Frontiers in Plant Science* 7: 1029.

Joshi, S., Thoday-Kennedy, E., Daetwyler, H. D., Hayden, M., Spangenberg, G. & Kant, S. (2021). High-throughput phenotyping to dissect genotypic differences in safflower for drought tolerance. *PloS One* 16(7): e0254908.

Kaiser, W. M. & Brendle-Behnisch, E. (1991). Rapid modulation of spinach leaf nitrate reductase activity by photosynthesis: I. Modulation in vivo by CO2 availability. *Plant Physiology* 96(2): 363-367.

Kaiser, W. M. & Huber, S. C. (2001). Post-translational regulation of nitrate reductase: mechanism, physiological relevance and environmental triggers. *Journal of Experimental Botany* 52(363): 1981-1989.

Kamanga, R.M. (2020). Screening and differential physiological responses of tomato (*Solanum lycopersicum* L.) to drought stress. *Plant Physiology Reports* 25: 472-482.

Kan, I. (2008). Yield quality and irrigation with saline water under environmental limitations: the case of processing tomatoes in California. *Agricultural Economics* 38: 57-66.

Kant, S., Bi, Y.-M. & Rothstein, S. J. (2011). Understanding plant response to nitrogen limitation for the improvement of crop nitrogen use efficiency. *Journal of Experimental Botany* 62(4): 1499-1509.

Kapilan, R., Vaziri, M. & Zwiazek, J. J. (2018). Regulation of aquaporins in plants under stress. *Biological research* 51(1): 1-11.

Kapoor, D., Bhardwaj, S., Landi, M., Sharma, A., Ramakrishnan, M. & Sharma, A. (2020). The impact of drought in plant metabolism: How to exploit tolerance mechanisms to increase crop production. *Applied Sciences* 10(16): 5692.

Kavoosi, G., Balotf, S., Eshghi, H. & Hasani, H. (2014). Analysis of nitrate reductase mRNA expression and nitrate reductase activity in response to nitrogen supply. *Molecular Biology Research Communications* 3(2): 75.

Kaya, C., Tuna, L. & Higgs, D. (2006). Effect of silicon on plant growth and mineral nutrition of maize grown under water-stress conditions. *Journal of Plant Nutrition* 29(8): 1469-1480.

Khan, M., Khan, M. J., Ahmad, S., Ali, A., Khan, N. & Fahad, M. A. (2020). Effect of different nitrogen doses and deficit irrigation on nitrogen use efficiency and growth parameters of tomato crop under drip irrigation system. *Sarhad Journal of Agriculture* 36(1): 319-323.

Khan, S. H., Arsalan, K., Uzma, L., Shah, A. S., Khan, M. A., Muhammad, B. & Ali, M. U. (2015). Effect of drought stress on tomato cv. Bombino. *Journal of Food Processing and Technology* 6(7).

Khapte, P., Kumar, P., Burman, U. & Kumar, P. (2019). Deficit irrigation in tomato: Agronomical and physio-biochemical implications. *Scientia Horticulturae* 248: 256-264.

Khavari-Nejad, R. A., Najafi, F. & Tofighi, C. (2009). Diverse responses of tomato to N and P deficiency. *International Journal of Agriculture and Biology* 11(2): 209-213.

Kiba, T., Kudo, T., Kojima, M. & Sakakibara, H. (2011). Hormonal control of nitrogen acquisition: roles of auxin, abscisic acid, and cytokinin. *Journal of Experimental Botany* 62(4): 1399-1409.

Kim, Y. H., Khan, A. L., Waqas, M. & Lee, I. J. (2017). Silicon Regulates Antioxidant Activities of Crop Plants under Abiotic-Induced Oxidative Stress: A Review. *Front Plant Sci* 8: 510.

Kirkby, E. A. & Knight, A. H. (1977). Influence of the level of nitrate nutrition on ion uptake and assimilation, organic acid accumulation, and cation-anion balance in whole tomato plants. *Plant Physiology* 60(3): 349-353.

Klunklin, W. & Savage, G. (2017). Effect on quality characteristics of tomatoes grown under well-watered and drought stress conditions. *Foods* 6(8)-56.

Koevoets, I. T., Venema, J. H., Elzenga, J. T. M. & Testerink, C. (2016). Roots withstanding their environment: exploiting root system architecture responses to abiotic stress to improve crop tolerance. *Frontiers in Plant Science* 7; 1335.

Koleška, I., Hasanagić, D., Todorović, V., Murtić, S., Klokić, I., Parađiković, N. & Kukavica, B. (2017). Biostimulant prevents yield loss and reduces oxidative damage in tomato plants grown on reduced NPK nutrition. *Journal of Plant Interactions* 12(1): 209-218.

Kosová, K., Vítámvás, P., Urban, M. O., Prášil, I. T. & Renaut, J. (2018). Plant abiotic stress proteomics: the major factors determining alterations in cellular proteome. *Frontiers in Plant Science* 9: 122.

Kostic, L., Nikolic, N., Bosnic, D., Samardzic, J. & Nikolic, M. (2017). Silicon increases phosphorus (P) uptake by wheat under low P acid soil conditions. *Plant and Soil* 419(1): 447-455.

Ková ik, J., Rep ák, M. & Korn, I. (2006). Nitrogen deficiency induced changes of free amino acids and coumarin contents in the leaves of Matricaria chamomilla. *Acta Physiologiae Plantarum* 28(2): 159-164.

Kovinich, N., Kayanja, G., Chanoca, A., Otegui, M. S. & Grotewold, E. (2015). Abiotic stresses induce different localizations of anthocyanins in Arabidopsis. *Plant Signal & Behaviour* 10(7): e1027850.

Krapp, A., Berthomé, R., Orsel, M., Mercey-Boutet, S., Yu, A., Castaings, L., Elftieh, S., Major, H., Renou, J. P. & Daniel-Vedele, F. (2011). Arabidopsis roots and shoots show distinct temporal adaptation patterns toward nitrogen starvation. *Plant Physiology* 157(3): 1255-1282.

Kubalt, K. (2016). The role of phenolic compounds in plant resistance.

Kumar, A., Singh, U. M., Manohar, M. & Gaur, V. S. (2014). Calcium transport from source to sink: understanding the mechanism(s) of acquisition, translocation, and accumulation for crop biofortification. *Acta Physiologiae Plantarum* 37(1): 1722.

Kumar, S., Sachdeva, S., Bhat, K. & Vats, S. (2018).Plant responses to drought stress: physiological, biochemical and molecular basis. In *Biotic and abiotic stress tolerance in plants*, 1-25: Springer.

Kumar, V., Sharma, A., Kaur, R., Thukral, A. K., Bhardwaj, R. & Ahmad, P. (2017). Differential distribution of amino acids in plants. *Amino acids* 49(5): 821-869.

Kyalo Willy, D., Muyanga, M. & Jayne, T. (2019). Can economic and environmental benefits associated with agricultural intensification be sustained at high population densities? A farm level empirical analysis. *Land Use Policy* 81: 100-110.

Lacrampe, N., Lopez-Lauri, F., Lugan, R., Colombié, S., Olivares, J., Nicot, P. C. & Lecompte, F. (2020). Regulation of sugar metabolism genes in the nitrogen-dependent susceptibility of tomato stems to Botrytis cinerea. *Annals of Botany* 127(1): 143-154.

Lam, H. M., Peng, S. & Coruzzi, G. M. (1994). Metabolic regulation of the gene encoding glutamine-dependent asparagine synthetase in *Arabidopsis thaliana*. *Plant Physiology* 106(4): 1347-1357.

Lamaoui, M., Jemo, M., Datla, R. & Bekkaoui, F. (2018). Heat and drought stresses in crops and approaches for their mitigation. *Frontiers in chemistry* 6: 26.

Larbat, R., Le Bot, J., Bourgaud, F., Robin, C. & Adamowicz, S. (2012). Organ-specific responses of tomato growth and phenolic metabolism to nitrate limitation. Plant Biology14(5): 760-769.

Larbat, R., Paris, C., Le Bot, J. & Adamowicz, S. (2014). Phenolic characterization and variability in leaves, stems and roots of micro-tom and patio tomatoes, in response to nitrogen limitation. *Plant Science* 224: 62-73.

Lata-Tenesaca, L. F., de Mello Prado, R., de Cássia Piccolo, M., da Silva, D. L. & da Silva, J. L. F. (2021). Silicon modifies C:N:P stoichiometry, and increases nutrient use efficiency and productivity of quinoa. *Scientific Reports* 11(1): 9893.

Lauter, F. R., Ninnemann, O., Bucher, M., Riesmeier, J. W. & Frommer, W. B. (1996). Preferential expression of an ammonium transporter and of two putative nitrate transporters in root hairs of tomato. *Proceedings of the National Academy of Sciences* 93(15), 8139-8144.

Lawlor, D. W. & Cornic, G., 2002. Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. *Plant, Cell & Environment* 25: 275-294.

Lawlor, D. W. & Tezara, W., 2009. Causes of decreased photosynthetic rate and metabolic capacity in water-deficient leaf cells: a critical evaluation of mechanisms and integration of pro-cesses. *Annals of Botany* 103(4), 561-579.

Laxa, M., Liebthal, M., Telman, W., Chibani, K. & Dietz, K.-J. (2019). The role of the plant antioxidant system in drought tolerance. *Antioxidants* 8(4): 94.

Le Gouis, J., Oury, F.-X. & Charmet, G. (2020). How changes in climate and agricultural practices influenced wheat production in Western Europe. *Journal of Cereal Science* 93: 102960.

Lea, P. J. & Azevedo, R. A. (2006). Nitrogen use efficiency. 1. Uptake of nitrogen from the soil. *Annals of Applied Biology* 149(3): 243-247.

Lea, P. J., Sodek, L., Parry, M. A. J., Shewry, P. R. & Halford, N. G. (2007). Asparagine in plants. *Annals of Applied Biology* 150(1): 1-26.

Leal, A. J. F., Barreto, R. F., de Mello Prado, R., da Silva Junior, G. B., Barreto, C. F., Santos, L. C. N. d. & Campos, C. N. S. (2021). Silicon attenuates abiotic stress caused by ammonium toxicity but not nitrogen deficiency in cotton plants. *Journal of Agronomy and Crop Science* 207(4): 774-781.

Lemaître, T., Gaufichon, L., Boutet-Mercey, S., Christ, A. & Masclaux-Daubresse, C. (2008). Enzymatic and metabolic diagnostic of nitrogen deficiency *in Arabidopsis thaliana* Wassileskija accession. *Plant and Cell Physiology* 49(7): 1056-1065.

Li, C., Yan, J. M., Li, Y. Z., Zhang, Z. C., Wang, Q. L. & Liang, Y. (2013). Silencing the *SpMPK1, SpMPK2,* and *SpMPK3* genes in tomato reduces abscisic acid-mediated drought tolerance. *International Journal of Molecular Sciences* 14(11): 21983-21996.

Li, D., Ma, W., Wei, J., Mao, Y., Peng, Z., Zhang, J., Kong, X., Han, Q., Fan, W., Yang, Y., Chen, J., Wu, L., Rengel, Z., Cui, X. & Chen, Q. (2020). Magnesium promotes root growth and increases aluminum tolerance via modulation of nitric oxide production in Arabidopsis. *Plant and Soil* 457(1): 83-95.

Li, J., Chen, C., Wei, J., Pan, Y., Su, C. & Zhang, X. (2019a). *SpPKE1*, a multiple stress-responsive gene confers salt tolerance in tomato and tobacco. *International Journal of Molecular Science* 20:(10): 2478.

Li, J., Wang, Y., Wei, J., Pan, Y., Su, C. & Zhang, X. (2018). A tomato proline-, lysine-, and glutamic- rich type gene *SpPKE1* positively regulates drought stress

tolerance. *Biochemical and Biophysical Research Communications* 499(4): 777-782.

Li, R., Liu, C., Zhao, R., Wang, L., Chen, L., Yu, W., Zhang, S., Sheng, J. & Shen, L. (2019b). CRISPR/Cas9-Mediated *SlNPR1* mutagenesis reduces tomato plant drought tolerance. *BMC Plant Biology* 19(1): 38.

Li, R., Wang, J., Li, S., Zhang, L., Qi, C., Weeda, S., Zhao, B., Ren, S. & Guo, Y. D. (2016). Plasma membrane intrinsic proteins *SlPIP2;1*, *SlPIP2;7* and *SlPIP2;5* conferring enhanced drought stress tolerance in tomato. *Scientific Reports* 6: 31814.

Li, W., Wang, Y., Okamoto, M., Crawford, N. M., Siddiqi, M. Y. & Glass, A. D. M. (2007). Dissection of the *AtNRT2.1:AtNRT2.2* inducible high-affinity nitrate transporter gene cluster. *Plant Physiology* 143(1): 425-433.

Liang, G., Liu, J., Zhang, J. & Guo, J. (2020). Effects of drought stress on photosynthetic and physiological parameters of tomato. *Journal of the American Society for Horticultural Science* 145(1): 12-17.

Liang, L., Ridoutt, B. G., Lal, R., Wang, D., Wu, W., Peng, P., Hang, S., Wang, L. & Zhao, G. (2019). Nitrogen footprint and nitrogen use efficiency of greenhouse tomato production in North China. *Journal of cleaner production* 208: 285-296.

Liang, S., Li, Y., Zhang, X., Sun, Z., Sun, N., Duan, Y., Xu, M. & Wu, L. (2018). Response of crop yield and nitrogen use efficiency for wheat-maize cropping system to future climate change in northern China. *Agricultural and Forest Meteorology* 262: 310-321.

Liang, Y., Sun, W., Zhu, Y.-G. & Christie, P. (2007). Mechanisms of silicon-mediated alleviation of abiotic stresses in higher plants: A review. *Environmental Pollution* 147(2): 422-428.

Líška, D., Soukup, M., Lukačová, Z., Bokor, B., Vaculík, M., Tripathi, D., Singh, V., Ahmad, P., Chauhan, D. & Prasad, S. (2017). Mechanisms of silicon-mediated alleviation of abiotic stress in plants: recent advances and future perspective. *Silicon in plants: advances and future prospects*: 1-28.

Liu, B., Soundararajan, P. & Manivannan, A. (2019a). Mechanisms of Silicon-Mediated Amelioration of Salt Stress in Plants. *Plants (Basel)* 8(9).

Liu, H. S. & Li, F. M. (2005). Root respiration, photosynthesis and grain yield of two spring wheat in response to soil drying. *Plant Growth Regulation* 46: 233-240.

Liu, J., Chen, F., Olokhnuud, C., Glass, A. D. M., Tong, Y., Zhang, F. & Mi, G. (2009). Root size and nitrogen-uptake activity in two maize (*Zea mays*) inbred lines differing in nitrogen-use efficiency. *Journal of Plant Nutrition and Soil Science* 172(2): 230-236.

Liu, J., Ma, K., Ciais, P. & Polasky, S. (2016). Reducing human nitrogen use for food production. *Scientific Reports* 6(1): 1-14.

Liu, L., Wang, J., Han, Z., Sun, X., Li, H., Zhang, J., & Lu, Y. (2016.) Molecular analyses of tomato GS, GOGAT And GDH gene families and their response to abiotic stresses. *Acta Physiologiae Plantarum* 38: 229.

Liu, M., Yu, H., Zhao, G., Huang, Q., Lu, Y. & Ouyang, B. (2017). Profiling of drought-responsive microrna and mrna in tomato using high-throughput sequencing. *BMC Genomics* 18(1): 481.

Liu, M., Yu, H., Zhao, G., Huang, Q., Lu, Y. & Ouyang, B. (2018). Identification of drought-responsive micrornas in tomato using high-throughput sequencing. Functional & Integrative Genomics 18: 67-78.

Liu, P., Yin, L., Deng, X., Wang, S., Tanaka, K. & Zhang, S. (2014). Aquaporin-mediated increase in root hydraulic conductance is involved in silicon-induced improved root water uptake under osmotic stress in *Sorghum bicolor* L. *Journal of ExperimentalBbotany* 65(17): 4747-4756.

Liu, Q., Ma, H., Sun, Z., Lin, X. & Zhou, X. (2019b). Translocation efficiencies and allocation of nitrogen, phosphorous and potassium in rice as affected by silicon fertilizer under high daytime temperature. *Journal of Agronomy and Crop Science* 205(2): 188-201.

Livak, K. J. & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *Methods* 25(4): 402-408.

Lovisolo, C. & Schubert, A. (1998). Effects of water stress on vessel size and xylem hydraulic conductivity in Vitis vinifera L. *Journal of Experimental Botany* 49(321): 693-700.

Luna, A. M., García, E. R., Servín, J. L. C., Herrera, A. L. & Arellano, J. S. (2014). Evaluation of different concentrations of nitrogen for tomato seedling production (*Lycopersicon esculentum* Mill.). *Universal Journal of Agricultural Research* 2(8): 305-312.

Lux, A., Lukačová, Z., Vaculík, M., Švubová, R., Kohanová, J., Soukup, M., Martinka, M. & Bokor, B. (2020). Silicification of Root Tissues. *Plants* 9(1): 111.

Ma, C. C., Li, Q. F., Gao, Y. B. & Xin, T. R. (2004). Effects of silicon application on drought resistance of cucumber plants. *Soil Science and Plant Nutrition* 50(5): 623-632.

Macduff, J., Jarvis, S., Larsson, C. & Oscarson, P. (1993). Plant growth in relation to the supply and uptake of NO_3^-: a comparison between relative addition rate and external concentration as driving variables. *Journal of Experimental Botany* 44(9): 1475-1484.

Machado, J., Fernandes, A., Fernandes, T., Heuvelink, E., Vasconcelos, M. & Carvalho, S. (2022). Drought and nitrogen stress effects and tolerance mechanisms in tomato: a review. *Plant nutrition and food security in the era of climate change*: 315-359.

Machado, J., Vasconcelos, M. W., Soares, C., Fidalgo, F., Heuvelink, E. & Carvalho, S. M. P. (2023a). Enzymatic and Non-Enzymatic Antioxidant Responses of Young Tomato Plants (cv. Micro-Tom) to Single and Combined Mild Nitrogen and Water Deficit: Not the Sum of the Parts. *Antioxidants* 12(2): 375.

Machado, J., Vasconcelos, M. W., Soares, C., Fidalgo, F., Heuvelink, E. & Carvalho, S. M. P. (2023b). Young Tomato Plants Respond Differently under Single or Combined Mild Nitrogen and Water Deficit: An Insight into Morphophysiological Responses and Primary Metabolism. *Plants* 12(5): 1181.

Madrid, R., Barba, E. M., Sánchez, A. & García, A. L. (2009). Effects of organic fertilisers and irrigation level on physical and chemical quality of industrial tomato fruit (cv. Nautilus). *Journal of the Science of Food and Agriculture* 89: 2608-2615.

Maillard, A., Ali, N., Schwarzenberg, A., Jamois, F., Yvin, J.-C. & Hosseini, S. A. (2018). Silicon transcriptionally regulates sulfur and ABA metabolism and delays leaf senescence in barley under combined sulfur deficiency and osmotic stress. *Environmental and Experimental Botany* 155: 394-410.

Makhadmeh, I., Albalasmeh, A. A., Ali, M., Thabet, S. G., Darabseh, W. A., Jaradat, S. & Alqudah, A. M. (2022). Molecular Characterization of Tomato (*Solanum lycopersicum* L.) Accessions under Drought Stress. *Horticulturae* 8(7): 600.

Malagoli, P., Laine, P., Rossato, L. & Ourry, A. (2005). Dynamics of nitrogen uptake and mobilization in field-grown winter oilseed rape (*Brassica napus*) from stem extension to harvest. II. An 15N-labelling-based simulation model of N partitioning between vegetative and reproductive tissues. *Annals of Bbotany* 95(7): 1187-1198.

Malik, M. A., Wani, A. H., Mir, S. H., Rehman, I. U., Tahir, I., Ahmad, P. & Rashid, I. (2021). Elucidating the role of silicon in drought stress tolerance in plants. *Plant Physiology and Biochemistry* 165: 187-195.

Manoj, K. & Uday, D. (2007). In vitro screening of tomato genotypes for drought resistance using polyethylene glycol. *African Journal of Biotechnology* 5: 1488-1493.

Mariz-Ponte, N., Mendes, R. J., Sario, S., Correia, C. V., Correia, C. M., Moutinho-Pereira, J., Melo, P., Dias, M. C. & Santos, C. (2021). Physiological, biochemical and molecular assessment of UV-A and UV-B supplementation in *Solanum lycopersicum*. *Plants* 10(5): 918.

Martin, T., Oswald, O. & Graham, I. A. (2002). Arabidopsis seedling growth, storage lipid mobilization, and photosynthetic gene expression are regulated by carbon: nitrogen availability. *Plant Physiology* 128(2): 472-481.

Martínez-Cuenca, M.-R., Pereira-Dias, L., Soler, S., López-Serrano, L., Alonso, D., Calatayud, Á. & Díez, M. J. (2020). Adaptation to water and salt stresses of solanum pimpinellifolium and *Solanum lycopersicum* var. cerasiforme. *Agronomy* 10(8): 1169.

Martinez, V., Mestre, T. C., Rubio, F., Girones-Vilaplana, A., Moreno, D. A., Mittler, R. & Rivero, R. M. (2016). Accumulation of flavonols over hydroxycinnamic acids favors oxidative damage protection under abiotic stress. *Frontiers in Plant Science* 7: 838.

Martinez, V., Nieves-Cordones, M., Lopez-Delacalle, M., Rodenas, R., Mestre, T. C., Garcia-Sanchez, F., Rubio, F., Nortes, P. A., Mittler, R. & Rivero, R. M. (2018). Tolerance to stress combination in tomato plants: New insights in the protective role of melatonin. *Molecules* 23(3): 535.

Masclaux-Daubresse, C., Daniel-Vedele, F., Dechorgnat, J., Chardon, F., Gaufichon, L. & Suzuki, A. (2010). Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. *Annals of Botany* 105(7): 1141-1157.

Masclaux-Daubresse, C., Reisdorf-Cren, M. & Orsel, M. (2008). Leaf nitrogen remobilisation for plant development and grain filling. *Plant Biology* 10: 23-36.

Matoh, T., Murata, S. & Takahashi, E. (1991). Effect of silicate application on photosynthesis of rice [Oryza sativa] plants. *Japanese Journal of Soil Science and Plant Nutrition (Japan)*.

Matsunami, M., Toyofuku, K., Kimura, N. & Ogawa, A. (2020). Osmotic stress leads to significant changes in rice root metabolic profiles between tolerant and sensitive genotypes. *Plants* 9(11): 1503.

Miao, B.-H., Han, X.-G. & Zhang, W.-H. (2010). The ameliorative effect of silicon on soybean seedlings grown in potassium-deficient medium. *Annals of Botany* 105(6): 967-973.

Millones-Chanamé, C. E., Souza de Oliveiras, A. M., Castro, E. M. & Maluf, W. R. (2019). Inheritance of blossom end rot resistance induced by drought stress and of associated stomatal densities in tomatoes. *Euphytica* 215: 120.

Mills, H. & Jones Jr, J. B. (1996). Plant analysis handbook II. Athens. *GA: MicroMacro Publishing*: 185-414.

Minden, V., Schaller, J. & Olde Venterink, H. (2021). Plants increase silicon content as a response to nitrogen or phosphorus limitation: a case study with *Holcus lanatus. Plant and Soil* 462(1): 95-108.

Ming, D., Pei, Z., Naeem, M., Gong, H. & Zhou, W. (2012). Silicon alleviates PEG-induced water-deficit stress in upland rice seedlings by enhancing osmotic adjustment. *Journal of Agronomy and Crop Science* 198(1): 14-26.

Mingo, D. M., Theobald, J. C., Bacon, M. A., Davies, W. J. & Dodd, I. C. (2004). Biomass allocation in tomato (*Lycopersicon esculentum*) plants grown under partial rootzone drying: enhancement of root growth. *Functional Plant Biology* 31: 971-978.

Mittler, R. (2006). Abiotic stress, the field environment and stress combination. *Trends in plant science* 11(1): 15-19.

Mittler, R., Vanderauwera, S., Suzuki, N., Miller, G., Tognetti, V. B., Vandepoele, K., Gollery, M., Shulaev, V. & Van Breusegem, F. (2011). ROS signaling: the new wave? *Trends in Plant Science* 16(6): 300-309.

Moles, T. M., Mariotti, L., De Pedro, L. F., Guglielminetti, L., Picciarelli, P. & Scartazza, A. (2018). Drought induced changes of leaf-to-root relationships in two tomato genotypes. *Plant Physiology and Biochemistry* 128: 24-31.

Monforte, A. J. (2020). Time to exploit phenotypic plasticity. *Journal of experimental botany* 71(18): 5295-5297.

Montagu, K. D. & Goh, K. M. (1990). Effects of forms and rates of organic and inorganic nitrogen fertilisers on the yield and some quality indices of tomatoes (*Lycopersicon esculentum* Miller). *New Zealand Journal of Crop and Horticultural Science* 18(1): 31-37.

Montesinos-Pereira, D., Barrameda-Medina, Y., Romero, L., Ruiz, J. M. & Sánchez-Rodríguez, E. (2014). Genotype differences in the metabolism of proline and polyamines under moderate drought in tomato plants. *Plant Biol. (Stuttg.)* 16 (6): 1050-1057.

Mu, X. & Chen, Y. (2021). The physiological response of photosynthesis to nitrogen deficiency. *Plant Physiology and Biochemistry* 158: 76-82.

Muhammad, T., Zhang, J., Ma, Y., Li, Y., Zhang, F., Zhang, Y. & Liang, Y. (2019). Overexpression of a mitogen-activated protein kinase *SlMAPK3* positively regulates tomato tolerance to cadmium and drought stress. *Molecules* 24(3).

Munir, S., Liu, H., Xing, Y., Hussain, S., Ouyang, B., Zhang, Y., Li, H. & Ye, Z. (2016). Overexpression of calmodulin-like (*ShCML44*) stress-responsive gene from *Solanum habrochaites* enhances tolerance to multiple abiotic stresses. *Scientific Reports* 6: 31772.

Murshed, R., Lopez-Lauri, F. & Sallanon, H. (2013). Effect of water stress on antioxidant systems and oxidative parameters in fruits of tomato (*Solanum Lycopersicon L*, cv. Micro-tom). *Physiology and Molecular Biology of Plants* 19(3): 363-378.

Muscolo, A., Junker, A., Klukas, C., Weigelt-Fischer, K., Riewe, D. & Altmann, T. (2015). Phenotypic and metabolic responses to drought and salinity of four contrasting lentil accessions. *J Exp Bot* 66(18): 5467-5480.

Nacry, P., Bouguyon, E. & Gojon, A. (2013). Nitrogen acquisition by roots: physiological and developmental mechanisms ensuring plant adaptation to a fluctuating resource. *Plant and Soil* 370(1): 1-29.

Nadeem, M., Li, J., Yahya, M., Sher, A., Ma, C., Wang, X. & Qiu, L. (2019). Research progress and perspective on drought stress in legumes: a review. *International Journal of Molecular Sciences* 20(10).

Nahar, K. & Gretzmache, R. (2011). Response of shoot and root development of seven tomato cultivars in hydrophonic system under water stress. *Academic Journal of Plant Sciences* 4(2): 57-63.

Nahar, K. & Ullah, S. (2017). Fruit quality and osmotic adjustment of four tomato cultivars under drought stress. *Asian Journal of Soil Science and Plant Nutrition* 2: 1-8.

Nahar, K. & Ullah, S. (2018). Drought stress effects on plant water relations, growth, fruit quality and osmotic adjustment of tomato (*Solanum Lycopersicum*) under subtropical condition. *Asian Journal of Agricultural and Horticultural Research* 1(2):114.

Nahar, K., Ullah, S. & Islam, N. (2011). Osmotic adjustment and quality response of five tomato cultivars (*Lycopersicon esculentum* Mill) following water deficit stress under subtropical climate. *Asian Journal of Plant Sciences* 10(2): 153-157.

Nakano, Y. & Asada, K. (1981). Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant and Cell Physiology* 22(5): 867-880.

Nangare, D. D., Singh, Y., Kumar, P. S. & Minhas, P. S. (2016). Growth, fruit yield and quality of tomato (*Lycopersicon esculentum* Mill.) as affected by deficit irrigation regulated on phenological basis. *Agricultural Water Management* 171: 73-79.

Nawaz, H., Zubair, M. & Derawadan, H. (2012). Interactive effects of nitrogen, phosphorus and zinc on growth and yield of tomato (*Solanum lycopersicum*). *African Journal of Agricultural Research* 7(26), 3792-3799.

Nemeskéri, E., Neményi, A., Bőcs, A., Pék, Z. & Helyes, L. (2019). Physiological Factors and their Relationship with the Productivity of Processing Tomato under Different Water Supplies. *Water* 11(3): 586.

Neu, S., Schaller, J. & Dudel, E. G. (2017). Silicon availability modifies nutrient use efficiency and content, C:N:P stoichiometry, and productivity of winter wheat (*Triticum aestivum* L.). *Scientific Reports* 7(1): 40829.

Nicoue, E. E., Savard, S. & Belkacemi, K. (2007). Anthocyanins in wild blueberries of Quebec: extraction and identification. *Journal of Agricultural and Food Chemistry* 55(14): 5626-5635.

Nonami, H. (1998). Plant water relations and control of cell elongation at low water potentials. *Journal of Plant Research* 111: 373-382.

Nunes da Silva, M., Machado, J., Osorio, J., Duarte, R. & Santos, C. S. (2022). Non-essential elements and their role in sustainable agriculture. *Agronomy* 12(4): 888.

Nunes-Nesi, A., Fernie, A. R. & Stitt, M. (2010). Metabolic and signaling aspects underpinning the regulation of plant carbon nitrogen interactions. *Molecular Plant* 3(6): 973-996.

Okunlola, O., Olatunji, A., Afolabi, M. & Gbadegesin, K. (2015). Effects of nitrogen nutritional stress on the morphological and yield parameters of tomato (*Solanum lycopersicum* L.). *Sciences in Cold and Arid Regions* 7(2): 137-145.

Oliveira, K. S., de Mello Prado, R., Checchio, M. V. & Gratão, P. L. (2021). Silicon via nutrient solution modulates deficient and sufficient manganese sugar and energy cane antioxidant systems. *Scientific Reports* 11(1): 1-14.

Ono, F., Frommer, W. B. & Wirén, N. (2000). Coordinated diurnal regulation of low- and high- affinity nitrate transporters in tomato. *Plant Biology* 2(1): 1723.

Orellana, S., Yañez, M., Espinoza, A., Verdugo, I., González, E., Ruiz-Lara, S. & Casaretto, J. A. (2010). The transcription factor *SlAREB1* confers drought, salt stress tolerance and regulates biotic and abiotic stress-related genes in tomato. *Plant, Cell & Environment* 33(12): 2191-2208.

Osakabe, Y., Osakabe, K., Shinozaki, K. & Tran, L.-S. P. (2014). Response of plants to water stress. *Frontiers in Plant Science* 5: 86.

Ozturk, M., Turkyilmaz Unal, B., García-Caparrós, P., Khursheed, A., Gul, A. & Hasanuzzaman, M. (2021). Osmoregulation and its actions during the drought stress in plants. *Physiol Plant* 172(2): 1321-1335.

Pan, Y., Seymour, G. B., Lu, C., Hu, Z., Chen, X. & Chen, G. (2012). An ethylene response factor (*ERF5*) promoting adaptation to drought and salt tolerance in tomato. *Plant Cell Reports* 31: 349-360.

Pandey, P., Ramegowda, V. & Senthil-Kumar, M. (2015). Shared and unique responses of plants to multiple individual stresses and stress combinations: physiological and molecular mechanisms. *Frontiers in plant science* 6: 723.

Parajuli, S., Ojha, B. R. & Ferrara, G. O. (2018). Quantification of secondary traits for drought and low nitrogen stress tolerance in inbreds and hybrids of maize (*Zea mays* L.). *Journal of Plant Breeding and Genetics* 2(1): 106.

Parkash, V. & Singh, S. (2020). A review on potential plant-based water stress indicators for vegetable crops. *Sustainability* 12(10): 3945.

Parry, M. A. J., Andralojc, P. J., Khan, S., Lea, P. J. & Keys, A. J. (2002). Rubisco activity: effects of drought stress. *Annals of Botany* 89(7): 833-839.

Parveen, A., Liu, W., Hussain, S., Asghar, J., Perveen, S. & Xiong, Y. (2019). Silicon priming regulates morpho-physiological growth and oxidative metabolism in maize under drought stress. *Plants* 8(10): 431.

Pascual, L. S., Segarra-Medina, C., Gómez-Cadenas, A., López-Climent, M. F., Vives-Peris, V. & Zandalinas, S. I. (2022). Climate change-associated multifactorial stress combination: A present challenge for our ecosystems. *Journal of Plant Physiology* 276: 153764.

Patanè, C., Scordia, D., Testa, G. & Cosentino, S. L. (2016). Physiological screening for drought tolerance in mediterranean long-storage tomato. *Plant Science* 249: 2534.

Patanè, C., Tringali, S. & Sortino, O. (2011). Effects of deficit irrigation on biomass, yield, water productivity and fruit quality of processing tomato under semi-arid Mediterranean climate conditions. *Scientia Horticulturae* 129(4): 590-596.

Pavlovic, J., Kostic, L., Bosnic, P., Kirkby, E. A. & Nikolic, M. (2021). Interactions of Silicon With Essential and Beneficial Elements in Plants. *Front Plant Sci* 12: 697592.

Pavlovic, J., Samardzic, J., Maksimović, V., Timotijevic, G., Stevic, N., Laursen, K. H., Hansen, T. H., Husted, S., Schjoerring, J. K. & Liang, Y. (2013). Silicon alleviates iron deficiency in cucumber by promoting mobilization of iron in the root apoplast. *New Phytologist* 198(4): 1096-1107.

Peng, M., Bi, Y.-M., Zhu, T. & Rothstein, S. J. (2007). Genome-wide analysis of Arabidopsis responsive transcriptome to nitrogen limitation and its regulation by the ubiquitin ligase gene NLA. *Plant Molecular Biology* 65(6): 775-797.

Perchlik, M. & Tegeder, M. (2018). Leaf Amino acid supply affects photosynthetic and plant nitrogen use efficiency under nitrogen stress. *Plant Physiology* 178(1): 174-188.

Pereira, B. d. J., Cecílio Filho, A. B. & La Scala, N. (2021). Greenhouse gas emissions and carbon footprint of cucumber, tomato and lettuce production using two cropping systems. *Journal of cCeanerPproduction* 282: 124517.

Pervez, M. A., Ayub, C. M., Khan, H. A., Shahid, M. A. & Ashraf, I. (2009). Effect of drought stress on growth, yield and seed quality of tomato (*Lycopersicon esculentum* L.). *Pakistan Journal of Agricultural Sciences* 46(3): 174-178.

Pinheiro, C. & Chaves, M. M. (2011). Photosynthesis and drought: can we make metabolic connections from available data. *Journal of Experimental Botany* 62(3): 869-882.

Pirasteh-Anosheh, H., Saed-Moucheshi, A., Pakniyat, H. & Pessarakli, M. (2016).Stomatal responses to drought stress. In *Water Stress and Crop Plants*, 24-40.

Pirasteh-Anosheh, H., Saed-Moucheshi, A., Pakniyat, H. & Pessarakli, M. (2016). Stomatal responses to drought stress. *Water stress and crop plants: A sustainable approach* 1: 24-40.

Plett, D. C., Ranathunge, K., Melino, V. J., Kuya, N., Uga, Y. & Kronzucker, H. J. (2020). The intersection of nitrogen nutrition and water use in plants: new paths toward improved crop productivity. *Journal of experimental botany* 71(15): 4452-4468.

Poiroux-Gonord, F., Bidel, L.P.R., Fanciullino, A.-L., Gautier, H., Lauri-Lopez, F. & Urban, L. (2010). Health benefits of vitamins and secondary metabolites of fruits and vegetables and prospects to increase their concentrations by agronomic approaches. *Journal of Agricultural and Food Chemistry* 58(23): 12065-12082.

Pokluda, R., Petříková, K., Abdelaziz, M. E. & Jezdinský, A. (2010). Effect of water stress on selected physiological characteristics of tomatoes. Acta *Universitatis Agriculturae et Silviculturae Mendelianae Brunensis* 58: 131-138.

Polesskaya, O., Kashirina, E. & Alekhina, N. (2004). Changes in the activity of antioxidant enzymes in wheat leaves and roots as a function of nitrogen source and supply. *Russian Journal of Plant Physiology* 51(5): 615-620.

Qaderi, M. M., Martel, A. B. & Dixon, S. L. (2019). Environmental factors influence plant vascular system and water regulation. *Plants* 8(3): 65.

Qazi, H. A., Jan, N., Ramazan, S. & John, R. (2019).Protein modification in plants in response to abiotic stress. In *Protein modificomics*, 171-201: Elsevier.

Qi, H.Y., Li, T.L., Zhang, J., Wang, L., Chen, Y.H., 2003. Effects on sucrose metabolism, dry matter distribution and fruit quality of tomato under water deficit. Agric. Sci. China 2, 12531258.

Rai, G. K., Parveen, A., Jamwal, G., Basu, U., Kumar, R. R., Rai, P. K., Sharma, J. P., Alalawy, A. I., Al-Duais, M. A. & Hossain, M. A. (2021). Leaf Proteome Response to Drought Stress and Antioxidant Potential in Tomato (Solanum lycopersicum L.). *Atmosphere* 12(8): 1021.

Raja, V., Qadir, S. U., Alyemeni, M. N. & Ahmad, P. (2020). Impact of drought and heat stress individually and in combination on physio-biochemical parameters, antioxidant responses, and gene expression in Solanum lycopersicum. *3 Biotech* 10(5): 1-18.

Rajagopal, V. & Rao, I. (1974). Changes in the endogenous level of auxins and gibberellin-like substances in the shoot apices of nitrogen-deficient tomato plants (Lycopersicon esculentum Mill). *Australian Journal of Botany* 22(3): 429-435.

Ramos, P. A., Moreirinha, C., Silva, S., Costa, E. M., Veiga, M., Coscueta, E., Santos, S. A., Almeida, A., Pintado, M. M. & Freire, C. S. (2019). The health-promoting potential of Salix spp. bark polar extracts: Key insights on phenolic composition and in vitro bioactivity and biocompatibility. *Antioxidants* 8(12): 609.

Rayar, A.J., van Hai, T., 1977. Effect of ammonium on uptake of phosphorus, potassium, calcium and magnesium by intact soybean plants. Plant Soil 48, 8187. Available from: https://doi.org/10.1007/BF00015159.

Rivero, R. M., Mestre, T. C., Mittler, R., Rubio, F., Garcia-Sanchez, F. & Martinez, V. (2014). The combined effect of salinity and heat reveals a specific physiological, biochemical and molecular response in tomato plants. *Plant, Cell & Environment* 37(5): 1059-1073.

Rizwan, M., Ali, S., Ibrahim, M., Farid, M., Adrees, M., Bharwana, S. A., Zia-ur-Rehman, M., Qayyum, M. F. & Abbas, F. (2015). Mechanisms of silicon-mediated alleviation of drought and salt stress in plants: a review. *Environmental Science and Pollution Research* 22(20): 15416-15431.

Robredo, A., Pérez-López, U., Miranda-Apodaca, J., Lacuesta, M., Mena-Petite, A. & Muñoz-Rueda, A. (2011). Elevated CO2 reduces the drought effect on nitrogen metabolism in barley plants during drought and subsequent recovery. *Environmental and experimental botany* 71(3): 399-408.

Roohanitaziani, R., de Maagd, R. A., Lammers, M., Molthoff, J., Meijer-Dekens, F., van Kaauwen, M. P. W., Finkers, R., Tikunov, Y., Visser, R. G. F. & Bovy, A. G. (2020). Exploration of a Resequenced Tomato Core Collection for Phenotypic and Genotypic Variation in Plant Growth and Fruit Quality Traits. *Genes* 11(11): 1278.

Roohanitaziani, R., Lammers, M., Molthoff, J., Tikunov, Y., Meijer-Dekens, F., Visser, R. G. F., van Arkel, J., Finkers, R., de Maagd, R. A. & Bovy, A. G. (2022). Phenotyping of a diverse tomato collection for postharvest shelf-life. *Postharvest Biology and Technology* 188: 111908.

Rouphael, Y., Cardarelli, M., Schwarz, D., Franken, P. & Colla, G. (2012).Effects of Drought on Nutrient Uptake and Assimilation in Vegetable Crops. In *Plant Responses to Drought Stress: From Morphological to Molecular Features*, 171-195 (Ed R. Aroca). Berlin, Heidelberg: Springer Berlin Heidelberg.

Ru, C., Hu, X., Chen, D., Wang, W. & Song, T. (2022). Heat and drought priming induce tolerance to subsequent heat and drought stress by regulating leaf photosynthesis, root morphology, and antioxidant defense in maize seedlings. *Environmental and experimental botany* 202: 105010.

Ruan, J., Haerdter, R. & Gerendás, J. (2010). Impact of nitrogen supply on carbon/nitrogen allocation: a case study on amino acids and catechins in green tea [Camellia sinensis (L.) O. Kuntze] plants*. *Plant Biology* 12(5): 724-734.

Rubio-Wilhelmi, M., Sanchez-Rodriguez, E., Rosales, M., Begona, B., Rios, J., Romero, L., Blumwald, E. & Ruiz, J. (2011). Effect of cytokinins on oxidative stress in tobacco plants under nitrogen deficiency. *Environmental and experimental botany* 72(2): 167-173.

Ruggiero, A., Punzo, P., Van Oosten, M. J., Cirillo, V., Esposito, S., Costa, A., Maggio, A., Grillo, S. & Batelli, G. (2022). Transcriptomic and splicing changes underlying tomato responses to combined water and nutrient stress. *Front Plant Sci* 13: 974048.

Safavi-Rizi, V., Franzaring, J., Fangmeier, A. & Kunze, R. (2018). Divergent N deficiency-dependent senescence and transcriptome response in developmentally old and young Brassica napus leaves. *Frontiers in plant science* 9: 48.

Sakuraba, Y. (2022). Molecular basis of nitrogen starvation-induced leaf senescence. *Front Plant Sci* 13: 1013304.

Salehi-Lisar, S.Y., Rahman, I.M., Hossain, M.M., Motafakkerazad, R. (2012). Water stress in plants: causes, effects and responses. In: IntechOpen. https://doi.org/10.5772/39363 Available from https://www.intechopen.com/books/water-stress/water-stress-in-plants-causes-effects-and-responses

Sánchez-Bermúdez, M., Del Pozo, J. C. & Pernas, M. (2022). Effects of Combined Abiotic Stresses Related to Climate Change on Root Growth in Crops. *Front Plant Sci* 13: 918537.

Sanchez-Casas, P. & Klessig, D. F. (1994). A salicylic acid-binding activity and a salicylic acid-inhibitable catalase activity are present in a variety of plant species. *Plant physiology* 106(4): 1675-1679.

Sánchez-Rodríguez, E., del Mar Rubio-Wilhelmi, M., Ríos, J. J., Blasco, B., Rosales, M. Á., Melgarejo, R., Romero, L. & Ruiz, J. M. (2011a). Ammonia production and assimilation: its importance as a tolerance mechanism during moderate water deficit in tomato plants. *Journal of plant physiology* 168(8): 816-823.

Sánchez-Rodríguez, E., Moreno, D. A., Ferreres, F., del Mar Rubio-Wilhelmi, M. & Ruiz, J. M. (2011b). Differential responses of five cherry tomato varieties to water stress: changes on phenolic metabolites and related enzymes. *Phytochemistry* 72(8): 723-729.

Sánchez-Rodríguez, E., Rubio-Wilhelmi, M. M., Cervilla, L. M., Blasco, B., Rios, J. J., Rosales, M. A., Romero, L. & Ruiz, J. M. (2010a). Genotypic differences in some physiological parameters symptomatic for oxidative stress under moderate drought in tomato plants. *Plant Science* 178(1): 30-40.

Sánchez-Rodríguez, E., Rubio-Wilhelmi, M., Blasco, B., Constán-Aguilar, C., Romero, L. & Ruiz, J. (2011c). Variation in the use efficiency of N under moderate water deficit in tomato plants (Solanum lycopersicum) differing in their tolerance to drought. *Acta Physiologiae Plantarum* 33(5): 1861-1865.

Sánchez-Rodríguez, E., Rubio-Wilhelmi, Md. M., Cervilla, L. M., Blasco, B., Ríos, J. J., Leyva, R., Romero, l. & Ruiz, J. M. (2010b). Study of the ionome and uptake fluxes in cherry tomato plants under moderate water stress conditions. *Plant & Soil* 335(1-2): 339-347.

Sandhu, R. K., Boyd, N. S., Zotarelli, L., Agehara, S. & Peres, N. (2021). Effect of planting density on the yield and growth of intercropped tomatoes and peppers in Florida. *HortScience* 56(2): 286-290.

Sardans, J., Rivas-Ubach, A., Estiarte, M., Ogaya, R. & Peñuelas, J. (2013). Field-simulated droughts affect elemental leaf stoichiometry in Mediterranean forests and shrublands. *Acta Oecologica* 50: 20-31.

Sattar, A., Sher, A., Ijaz, M., Ul-Allah, S., Butt, M., Irfan, M., Rizwan, M. S., Ali, H. & Cheema, M. A. (2020). Interactive effect of biochar and silicon on improving morpho-physiological and biochemical attributes of maize by reducing drought hazards. *Journal of Soil Science and Plant Nutrition* 20(4): 1819-1826.

Saud, S., Li, X., Chen, Y., Zhang, L., Fahad, S., Hussain, S., Sadiq, A. & Chen, Y. (2014). Silicon application increases drought tolerance of Kentucky bluegrass by improving plant water relations and morphophysiological functions. *The Scientific World Journal* 2014.

Schebesta, H. & Candel, J. J. L. (2020). Game-changing potential of the EU's Farm to Fork Strategy. *Nature Food* 1(10): 586-588.

Scholberg, J., McNeal, B. L., Boote, K. J., Jones, J. W., Locascio, S. J. & Olson, S. M. (2000). Nitrogen stress effects on growth and nitrogen accumulation by field-grown tomato. *Agronomy Journal* 92(1): 159-167.

Selim, A.-F. H. & El-Nady, M. F. (2011). Physio-anatomical responses of drought stressed tomato plants to magnetic field. *Acta Astronautica* 69(7-8): 387-396.

Senthilkumar, M., Sadashiva, A. T. & Laxmanan, V. (2017). Impact of water stress on root architecture in tomato (*Solanum lycopersicum* Mill). *International Journal of Current Microbiology and Applied Sciences* 6(7): 2095-2103.

Shafqat, W., Mazrou, Y. S. A., Sami-ur-Rehman, Nehela, Y., Ikram, S., Bibi, S., Naqvi, S. A., Hameed, M. & Jaskani, M. J. (2021). Effect of three water regimes on the physiological and anatomical structure of stem and leaves of different citrus rootstocks with distinct degrees of tolerance to drought stress. *Horticulturae* 7(12): 554.

Shangguan, Z. P., Shao, M. A. & Dyckmans, J. (2000). Nitrogen nutrition and water stress effects on leaf photosynthetic gas exchange and water use efficiency in winter wheat. *Environmental and Experimental Botany* 44(2): 141-149.

Shao, H.-B., Chu, L.-Y., Jaleel, C. A. & Zhao, C.-X. (2008). Water-deficit stress-induced anatomical changes in higher plants. *Comptes Rendus Biologies* 331(3): 215-225.

Shapiro, B. M. & Stadtman, E. (1970).[130] Glutamine synthetase (*Escherichia coli*). In *Methods in enzymology*, Vol. 17, 910-922: Elsevier.

Sharma, S., Villamor, J. G. & Verslues, P. E. (2011). Essential role of tissue-specific proline synthesis and catabolism in growth and redox balance at low water potential. *Plant Physiology* 157(1): 292-304.

Shen, X., Zhou, Y., Duan, L., Li, Z., Eneji, A. E. & Li, J. (2010). Silicon effects on photosynthesis and antioxidant parameters of soybean seedlings under drought and ultraviolet-B radiation. *Journal of Plant Physiology* 167(15): 1248-1252.

Shi, Y., Zhang, Y., Han, W., Feng, R., Hu, Y., Guo, J. & Gong, H. (2016). Silicon enhances water stress tolerance by improving root hydraulic conductance in *Solanum lycopersicum* L. *Frontiers in Plant Science* 7: 196.

Shi, Y., Zhang, Y., Yao, H., Wu, J., Sun, H. & Gong, H. (2014). Silicon improves seed germination and alleviates oxidative stress of bud seedlings in tomato under water deficit stress. *Plant Physiology and Biochemistry* 78: 27-36.

Shikata, M. & Ezura, H. (2016). Micro-Tom tomato as an alternative plant model system: mutant collection and efficient transformation. *Methods in Molecular Biology* 1363: 47-55.

Shirasawa, K., Isobe, S., Hirakawa, H., Asamizu, E., Fukuoka, H., Just, D., Rothan, C., Sasamoto, S., Fujishiro, T., Kishida, Y., Kohara, M., Tsuruoka, H., Wada, T., Nakamura, Y., Sato, S. & Tabata, S. (2010). SNP Discovery and linkage map construction in cultivated tomato. *DNA Research* 17(6): 381-391.

Sibomana, I., Aguyoh, J. & Opiyo, A. (2013). Water stress affects growth and yield of container grown tomato (*Lycopersicon esculentum* Mill) plants. *Global Journal of Biotechnology & Biochemistry* 2(4): 461-466.

Siddiqi, M. Y., Malhotra, B., Min, X. & Glass, A. D. M. (2002). Effects of ammonium and inorganic carbon enrichment on growth and yield of a hydroponic tomato crop. *Journal of Plant Nutrition and Soil Science* 165(2): 191.

Silva, J. L. F. d. & Prado, R. d. M. (2021). Elucidating the action mechanisms of silicon in the mitigation of phosphorus deficiency and enhancement of its response in sorghum plants. *Journal of Plant Nutrition* 44(17): 2572-2582.

Sinclair, T. R. & Muchow, R. C. (2001). System analysis of plant traits to increase grain yield on limited water supplies. *Agronomy Journal* 93(2):263.

Singh, M., Singh, V. P. & Prasad, S. M. (2019). Nitrogen alleviates salinity toxicity in *Solanum lycopersicum* seedlings by regulating ROS homeostasis. *Plant Physiology and Biochemistry* 141: 466-476.

Siracusa, L., Patanè, C., Rizzo, V., Cosentino, S. L. & Ruberto, G. (2018). Targeted secondary metabolic and physico-chemical traits analysis to assess genetic variability within a germplasm collection of “long storage” tomatoes. *Food Chemistry* 244: 275-283.

Sivakumar, R. & Srividhya, S. (2016). Impact of drought on flowering, yield and quality parameters in diverse genotypes of tomato (*Solanum lycopersicum* L.). *Advances in Horticultural Science* 30: 311.

Sivakumar, R., Nandhitha, G. K. & Nithila, S. (2018). Impact of drought on chlorophyll, soluble protein, abscisic acid, yield and quality characters of contrasting genotypes of tomato (*Solanum lycopersicum*). *Current Journal of Applied Science and Technology* 21(5), 110.

Slavík, B. & Jarvis, M. S. (1974). Methods of studying plant water relations.

Smart, D. R. & Bloom, A. J. (1993). Relationships between the kinetics of NH_4^+ and NO_3^- absorption and growth in the cultivated tomato (*Lycopersicon esculentum* Mill, cv T-5). *Plant, Cell & Environment* 16(3): 259-267.

Soares, C., Carvalho, M. E., Azevedo, R. A. & Fidalgo, F. (2019a). Plants facing oxidative challenges—A little help from the antioxidant networks. *Environmental and Experimental Botany* 161: 4-25.

Soares, C., Pereira, R., Spormann, S. & Fidalgo, F. (2019b). Is soil contamination by a glyphosate commercial formulation truly harmless to non-target plants? – Evaluation of oxidative damage and antioxidant responses in tomato. *Environmental Pollution* 247: 256-265.

Song, J., Wang, Y., Pan, Y., Pang, J., Zhang, X., Fan, J. & Zhang, Y. (2019). The influence of nitrogen availability on anatomical and physiological responses of Populus alba × P. glandulosa to drought stress. *BMC Plant Biology* 19(1): 63.

Sonneveld, C. & Voogt, W. (2009).Tissue Tests. In *Plant Nutrition of Greenhouse Crops*, 83-101 Dordrecht: Springer Netherlands.

Sonobe, K., Hattori, T., An, P., Tsuji, W., Eneji, A. E., Kobayashi, S., Kawamura, Y., Tanaka, K. & Inanaga, S. (2010). Effect of silicon application on sorghum root responses to water stress. *Journal of Plant Nutrition* 34(1): 71-82.

Sousa, B., Rodrigues, F., Soares, C., Martins, M., Azenha, M., Lino-Neto, T., Santos, C., Cunha, A. & Fidalgo, F. (2022). Impact of combined heat and salt stresses on tomato plants—insights into nutrient uptake and redox homeostasis. *Antioxidants* 11(3): 478.

Sousaraei, N., Mashayekhi, K., Mousavizadeh, S. J., Akbarpour, V., Medina, J. & Aliniaeifard, S. (2021). Screening of tomato landraces for drought tolerance based on growth and chlorophyll fluorescence analyses. *Horticulture, Environment, and Biotechnology* 62(4): 521-535.

Soval-Villa, M., Wood, C. & Guertal, E. (2002). Tomato leaf chlorophyll meter readings as affected by variety, nitrogen form, and nighttime nutrient solution strength. *Journal of Plant Nutrition* 25(10): 2129-2142.

Spollen, W. G. & Sharp, R. E. (1991). Spatial distribution of turgor and root growth at low water potentials. *Plant Physiology* 96(2): 438-443.

Spormann, S., Soares, C., Martins, V., Azenha, M., Gerós, H. & Fidalgo, F. (2022). Early activation of antioxidant responses in ni-stressed tomato cultivars determines their resilience under co-exposure to drought. *Journal of Plant Growth Regulation*: 1-15.

Srinivasa Rao, N. K., Bhatt, R. M. & Sadasivan, A. T., 2001. Tolerance to water stress in tomato cultivars. *Photosynthetica* 38(3): 465-467.

Sun, X., Chen, F., Yuan, L. & Mi, G. (2020a). The physiological mechanism underlying root elongation in response to nitrogen deficiency in crop plants. *Planta* 251(4): 84.

Sun, Y., Wang, C., Chen, H. Y. H. & Ruan, H. (2020b). Response of plants to water stress: a meta- analysis. Frontiers in Plant Science 11(978): 1-18.

Sung, J., Lee, S., Lee, Y., Ha, S., Song, B., Kim, T., Waters, B. M. & Krishnan, H. B. (2015). Metabolomic profiling from leaves and roots of tomato (Solanum lycopersicum L.) plants grown under nitrogen, phosphorus or potassium-deficient condition. *Plant Sci* 241: 55-64.

Suniaga Quijada, J., Bonafous, M. & Dumas, Y. (1992). Growth and development of young tomato plants under nitrogen deficiency. *In: International Symposium on Processing Tomatoes*, Mendoza, Argentina, pp. 159-164.

Szabados, L. & Savouré, A. (2010). Proline: a multifunctional amino acid. *Trends in Plant Science* 15(2): 89-97.

Tabatabaie, S. J., Gregory, P. J. & Hadley, P. (2004). Uneven distribution of nutrients in the root zone affects the incidence of blossom end rot and concentration of calcium and potassium in fruits of tomato. *Plant Soil Dordrecht* 258(2): 169-178

Taiz, L., Zeiger, E., Møller, I. M. & Murphy, A. (2015). *Plant physiology and development.* Sinauer Associates Incorporated.

Tajima, R. (2021). Importance of individual root traits to understand crop root system in agronomic and environmental contexts. *Breeding Science* 71(1): 13-19.

Takács, S., Pék, Z., Csányi, D., Daood, H. G., Szuvandzsiev, P., Palotás, G. & Helyes, L. (2020) Influence of water stress levels on the yield and lycopene content of tomato. *Water* 12(8): 2165.

Tamburino, R., Vitale, M., Ruggiero, A., Sassi, M., Sannino, L., Arena, S., Costa, A., Batelli, G., Zambrano, N. & Scaloni, A. (2017). Chloroplast proteome response to drought stress and recovery in tomato (*Solanum lycopersicum* L.). *BMC Plant Biology* 17(1): 1-14.

Tapia, G., Mendez, J. & Inostroza, L. (2016). Different combinations of morpho-physiological traits are responsable for tolerance to drought in wild tomatoes *Solanum chilense* and *Solanum peruvianum*. *Plant Biology* 18(3): 406-416.

Tavallali, V., Esmaili, S. & Karimi, S. (2018). Nitrogen and potassium requirements of tomato plants for the optimization of fruit quality and antioxidative capacity during storage. *Journal of Food Measurement and Characterization* 12: 755-762.

Tei, F., Benincasa, P. & Guiducci, M. (2002). Effect of N availability on growth, N uptake, light interception and photosynthetic activity in processing tomato. *Acta Horticulturae* 571: 209-216.

ten Hoopen, F., Cuin, T. A., Pedas, P., Hegelund, J. N., Shabala, S., Schjoerring, J. K. & Jahn, T. P. (2010). Competition between uptake of ammonium and potassium in barley and Arabidopsis roots: molecular mechanisms and physiological consequences. *Journal of Experimental Botany* 61(9): 2303-2315.

Tewari, R. K., Kumar, P. & Sharma, P. N. (2007). Oxidative stress and antioxidant responses in young leaves of mulberry plants grown under nitrogen, phosphorus or potassium deficiency. *Journal of Integrative Plant Biology* 49(3): 313-322.

Tezara, W., Mitchell, V. J., Driscoll, S. D. & Lawlor, D. W. (1999). Water stress inhibits plant photosynthesis by decreasing coupling factor and ATP. *Nature* 401: 914-917.

The, S. V., Snyder, R. & Tegeder, M. (2020). Targeting nitrogen metabolism and transport processes to improve plant nitrogen use efficiency. *Frontiers in Plant Science* 11: 628366.

Thipyapong, P., Melkonian, J., Wolfe, D. W. & Steffens, J. C. (2004). Suppression of polyphenol oxidases increases stress tolerance in tomato. *Plant Science* 167: 69-3703.

Thirumalaikumar, V.P., Devkar, V., Mehterov, N., Ali, S., Ozgur, R., Turkan, I., Mueller-Roeber, B. & Balazadeh, S. (2018). NAC transcription factor *JUNGBRUNNEN1* enhances drought tolerance in tomato. *Plant Biotechnology Journal* 16(2): 354-366.

Thompson, A. R., Doelling, J. H., Suttangkakul, A. & Vierstra, R. D. (2005). Autophagic nutrient recycling in Arabidopsis directed by the ATG8 and ATG12 conjugation pathways. *Plant Physiology* 138(4): 2097-2110.

Thorne, S. J., Hartley, S. E. & Maathuis, F. J. M. (2020). Is Silicon a panacea for alleviating drought and salt stress in crops? *Frontiers in Plant Science* 11: 1221.

Tian, J., Guo, S. R., Sun, J., Wang, L.-P., Yang, Y. & Li, B. (2011.) Effects of exogenous spermidine on nitrogen metabolism of cucumber seedlings under high temperature stress. *Chinese Journal of Ecology* 30(10): 2197-2202.

Tränkner, M., Tavakol, E. & Jákli, B. (2018). Functioning of potassium and magnesium in photosynthesis, photosynthate translocation and photoprotection. *Physiologia Plantarum* 163(3): 414-431.

Tripathi, P., Subedi, S., Khan, A. L., Chung, Y.-S. & Kim, Y. (2021). Silicon Effects on the Root System of Diverse Crop Species Using Root Phenotyping Technology. *Plants* 10(5): 885.

Tripodi, P., Figàs, M. R., Leteo, F., Soler, S., Díez, M. J., Campanelli, G., Cardi, T. & Prohens, J. (2022). Genotypic and environmental effects on morpho-physiological and agronomic performances of a tomato diversity panel in relation to nitrogen and water stress under organic farming. *Frontiers in plant science*: 2190.

Tubana, B. S., Babu, T. & Datnoff, L. E. (2016). A review of silicon in soils and plants and its role in US agriculture: history and future perspectives. *Soil Science* 181(9/10): 393-411.

Ullah, H., Santiago-Arenas, R., Ferdous, Z., Attia, A. & Datta, A. (2019).Chapter Two - Improving water use efficiency, nitrogen use efficiency, and radiation use efficiency in field crops under drought stress: A review. In *Advances in Agronomy*, Vol. 156, 109-157 (Ed D. L. Sparks). Academic Press.

Ullah, U., Ashraf, M., Shahzad, S. M., Siddiqui, A. R., Piracha, M. A. & Suleman, M. (2016). Growth behavior of tomato (*Solanum lycopersicum* L.) under drought stress in the presence of silicon and plant growth promoting rhizobacteria. *Soil & Environment* 35(1).

Urbanczyk-Wochniak, E. & Fernie, A. R. (2005). Metabolic profiling reveals altered nitrogen nutrient regimes have diverse effects on the metabolism of hydroponically-grown tomato (*Solanum lycopersicum*) plants. *Journal of Experimental Botany* 56(410): 309-321.

Vaerman, J., Saussoy, P. & Ingargiola, I. (2004). Evaluation of real-time PCR data. *Journal of Biological Regulators and Homeostatic Agents* 18(2): 212-214.

van Dijk, M., Morley, T., Rau, M. L. & Saghai, Y. (2021). A meta-analysis of projected global food demand and population at risk of hunger for the period 2010–2050. *Nature Food* 2(7): 494-501.

Vega-Mas, I., Rossi, M. T., Gupta, K. J., González-Murua, C., Ratcliffe, R. G., Estavillo, J. M. & González-Moro, M. B. (2019). Tomato roots exhibit in vivo glutamate dehydrogenase aminating capacity in response to excess ammonium supply. *Journal of Plant Physiology* 239: 83-91.

Vega, I., Nikolic, M., Pontigo, S., Godoy, K., Mora, M. d. L. L. & Cartes, P. (2019). Silicon Improves the Production of High Antioxidant or Structural Phenolic Compounds in Barley Cultivars under Aluminum Stress. *Agronomy* 9(7): 388.

Végh, K. R. (1991).Effect of soil water and nutrient supply on root characteristics and nutrient uptake of plants. In *Developments in Agricultural and Managed Forest Ecology*, Vol. 24, 143-148: Elsevier.

Verma, K. K., Liu, X.-H., Wu, K.-C., Singh, R. K., Song, Q.-Q., Malviya, M. K., Song, X.-P., Singh, P., Verma, C. L. & Li, Y.-R. (2020). The impact of silicon on photosynthetic and biochemical responses of sugarcane under different soil moisture levels. *Silicon* 12(6): 1355-1367.

Verma, K. K., Song, X.-P., Lin, B., Guo, D.-J., Singh, M., Rajput, V. D., Singh, R. K., Singh, P., Sharma, A., Malviya, M. K., Chen, G.-L. & Li, Y.-R. (2022). Silicon Induced Drought Tolerance in Crop Plants: Physiological Adaptation Strategies. *Silicon* 14(6): 2473-2487.

Vernoux, T., Besnard, F. & Traas, J. (2010). Auxin at the shoot apical meristem. *Cold Spring Harbor Perspectives in Biology* 2(4): 1487.

Von Wiren, N., Lauter, F.-R., Ninnemann, O., Gillissen, B., Walch-Liu, P., Engels, C., Jost, W & Frommer, W. B. (2000). Differential regulation of three functional ammonium transporter genes by nitrogen in root hairs and by light in leaves of tomato. *Plant Journal* 21(2): 167-175.

Vos, J., Van Der Putten, P. & Birch, C. (2005). Effect of nitrogen supply on leaf appearance, leaf growth, leaf nitrogen economy and photosynthetic capacity in maize (*Zea mays* L.). *Field Crops Research* 93(1): 64-73.

Wang, F., Kang, S., Du, T., Li, F. & Qiu, R., (2011). Determination of comprehensive quality index for tomato and its response to different irrigation treatments. *Agricultural Water Management* 98: 1228.1238.

Wang, H., Fan, H. & Yao, H. (2020). Effects of elevated CO_2 on tomato (*Lycopersicon esculentum* Mill.) growth and rhizosphere soil microbial community structure and functionality. *Agronomy* 10: 1752.

Wang, H., Inukai, Y. & Yamauchi, A. (2006). Root development and nutrient uptake. *Critical Reviews in Plant Sciences* 25(3): 279-301.

Wang, L., Chen, L., Li, R., Zhao, R., Yang, M., Sheng, J. & Shen, L. (2017). Reduced drought tolerance by CRISPR/Cas9-mediated *SlMAPK3* mutagenesis in tomato plants. *Journal of Agricultural and Food Chemistry* 65(39): 8674-8682.

Wang, M., Wang, R., Mur, L. A. J., Ruan, J., Shen, Q. & Guo, S. (2021). Functions of silicon in plant drought stress responses. *Horticulture Research* 8.

Wang, P., Sun, X., Jia, X., Wang, N., Gong, X. & Ma, F. (2016). Characterization of an autophagy- related gene *MdATG8i* from apple. *Frontiers in Plant Science* 7: 720

Wang, R., Min, J., Kronzucker, H. J., Li, Y. & Shi, W. (2019a). N and P runoff losses in China's vegetable production systems: Loss characteristics, impact, and management practices. *Science of the Total Environment* 663: 971-979.

Wang, Y.-H. (2001). Nitrate-induced genes in tomato roots. Array analysis reveals novel genes that may play a role in nitrogen nutrition. *Plant Physiology* 127(1): 345-359.

Wang, Y.-T., Huang, S.-W., Liu, R.-L. & Jin, J.-Y. (2007). Effects of nitrogen application on flavor compounds of cherry tomato fruits. *Journal of Plant Nutrition and Soil Science* 170(4), 461-468.

Wang, Y., Cai, S., Yin, L., Shi, K., Xia, X., Zhou, Y., Jingguan, Y. & Zhou, J. (2015). Tomato *HsFA1a* plays a critical role in plant drought tolerance by activating ATG genes and inducing Autophagy. *Autophagy* 11(11): 2033-2047.

Wang, Y., Zhang, B., Jiang, D. & Chen, G. (2019b). Silicon improves photosynthetic performance by optimizing thylakoid membrane protein components in rice under drought stress. *Environmental and Experimental Botany* 158: 117-124.

Wani, S. H., Singh, N. B., Haribhushan, A. & Mir, J. I. (2013). Compatible solute engineering in plants for abiotic stress tolerance: role of glycine betaine. *Current Genomics* 14(3): 1571-65.

Waraich, E. A., Ahmad, R. & Ashraf, M. (2011). Role of mineral nutrition in alleviation of drought stress in plants. *Australian Journal of Crop Science* 5(6): 764-777.

Warner, J., Zhang, T.Q. & Hao, X. (2004). Effects of nitrogen fertilization on fruit yield and quality of processing tomatoes. *Canadian Journal of Plant Sciences* 84(3), 865-871.

Wei, S., Wang, X., Shi, D., Li, Y., Zhang, J., Liu, P. Zhao, B. & Dong, S. (2016). The mechanisms of low nitrogen induced weakened photosynthesis in summer maize (*Zea mays* L.) under field conditions. *Plant Physiology and Biochemistry* 105: 118-128.

White, P. J. (2012).Chapter 3 - Long-distance transport in the xylem and phloem. in *marschner's mineral nutrition of higher plants (Third Edition)*, 49-70 (Ed P. Marschner). San Diego: Academic Press.

Wingler, A. & Roitsch, T. (2008). Metabolic regulation of leaf senescence: interactions of sugar signalling with biotic and abiotic stress responses. *Plant Biology* 10: 50-62.

Winter, G., Todd, C. D., Trovato, M., Forlani, G. & Funck, D. (2015). Physiological implications of arginine metabolism in plants. *Frontiers in Plant Science* 6: 534.

Wu, X., Yu, Y., Baerson, S. R., Song, Y., Liang, G., Ding, C., Niu, J., Pan, Z. & Zeng, R. (2017). Interactions between nitrogen and silicon in rice and their effects on resistance toward the brown planthopper Nilaparvata lugens. *Frontiers in Plant Science* 8: 28.

Wu, Y. & Cosgrove, D. J. (2000). Adaptation of roots to low water potentials by changes in cell wall extensibility and cell wall proteins. *Journal of ExperimentalBbotany* 51(350): 1543-1553.

Wu, Y., Yan, S., Fan, J., Zhang, F., Zhao, W., Zheng, J., Guo, J., Xiang, Y. & Wu, L. (2022). Combined effects of irrigation level and fertilization practice on yield, economic benefit and water-nitrogen use efficiency of drip-irrigated greenhouse tomato. *Agricultural Water Management* 262: 107401.

Xu, L., Islam, F., Ali, B., Pei, Z., Li, J., Ghani, M. A. & Zhou, W. (2017). Silicon and water-deficit stress differentially modulate physiology and ultrastructure in wheat (*Triticum aestivum* L.). *3 Biotech* 7(4): 273.

Yang, T., Li, H., Tai, Y., Dong, C., Cheng, X., Xia, E., Chen, Z., Li, F., Wan, X. & Zhang, Z. (2020). Transcriptional regulation of amino acid metabolism in response to nitrogen deficiency and nitrogen forms in tea plant root (*Camellia sinensis* L.). *Scientific Reports* 10(1): 6868.

Yang, X., Lu, M., Wang, Y., Wang, Y., Liu, Z. & Chen, S. (2021). Response Mechanism of Plants to Drought Stress. *Horticulturae* 7(3): 50.

Yin, L., Wang, S., Liu, P., Wang, W., Cao, D., Deng, X. & Zhang, S. (2014). Silicon-mediated changes in polyamine and 1-aminocyclopropane-1-carboxylic acid are involved in silicon-induced drought resistance in *Sorghum bicolor* L. *Plant Physiology and Biochemistry* 80: 268-277.

Yoneyama, K., Xie, X., Kim, H., Nomura, T., Sekimoto, H., Yokota, T. & Yoneyama, K. (2012). How do nitrogen and phosphorus deficiencies affect strigolactone production and exudation? *Planta* 235: 1197:1207.

Yuan, X., Yang, Z., Li, Y., Liu, Q. & Han, W. (2016). Effects of different levels of water stress on leaf photosynthetic characteristics and antioxidant enzyme activities of greenhouse tomato. *Photosynthetica* 54(1): 28-39.

Zafar, H., Ali, A., Ali, J. S., Haq, I. U. & Zia, M. (2016). Effect of ZnO nanoparticles on Brassica nigra seedlings and stem explants: growth dynamics and antioxidative response. *Frontiers in Plant Science* 7: 535.

Zakari, S. A., Zaidi, S. H. R., Sunusi, M. & Dauda, K. D. (2021). Nitrogen deficiency regulates premature senescence by modulating flag leaf function, ROS homeostasis, and intercellular sugar concentration in rice during grain filling. *Journal of Genetic Engineering and Biotechnology* 19(1): 1-14.

Zandalinas, S. I., Balfagón, D., Arbona, V. & Gómez-Cadenas, A. (2017). Modulation of antioxidant defense system is associated with combined drought and heat stress tolerance in citrus. *Frontiers in Plant Science* 8: 953.

Zargar, S. M., Gupta, N., Nazir, M., Mahajan, R., Malik, F. A., Sofi, N. R., Shikari, A. b. & Salgotra, R. K. (2017). Impact of drought on photosynthesis: molecular perspective. *Plant Gene* 11: 154-159.

Zhang, F., Niu, J., Zhang, W., Chen, X., Li, C., Yuan, L. & Xie, J. (2010). Potassium nutrition of crops under varied regimes of nitrogen supply. *Plant and Soil* 335(1): 21-34.

Zhang, Y., Yu, S., Gong, H.-j., ZHAO, H.-l., LI, H.-l., HU, Y.-h. & WANG, Y.-c. (2018). Beneficial effects of silicon on photosynthesis of tomato seedlings under water stress. *Journal of Integrative Agriculture* 17(10): 2151-2159.

Zheng, N., Xiao, H., Zhang, Z., Gao, X. & Zhao, J. (2017). Rapid and sensitive method for determining free amino acids in plant tissue by high-performance liquid chromatography with fluorescence detection. *Acta Geochimica* 36(4): 680-696.

Zhou, R., Kong, L., Wu, Z., Rosenqvist, E., Wang, Y., Zhao, L., Zhao, T. & Ottosen, C.-O. (2019a). Physiological response of tomatoes at drought, heat and their combination followed by recovery. *Physiologia Plantarum* 165(2): 144-154.

Zhou, R., Kong, L., Wu, Z., Rosenqvist, E., Wang, Y., Zhao, L., Zhao, T. & Ottosen, C. O. (2019b). Physiological response of tomatoes at drought, heat and their combination followed by recovery. *Physiologia Plantarum* 165(2): 144-154.

Zhou, R., Kong, L., Yu, X., Ottosen, C.-O., Zhao, T., Jiang, F. & Wu, Z. (2019c). Oxidative damage and antioxidant mechanism in tomatoes responding to drought and heat stress. *Acta Physiologiae Plantarum* 41(2): 1-11.

Zhou, R., Yu, X., Ottosen, C.-O., Rosenqvist, E., Zhao, L., Wang, Y., Yu, W., Zhao, T. & Wu, Z. (2017). Drought stress had a predominant effect over heat stress on three tomato cultivars subjected to combined stress. *BMC Plant Biology* 17(1): 1-13.

Zhou, X-m., Zhao, P., Wang, W., Zou, J., Cheng, T-h., Peng, X-b. & Sun, M-x. (2015). A comprehensive, genome-wide analysis of autophagy-related genes identified in tobacco suggests a central role of autophagy in plant response to various environmental cues. *DNA Research* 22(4): 245-257.

Zhu, M., Meng, X., Cai, J., Li, G., Dong, T. & Li, Z., (2018). Basic leucine zipper transcription factor *SlbZIP1* mediates salt and drought stress tolerance in tomato. *BMC Plant Biology* 18(1): 83.

Zhu, Y. & Gong, H. (2014). Beneficial effects of silicon on salt and drought tolerance in plants. *Agronomy for sustainable development* 34(2): 455-472.

Živanović, B., Milić Komić, S., Tosti, T., Vidović, M., Prokić, L. & Veljović Jovanović, S. (2020). Leaf Soluble Sugars and Free Amino Acids as Important Components of Abscisic Acid—Mediated Drought Response in Tomato. *Plants* 9(9): 1147.