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Exploring the Impact of Abiotic Stress on Brassica and Tomato: Morphophysiology and Microbiome Dynamics

PhD thesis

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Contents

Abstract

Climate change and the increasing global demand for food necessitate the development of crop species that are both resilient to abiotic stresses and compatible with sustainable agricultural practices. In this thesis, two complementary approaches were investigated: the genetic basis of drought tolerance in Brassica species and the role of grafting and microbial amendments in shaping the rhizosphere microbiome and yield performance of tomato (*Solanum lycopersicum L.*). In the first part, transcriptomic analyses were performed on drought-sensitive *Brassica oleracea L. var. botrytis* × italica Sicilian landraces and drought-tolerant B. macrocarpa Guss populations subjected to optimal irrigation and water deficit conditions. Physiological measurements (for exapmple leaf area and SPAD index) confirmed differential responses between tolerant and sensitive genotypes. Comparative RNA-seq using both de novo and reference-based assemblies allowed the identification of key genes and pathways underlying drought stress response, including a transcription factor showing opposite expression trends between tolerant and sensitive genotypes, thus providing novel insights into the genetic determinants of drought resistance in Brassica crops. In the second part, tomato grafting with two commercial hybrid rootstocks combined with a microbial amendment was assessed under greenhouse conditions. Yield traits, root components, and rhizosphere microbiomes were analyzed through ITS and 16S rRNA amplicon sequencing. Results demonstrated that fungal community composition was primarily influenced by grafting combinations, whereas bacterial abundance responded more strongly to microbial treatment. Significant correlations between microbial taxa and plant morphometric traits highlighted the synergistic potential of grafting and microbial amendments in enhancing plant growth, mitigating soilborne stresses, and supporting sustainable horticultural

production. Collectively, the findings from both case studies provide valuable strategies for advancing climate-resilient and ecofriendly crop production systems. The research presented in this thesis has been published in peer-reviewed journals: on tomato grafting and microbial consortia in [1] (Agronomy), and on comparative transcriptomics of Brassica genotypes under drought stress in [2] (Plant Stress).

Chapter 1

Overview of studied crops

1.1 Introduction to the *Brassicaceae* Family

The horticultural crop *Brassica oleracea L.* has played an important role in global food systems for centuries, providing a source of leaf and root vegetables, feed and forage [3]. The predominantly temperate and herbaceous family *Brassicaceae* is part of the plant order Brassicales, which encompasses 18 families, all characterized by the presence of glucosinolates, a unique chemical feature that serves a protective function. While many individuals may be aware that various breeds of dogs belong to the same species, they often find it surprising to discover that the cultivated varieties of B. oleracea, such as broccoli (var. italica), Brussels sprouts (var. gemmifera), cabbage (var. capitata), cauliflower (var. botrytis), kale (var. acephala), and kohlrabi (var. gongylodes), also belong to a single species. B. oleracea plants contain a variety of vital and abundant nutrients, including glucosinolates that are specific to crucifers, which have numerous biological functions and show significant diversity. For instance, cabbage forms a head of leaves, while cauliflower and broccoli produce thick, undeveloped flower clusters known as curds. Sprouts feature buds that develop along their stems, Chinese kale has a tender stem, and kohlrabi produces a swollen stem that is tuberous. In the morphotypes of B. oleracea serves as a clear illustration of how the different parts of a plant species can be focused on during domestication, leading to high-yielding varieties with unique edible qualities [4].

1.1.1 Taxonomy, Domestication and Evolution

The *Brassicaceae* family showcases extensive diversity and includes two subfamilies: Aethionematoideae and Brassicoideae. Furthermore, the Brassicoideae are divided into five supertribes, which consist of the previously classified Brassicodae and the newly formed Arabodae, Heliophilodae, Hesperodae, and Camelinodae. Within the Brassicodae, the Brassiceae are categorized into 13 subtribes. Notably, the Brassiceae stands out with the greatest variety of genera, totaling 92. A considerable number of these species are economically significant, with *Brassica rapa var. oleifera DC.*, commonly known as rapeseed, being extensively grown in China for use as both an edible oil crop and an ornamental plant [5]. Moreover, the family *Brassicaceae* has a pivotal role in agriculture as a source of crucial crops such as cabbage, cauliflower, and mustard. The *Brassicaceae* family, which separated from other eudicots around. 5 to 60 million years ago, is no different. Researchers have also proposed that nearly half of the species in the *Brassicaceae* family may have emerged from recent polyploid events. The divergence of these species and their major polyploid features often coincide with times of climatic instability, implying that possessing multiple chromosome sets might provide an evolutionary benefit in swiftly shifting environments. This is especially clear in the development of the *Brassicaceae* family, highlighting the crucial role of polyploidization in promoting the variety and endurance of this plant group. Moreover, the *Brassicaceae* family is renowned for its frequent hybridization events and all the evolutionary complexity reflects the dynamic nature of the *Brassicaceae* genome over millions of years [6].

1.1.2 Phytochemical Composition and Nutritional Value

Brassicaceae vegetables are significant crops eaten around the globe because of their distinctive taste and widely acknowledged health benefits, which are closely linked to the substances they contain. Isothiocyanates (ITC) are the primary compounds known for giving them a spicy flavor. In addition to ITC, these vegetables are abundant in carotenoids, phenolic compounds, minerals, and vitamins. Therefore, the phytochemical characteristics of Brassica make them an excellent natural option enhance the nutritional value of processed foods. [7]. The *Brassicaceae* have a lot of attention be-cause in recent years their phytochemical compounds have been di-rectly linked to beneficial effects in human health. Antioxidants function through various mechanisms: (a) they remove substances

that start peroxidation, (b) they bind metal ions so that these ions cannot create reactive species or break down lipid peroxides, (c) they deactivate reactive oxygen species to stop peroxide formation, (d) they interrupt the chain reaction of auto-oxidation, and/or (e) they lower the concentrations of localized oxygen [8]. Even though there are expenses associated with the creation of all metabolites, it is widely believed that genes responsible for secondary metabolites are passed down because of the evolutionary benefits they provide to the plant, particularly in defense against environmental stress, pathogens, parasites, and herbivores. For example, Glucosinolates represent a group of secondary metabolites primarily found in plants of the order Capparales and have been thoroughly researched in brassicas. When plants experience mechanical injury, infection, or insect infestation, the destruction of cells reveals the glucosinolates stored within, making them accessible to degrading enzymes called myrosinases or thioglucosidases, as well as specific proteins that function as cofactors (such as epithio- and nitrile-specific proteins) [9]. A key factor in the effectiveness of the bioactive compounds found in vegetables from the *Brassicaceae* family is their proper processing and handling. These elements directly influence its nutritional and sensory characteristics, thereby affecting the bioactivity of its components [8].

1.1.3 Agricultural importance and Health-Promoting Applications of *Brassicaceae* in Human Nutrition

The market for functional foods that are made from *Brassicaceae* vegetables is quite new. Nonetheless, it holds intriguing possibilities to introduce novel food items and formats that can positively influence health. In this regard, consumers, particularly young adults and children, exhibit some resistance towards the organoleptic traits, such as the taste of various cruciferous vegetables like broccoli and radish. Consequently, various innovative products made from functional ingredients derived from cruciferous vegetables have been created to assist in incorporating this category of nutrients into the diet in different and unique forms (e.g. , smoothies, soups, breads). Thus, the production of these new food items can involve utilizing by-products or side-streams to achieve a more favorable environmental and socio-economic equilibrium. Moreover, various nutraceuticals such as broccoli capsules, tablets, or powders have been marketed to make up for the lack of this category of vegetables in the diet. It is indeed the case that some functional foods produce notable physiological effects; however,

it is important to emphasize that the newly created "functional food" products cannot substitute for the nutritional benefits of fresh food [7].

1.2 Introduction to the Tomato *(Solanum lycopersicum)* Family

The tomato (*Solanum lycopersicum*) is among the most researched cultivated plants. The process of its domestication has primarily gone through two phases, indicated by *S. Pimpinellifolium and S. Lycopersicum variety. Cerasiforme* serves as the ancestor, while the ongoing development of modern cultivars primarily focuses on the combinations of different traits to satisfy market demands. Tomato originales from Central and South America, is highly valued by many consumers for its great flavor and nutritional benefits. Consequently, it has emerged as one of the most significant crops in economic terms since the 16th century. Additionally, Asian developing nations account for over one-third of this total production. Due to their economic significance, scientists have focused more on agronomic traits that directly influence the final fruit yield, including branching patterns, inflorescence structure, and fruit growth [10]. Tomato, because of its adaptability and relatively small genome (950 Mb), is frequently utilized as a model plant species to explore the genetic foundations of complex traits. This makes it an excellent resource for modern "-omics" studies and genome-informed breeding, and it has been applied in both traditional and molecular genetics. Tomatoes are very sensitive to cold temperatures (0–12 °C), and many regions that cultivate tomatoes experience low (chilling) temperatures throughout the growing season, causing notable decreases in yield and color. The sensitivity of commercial varieties to low temperatures (cold stress) restricts their geographic range, cultivation practices, growing duration, and the timing of planting and harvesting. Because of the common occurrence of cold temperatures, there is only one cropping season in temperate regions. Fluctuations in surrounding temperature inevitably affect biological processes. Temperature influences the speed of both spontaneous and enzyme-driven chemical and physical reactions, the arrangement and movement of molecules, and the strength of molecular interactions. Each of these side effects interferes with metabolism and the processing of cellular signals in various manners. Cold stress prevents plants from realizing their complete genetic potential because it directly suppresses metabolic processes and

causes cold-related osmosis, which reduces water absorption and leads to cellular dehydration. Summarazing, cool temperatures adversely affect the growth and development of tomato plants during their entire life cycle [11].

1.2.1 Taxonomy, Domestication, and Morphology of Tomato (*Solanum lycopersicum*)

Tomato is an ideal plant species for studying the impact of domestication on microbiome assembly and functioning [12]. The process of domestication and later breeding led to various visible changes and also affected the natural and agricultural environments where tomatoes are grown. This encompasses the significant application of fertilizers and pesticides to promote the growth and wellbeing of tomatoes. For instance, in order to manage pests, diseases, and weeds in open-field tomato cultivation, the use of chemicals has become widespread because of their affordability and effectiveness in control [13]. Moreover, various categories of pesticides are utilized in different nations, including organophosphates, organochlorines, carbamates, triazines, pyrethroids, dithiocarbamates, benzimidazole, chloronitriles, liquid copper fungicides, and herbicides [14]. Therefore, different approaches are required for the effective management of pests and diseases in a sustainable manner. Furthermore, methods to enhance crop resilience against challenges presented by climate change, including high temperatures, drought, salinity, and soil degradation, will be crucial. This has resulted in a renewed focus on the plant microbiome, which is a significant but underutilized source of microorganisms that positively influence plant growth and well-being. For instance, introducing microbial groups into the tomato root zone can provide several advantages for the plant, including improved nutrient absorption and defense against harmful pathogens [15], [16].

1.2.2 Microbial Communities Associated with Tomato Plants

The microbiome is commonly defined as a characteristic microbial community that occupies a well-defined habitat with specific physico-chemical properties; the term includes not only the microorganisms themselves but also their "theater of activity" [17, 18]. More recent perspectives describe the microbiome as the dynamic and interactive assemblage of microorganisms (bacteria, archaea, fungi, viruses, and eukaryotes), their genomes, their metabolic products, and the

interactions they establish with each other and with their environment [18, 19].

The phytomicrobiome encompasses the microbial communities linked with plants, including both root systems and aerial structures. This includes various organisms such as fungi, viruses, bacteria, protists, protozoa, oomycetes, and microalgae [20]; [21]. The relationships between host plants and microorganisms change based on the environmental stress and stimuli [22]; [23]. Recent research indicates that alterations in microbial communities are not merely passive reactions by plants; rather, through millions of years of co-evolution, plants have also gained significant support from local microbes to manage stress [24]; [25]. Specifically, the "demand for assistance" strategy is a method used by plants that involves utilizing diverse environments when faced with biotic stresses. This strategy allows plants to uptake beneficial microorganisms from their surroundings through various chemical signals, thereby improving their capacity to withstand stress [26]; [27].

The interaction between plants and microorganisms within the rhizosphere—the soil region surrounding the roots—plays a fundamental role in facilitating nutrient uptake, enhancing plant growth, and improving the plant's ability to cope with various stress conditions [28]; [29]. In addition to promoting growth through the production of phytohormones, microorganisms also contribute to plant defense by providing protection against both biotic and abiotic stresses [30]; [31]). Plants actively support the proliferation of beneficial microbial communities by releasing significant amounts of root exudates rich in sugars and amino acids, which can account for up to 40 percent of the carbon fixed during photosynthesis [32]. Compared to free-living soil microbes, endophytic microorganisms—those residing inside plant tissues—tend to establish more intimate and beneficial associations with their plant hosts [33]. Through a combination of direct and indirect mechanisms, these microbial partners play a key role in promoting plant health and improving resilience to environmental challenges. Moreover, recent advances in molecular approaches to engineer the plant microbiome have been discussed, focusing on how these strategies can enhance plant defense responses against biotic and abiotic stress factors. Finally, plant-based microbiome engineering approaches are introduced as a promising tool to accurately predict and optimize microbiome functions for improved plant health and agricultural sustainability [34]. Nutrient absorption in plants primarily occurs through roots, which play a key role in interactions with plant growth-promoting rhizobacteria (PGPR). It's important to highlights the functions of PGPR in tomato plants, in-

cluding enhancing growth, fixing nitrogen and phosphate, and protecting against harmful pathogens by producing substances such as phytohormones and ammonia. These beneficial microbes contribute to increased crop yield by biologically controlling pests, thereby reducing the need for chemical pesticides that pose risks to human health and the environment. Rhizobacteria enhance tomato plant health and improve both yield and quality. Nevertheless, further research is needed to better understand the interactions between rhizobacteria, other crop plants, and microbial communities in the rhizosphere. Investigations into these relationships could lead to improved strategies for optimizing microbial benefits in tomato cultivation [35].

Microorganisms associated with tomato (*Solanum lycopersicum*) roots can be broadly classified as beneficial or pathogenic. Among the beneficial taxa, species of the genus *Bacillus*, such as *B. subtilis*, *B. amyloliquefaciens* and *B. licheniformis*, have been reported to promote plant growth, enhance nutrient uptake, and improve yield and stress tolerance [36, 37]. Likewise, *Pseudomonas* species are frequently enriched in healthy rhizospheres, where they suppress soil-borne pathogens and induce systemic resistance [38, 39]. Other root-associated beneficial genera include *Streptomyces*, *Flavobacterium*, *Acidovorax* and *Actinomadura*, which contribute to nutrient cycling, phytohormone production, and stress resilience during flowering and fruiting [40, 38]. In addition, the acidophilic bacterium *Acidobacterium capsulatum*, often enriched in tomato root-associated soil, has been shown to stimulate root growth and improve nutrient acquisition through exopolysaccharide-mediated adhesion and auxin-like activity [41]. In contrast, several pathogens colonize tomato roots, including *Clavibacter michiganensis* subsp. *michiganensis*, the causal agent of bacterial canker, *Ralstonia solanacearum*, responsible for bacterial wilt, and the fungus *Fusarium oxysporum* f. sp. *lycopersici*, which causes vascular wilt and root rot [42, 43]. Numerous inoculation studies have demonstrated the potential of beneficial bacteria to improve tomato performance: for example, application of *Bacillus amyloliquefaciens* strain GB03 enhanced growth and altered root exudates, thereby recruiting beneficial bacteria in the rhizosphere [44]; seed or root inoculation with strains of *Pseudomonas* and *Bacillus* significantly increased biomass, yield, and fruit quality under greenhouse conditions [45, 46]; and combined inoculation with bacterial and fungal strains, such as *Trichoderma afroharzianum* and *Funneliformis mosseae*, improved marketable yield and stress resilience in intensive tomato production systems [47, 37].

1.2.3 Role of Grafting in Improving Tomato Plant Resilience and Productivity

Grafting represents an efficient agricultural technique to enhance fruit quality under both optimal and saline growth conditions. Grafting is a widely used technique for vegetable crops in many Asian countries and certain regions of Europe. Originally, it was applied as a method to manage fusarium wilt in watermelon [48]. Over time, its application has been extended to protect cucurbit and solanaceous crops from a range of soilborne diseases. Nevertheless, despite its proven benefits in other parts of the world, grafting has yet to be broadly adopted in the United States. Several factors may explain this limited use, such as skepticism toward the technology, the prevalence of large-scale farming, the availability of methyl bromide exemptions, and concerns over the costs of grafting. Still, as awareness grows among American growers regarding the advantages of grafting, its adoption is expected to rise. Consequently, researchers should prepare for potential challenges that could emerge from the increased implementation of grafting in commercial agriculture [49]. Numerous rootstocks have been identified and selected from existing cultivars for use in different vegetable crops. More recently, breeding programs and seed companies have shown significant interest in developing new, improved rootstocks specifically suited for cultivation under particular environmental conditions. As a result, growers are increasingly required to carefully choose the rootstock that best meets the specific demands of their growing systems. In cucurbit crops, for example, the number of available rootstock varieties is steadily expanding, largely due to the rising popularity of grafted plants cultivated under a wide range of agricultural and environmental scenarios [50]. Generally, grafting is more commonly adopted in crops grown in protected environments such as greenhouses, compared to those grown in open fields. Furthermore, rootstocks derived from different species are often favored in order to take advantage of the benefits offered by genetic diversity.

Grafting has become a prevalent technique in tomato cultivation to mitigate damage from soilborne pathogens [51]. More recently, its use has expanded to enhance plant resilience under both low and high temperatures [52], alleviate iron chlorosis in calcareous soils [53], improve nutrient absorption [54], boost production of endogenous growth regulators [55], and optimize water efficiency [56]. Studies have also shown that selecting appropriate rootstocks can enhance salinity tolerance in tomatoes, as measured by yield under salt stress [57].

So, grafting is recognized as a valuable tool for commercial growers. Regarding fruit quality, evidence suggests that attributes such as sugar content and carotenoid levels can be influenced by rootstock through xylem transport [51]. Interestingly, grafting tobacco scions onto tomato rootstocks nearly eliminated foliar nicotine under both saline and optimal conditions [54]. However, the influence of rootstock on fruit quality is complex and varies depending on the specific scion-rootstock combination. For instance, grafted melon plants sometimes exhibit significant declines in flavor and texture—particularly under greenhouse fertigation—while certain tomato combinations show increased carotenoid content without affecting soluble solids or titratable acidity, both important quality metrics [58]. This variability suggests that fruit quality is largely governed by the scion genotype and its interaction with the chosen rootstock [58].

The findings suggest that fruit quality is influenced, at least in part, by the root system. Moreover, the beneficial impact of the rootstock on fruit quality appears to depend on the interaction between both shoot and root genotypes, as well as on the environmental conditions, whether stressful or favorable. This complexity makes the selection of the most suitable rootstock a challenging process [59].

1.3 Environmental Stress in Plants

Changing climate patterns pose a significant threat to life on Earth, as fulfilling the increasing food demand and achieving sustainable agriculture for a growing population becomes increasingly difficult. These climatic changes encompass events such as droughts, severe floods, earthquakes, and temperature fluctuations. Drought stress disrupts various physiological and biochemical processes, which negatively impacts plant growth and development. Although plants can tolerate limited water availability to some extent, this often results in a considerable reduction in total biomass and yield. Drought conditions affect nearly half of the world's semi-arid and arid regions. Key processes including photosynthesis and other vital physiological and biochemical activities are impaired under drought stress. Research has shown that drought induces oxidative stress in plants, leading to damage of cellular membranes and essential macromolecules like DNA, proteins, lipids, and photosynthetic pigments. To counteract oxidative damage, plants activate their inherent defense mechanisms and accumulate osmoprotective compounds, such as soluble proteins, proline, soluble sugars, and glycine betaine [60]. Understanding the nature of these stresses is essential for

developing strategies to improve plant resilience and ensure sustainable agricultural productivity. All of type of plants are exposed to various environmental challenges that adversely affect their growth, development, and productivity. These stresses are broadly classified as abiotic and biotic. Abiotic stresses are non-living factors such as drought, salinity, temperature extremes, heavy metals, radiation, flooding, and nutrient imbalances. Drought stress is among the most severe, affecting nearly half of the world's semi-arid and arid regions, disrupting key physiological and biochemical processes including photosynthesis and nutrient uptake, leading to decreased biomass and yield [60]; [61]; [62]. It also causes oxidative damage through reactive oxygen species [63]. Salinity stress results from excess soluble salts in soil, causing osmotic stress and ion toxicity, and plants respond by synthesizing osmoprotectants like proline and glycine betaine [64]; [65]. Temperature stress, both heat and cold, affects cellular homeostasis, membrane fluidity, and gene expression, triggering complex protective pathways [66]. Other abiotic stresses such as flooding create hypoxic conditions, and heavy metals interfere with nutrient metabolism, adding further challenges to plants [61]. Biotic stresses arise from living organisms including pathogens, insects, nematodes, and weeds, causing diseases and impairing plant health. Plants defend themselves through physical barriers, secondary metabolites, and immune responses to counteract these threats [63].

Brassica species and tomato plants frequently encounter a range of abiotic and biotic stresses that limit their development and yield potential. In Brassica crops, abiotic stresses such as drought, salinity, temperature extremes, and nutrient limitations disrupt essential physiological functions like photosynthesis and water use efficiency. Drought stress reduces stomatal opening and photosynthetic rates, resulting in diminished biomass and seed production [67]; [68]. Similarly, salinity induces ionic imbalance and osmotic challenges that negatively influence plant metabolism [69]. Exposure to low temperatures can impair membrane integrity and enzymatic activity, particularly in temperate climates [70]. Tomato plants experience similar abiotic stresses: drought causes reduced photosynthesis and oxidative damage, affecting fruit quantity and quality [60]; [52]. Salinity interferes with water absorption and root function, while extreme temperatures impact reproductive processes such as flowering and fruit set [71]; [72]. Both Brassica and tomato crops are also susceptible to biotic pressures from pathogens and pests, which activate complex defense mechanisms including hormone signaling and antioxidant production [73]; [74]. To mitigate these stresses, approaches

like grafting, cultivating tolerant varieties, and refined agricultural practices are increasingly important for sustaining productivity [51]; [58].

Comparative Transcriptome Analysis of B. oleracea var. italica and B. macrocarpa Ecotypes Under Drought Stress in Sicily: De Novo vs. Reference Genome Assembly

This study was undertaken to address the urgent need for improving drought stress resistance in crops, a key strategy to reduce water requirements under ongoing climate change. By comparing drought-sensitive and drought-tolerant *Brassica* genotypes through transcriptomic analysis, we aimed to identify crucial genes and pathways involved in stress adaptation, thereby contributing to the development of more resilient cultivars.

1.4 Technologies and approaches used

In this study, we employed high-throughput molecular approaches to investigate both the transcriptomic landscape and the microbial community composition. RNA sequencing (RNA-Seq) is a next-generation sequencing (NGS) technique that provides a comprehensive view of the transcriptome by quantifying RNA molecules present in a biological sample, enabling the detection of gene expression levels, alternative splicing, novel transcripts, and sequence variants [75, 76]. The general workflow includes RNA extraction, library preparation (poly(A) en-

richment or ribosomal RNA depletion), sequencing, and downstream computational analyses such as read alignment, transcript quantification, normalization, and differential expression testing, often using tools such as DESeq2 [77]. In parallel, 16S ribosomal RNA (rRNA) gene sequencing was used as an amplicon-based approach for profiling bacterial and archaeal communities. This method amplifies hypervariable regions of the conserved 16S rRNA gene, allowing taxonomic resolution at the genus or even species level, and has recently been enhanced by long-read sequencing platforms such as Oxford Nanopore Technologies [78, 79, 80]. The integration of RNA-Seq with 16S rRNA sequencing provides a complementary perspective: while 16S analysis identifies *who is there* in a microbial community, transcriptomics reveals *what they are doing*. Combined, these approaches enable a more comprehensive understanding of host–microbe interactions and their functional implications [81, 82].

1.5 Materials and Methods

1.5.1 Plant material and preliminary screening

The plant material consisted of 89 *Brassica* genotypes selected for preliminary screening from the active *Brassica* collection at the Department of Agriculture, Food and Environment (Di3A) of the University of Catania (UNICT). All accessions screened were part of the H2020 Breeding for Resilient, Efficient, Sustainable Organic Vegetable Production (BRESOV) project.

The trial took place at the University of Catania during the summer of 2020, under organic farming conditions. Plantlets were sown in cellular trays with one seed per hole, using the organic soil substrate BRILL® semina bio (Geotec, Italy) for sowing. Sowing occurred in the second decade of May 2020.

Plantlets received ample irrigation from the sowing date until the emission of the third true leaf. Subsequently, irrigation was withheld for a period of five days in June 2020, except for the control blocks. The comprehensive analysis involved six biological replicates for each accession, conducted under both irrigated and stressed conditions. These replicates were grown in an “open greenhouse” in July 2020, with an average temperature between 24.5 and 27.5°C.

Plantlets were assessed for various morphometric traits, including the total number of leaves, chlorotic leaves, and dry leaves. Chlorophyll content was measured using the SPAD-502 device (Konica Minolta Optics, Japan). Additionally,

plants were evaluated for their resilience to drought stress using a numerical scoring system, with a score of 0 indicating the most sensitive plantlets, 1 for intermediate tolerance, and 2 for highly tolerant ones.

1.5.2 Data analysis for preliminary screening

The genotype selection for subsequent analysis was conducted through morphological observations, assessment of adaptation to water deficit conditions, and by analyzing the variation in total leaf number and SPAD index values between stressed and irrigated control conditions.

The analysis was performed using the following formula:

$$\Delta L = \left(\frac{L_s}{L_c} - 1 \right) \times 100$$

where:

- ΔL indicates the variation in leaf number between the two conditions;
- L_s represents the number of leaves recorded under stress conditions;
- L_c refers to the number of leaves recorded under control (irrigated) conditions.

Similarly, the same formula was applied to the SPAD values:

$$\Delta S = \left(\frac{S_s}{S_c} - 1 \right) \times 100$$

where:

- ΔS indicates the variation in SPAD index between the two conditions;
- S_s represents the SPAD index recorded under stress conditions;
- S_c is the SPAD index recorded under control conditions.

Finally, ΔL and ΔS were statistically analyzed using SPSS software version 27 (IBM, Armonk, USA) by performing a **Principal Component Analysis (PCA)**.

1.5.3 Genotypes selection and stress imposition

Genotype selection and experimental setup

Four genotypes were selected among the initial 89 accessions based on their differential responses to stress imposition. The stressed plantlets were initially irrigated until total soil saturation and then allowed to recover for 18 days with daily irrigation in alveolar trays. After the recovery period, they were transplanted into 20 cm diameter pots (hydraulic capacity: 4.5 L) for subsequent stress application, which began ten days after transplanting.

The selected genotypes were tested under two distinct irrigation regimes (IR):

- **Irrigated (IRR)**: genotypes receiving regular irrigation (25 cl per pot per day);
- **Non-irrigated (NIR)**: genotypes subjected to seven days of water stress.

At the seventh day of treatment, plants were characterized for:

- total number of leaves;
- number of chlorotic and dry leaves;
- leaf chlorophyll content measured using SPAD (Konica Minolta Optics, Japan);
- morphometric traits, including leaf lamina area (cm^2), leaf length (cm), and leaf width (cm).

Fresh samples were collected and stored at $-80\,^{\circ}C$ for further biochemical analysis.

1.5.4 Chemical analysis

To assess drought stress tolerance or sensitivity, the leaves of the selected genotypes were analyzed for malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) content, which are metabolites associated with oxidative stress response.

- MDA content was determined according to the protocol by López-Hidalgo *et al.* [83];
- H_2O_2 content was measured following the method described by Velikova, Yordanov, and Edreva [84].

1.5.5 Transcriptomic analysis

RNA extraction

Leaves were kept frozen by the continuous addition of liquid nitrogen and ground using a pre-cooled mortar and pestle. Total RNA was extracted using the Spectrum Plant Total RNA Kit (Sigma-Aldrich®, Saint Louis, MO, USA). RNA degradation and contamination were monitored by electrophoresis on 1% agarose gel. Purity and concentration of the RNA were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) [85]. RNA integrity was evaluated using the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA) [86].

Library preparation

One microgram of RNA was used to prepare sequencing libraries (24 total: two irrigation conditions $\times$ four genotypes $\times$ three replicates). Libraries were prepared using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (New England Biolabs, Ipswich, MA, USA), following the manufacturer's instructions [85].

Briefly, mRNA was purified using poly-T oligo-attached magnetic beads. Fragmentation was carried out with divalent cations at elevated temperature. First-strand cDNA synthesis was performed with random hexamer primers and M-MuLV Reverse Transcriptase (RNase H-). Second-strand cDNA synthesis was done using DNA Polymerase I and RNase H. Overhangs were converted to blunt ends, and adapters with hairpin loops were ligated. Size selection (150–200 bp) was performed using AMPure XP beads (Beckman Coulter, Beverly, MA, USA). Libraries were treated with USER Enzyme (NEB), followed by PCR amplification using Phusion High-Fidelity DNA Polymerase and index primers. Final libraries were purified (AMPure XP) and checked on the Agilent Bioanalyzer 2100 [86].

Clustering and sequencing

Clustering and sequencing were performed by Novogene (UK). Cluster generation was done using a cBot Cluster Generation System with a PE Cluster Kit cBot-HS (Illumina), and sequencing was conducted on an Illumina HiSeq 2000 platform, producing paired-end reads (2 $\times$ 150 bp) [85, 87]. Raw reads were processed with in-house Perl scripts to remove adapters, poly-N sequences, and

low-quality reads. Quality metrics such as Q20, Q30, GC content, and duplication levels were calculated.

De novo assembly, annotation, and differential expression analysis

Transcriptome assembly was carried out using Trinity (v2.6.6, with `min_Kmer_Cov = 3` and `min_glue = 4`) [88]. Corset (v4.6) was used to cluster transcripts and retain the longest transcript per cluster as Unigene [89]. Assembly quality and gene prediction were assessed with BUSCO (v3.0.2) [90]. Functional annotation was performed using:

- Nr (NCBI, Diamond v0.8.22, e-value $\leq 1e^{-5}$) [91, 92],
- Nt (NCBI BLAST v2.9.0, e-value $\leq 1e^{-5}$) [92],
- Pfam (HMMER v3.1, e-value ≤ 0.01) [93],
- KOG/COG (Diamond v0.8.22, e-value $\leq 1e^{-5}$) [94, 91],
- Swiss-Prot (Diamond v0.8.22, e-value $\leq 1e^{-5}$) [95, 91],
- KEGG (Kyoto Encyclopedia of Genes and Genomes),(KAAS and Diamond) [96, 91],
- GO (Blast2GO, b2g4pipe v2.5, e-value $\leq 1e^{-6}$) [97]

Gene expression levels were estimated using RSEM (v1.2.28), and differentially expressed genes (DEGs) were identified with DESeq2 (v1.26, `padj` ≤ 0.05, $|\log_2 FC| \geq 1$) [85, 87].

Reference assembly

A custom RNA-seq pipeline was developed. Trimmomatic (v0.39) [98] was used for quality filtering and adapter trimming. STAR [99] was employed for alignment using the *Brassica oleracea* TO1000 genome from NCBI. Differential expression analysis was performed using edgeR [100] and limma [101] (R v4.2.2). Gene annotations were retrieved using UniProtR (v2.2.2) [102, 103]; if missing, BlastKoala [104] was used. Enrichment analysis was performed using clusterProfiler [105], AnnotationHub [106], Biomartr [107], and AnnotationDbi [108].

Bioinformatic analysis

Three biological replicates per genotype and irrigation condition (IRR and NIRR) were analyzed. Normality was assessed using the Shapiro–Wilk test, and homogeneity of variance with the Levene test. ANOVA or Kruskal–Wallis tests were applied based on these assumptions. Pairwise comparisons were performed with t-tests or Wilcoxon tests as appropriate. Principal Component Analysis (PCA) was performed on phenotypical and chemical data using the R package `Factoextra` (v1.0.6) [109], and graphical representations were generated using `ggplot2` (v3.4.2) [110] and `ggpubr` (v0.6.0) [109].

Investigated signatures

Three DEG-based transcriptomic signatures were generated to explore drought response:

- **Sensitive signature**: intersection of DEGs from BS (Sensitive) and BM (Medium Sensitive) under drought vs. control;
- **Tolerant signature**: intersection of DEGs from MF (Tolerant) and MM (Medium Tolerant) under drought vs. control;
- **Intersection signature**: obtained by subtracting DEGs of control from those of stressed plants and intersecting across tolerant vs. sensitive genotypes.

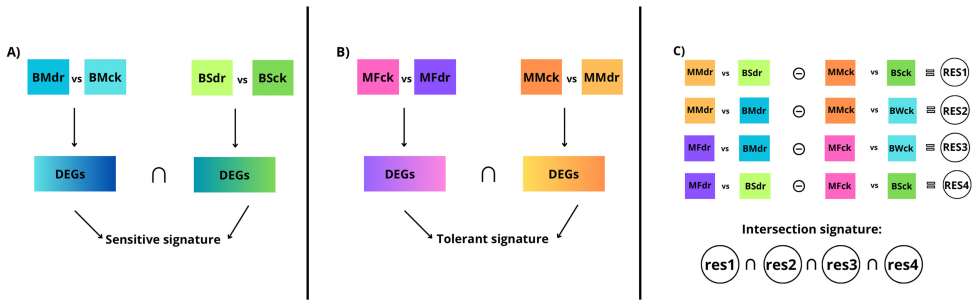

Figure 1.1: Descriptive diagram which explains how sensitive signature (A), tolerant signature (B) and intersection signature (C) have been obtained.

1.6 Results

1.6.1 Selection of the subset of sensitive and tolerant accession

The results concerning the preliminary screening cycle performed for the selection of the 2 most sensitive and tolerant genotypes, are summarized in Fig. 1.2. The selected genotypes were positioned at opposite ends of the PCA plot. This positioning indicated significant differences, with high ΔL and ΔS values observed for the tolerant *B. macrocarpa* Guss. accessions, while the two *B. oleracea var. italica* landraces exhibited lower values of ΔL and ΔS (Fig. 1.2). In the PCA plot, the accessions indicated as tolerant and selected are BM3 and BM4 (crop codes MM and MF). Conversely, the accessions selected as sensitive and indicated in the PCA plot, were BR15 and BR13 (crop codes BM and BS).

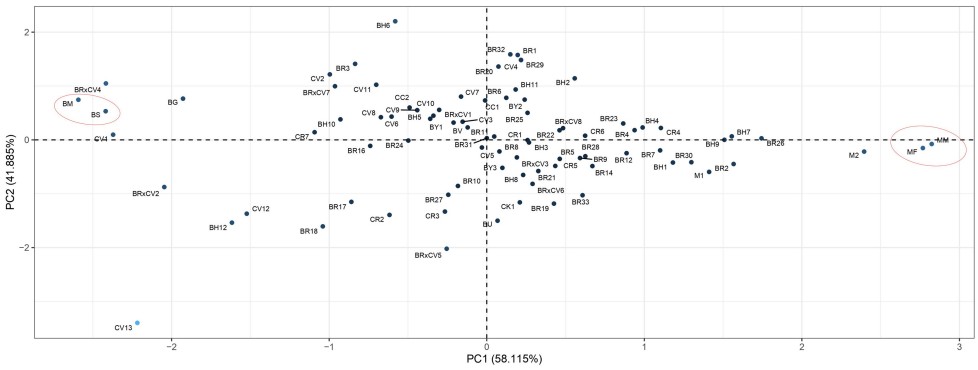

Figure 1.2: PCA plot of the 86 accessions evaluated in the preliminary screening cycle. Red circles indicate the tolerant and sensitive accessions selected (right and left circle, respectively).

1.6.2 Phenotypic and chemical analysis of the selected genotypes

Statistical analysis of phenotypic and chemical traits in the selected genotypes confirmed the visual evaluation hypothesis regarding the tolerance types of the cultivars. While the overall leaf count remained consistent between sensitive and tolerant NIR plants, a notable observation was the distinct wavy pattern in leaf shape (Fig. 1.3). Specifically, among the cultivars listed in Table 1.1, we observed a significantly greater leaf width in NIR BS and BM compared to MF

Table 1.1: Variation of the morphometric and chemical parameters in relation to not irrigated (NIR) tolerant and sensitive plants, as well for irrigated (IRR) tolerant and sensitive plants. ****,***, **, * indicate p value $p \leq 0.05$ 0.0001, 0.001, 0.01, 0.05, respectively. The values in the table represent the mean and standard deviation values of three biological replicates for each accession of each condition.

Samples	Total Leaves (n)	Chlorotic Leaves	Dry Leaves (g)	SPAD	Leaf Area (cm^2)	Leaf Lamina Length (cm)	Leaf Width (cm)	H_2O_2 (%)	MDA (ng/mg)
Tolerant stressed (MFdr and MMdr)	8 (0.89)	2.5 (0.84)	0.33 (0.52)	48.4 (7.14)	40.47 (1.36)	7.81 (0.42)	5.35 (0.31)	0.21 (0.12)	1.35 (0.5)
Sensitive stressed (BMsr and BSdr)	7.67 (1.03)	3.5 (0.55)	0.83 (0.75)	33.9 (3.1)	55.4 (4.31)	9.54 (0.48)	7.62 (0.69)	1.1 (0.43)	1.7 (0.65)
Tolerant control (MFck and MMck)	8.83 (1.33)	0 (0)	0.17 (0.41)	48.1 (6.38)	38.06 (1.87)	7.75 (0.43)	5.46 (0.43)	0.24 (0.24)	2.2 (0.96)
Sensitive control (BMck and BSck)	9.4 (0.55)	0.4 (0.58)	0.2 (0.45)	50.2 (5.78)	44.4 (5.14)	8.23 (0.25)	5.96 (0.76)	0.46 (0.15)	3.33 (1.77)
Sensitive stressed vs Tolerant stressed	ns	ns	ns	**	**	****	**	**	ns
Sensitive control vs Tolerant control	ns	ns	ns	ns	**	ns	ns	ns	ns

and MM. However, this pattern was not observed under IRR conditions for the same plants. A similar trend was observed for leaf lamina length, which was significantly smaller in NIR-tolerant plants compared to sensitive ones.

Notably, the differences were significant between MM and BS (p-value $<$ 0.001), and among MF and BS, and MF and BM (p-value $<$ 0.05), but these differences were not detected under IRR conditions. In contrast, within the irrigated genotype set, the SPAD index displayed no significant variations. However, among the NIR accessions, the SPAD index varied significantly between plants identified as tolerant and those identified as sensitive (Table 1.1). In particular, the SPAD index of MM was significantly higher than that of BM and BS, with a p-value $<$ 0.01. Regarding hydrogen peroxide content (H_2O_2), it is noteworthy that the most sensitive plant (BS) under IRR conditions exhibited a significantly higher level of this metabolite compared to the moderately tolerant IRR plant (MM). As shown in Table 1.2, leaf width, leaf area, and leaf lamina length increased significantly in NIR-sensitive plants compared to tolerant ones. The only parameter that was significantly different between sensitive and tolerant plants even under irrigated conditions was leaf area, which was already higher in sensitive plants. Concerning physiological traits, we observed significant changes in H_2O_2 and SPAD between sensitive and tolerant plants under NIR conditions but these traits remained stable under IRR conditions. We also analyzed MDA, but it remained unaltered between the two different irrigation conditions.

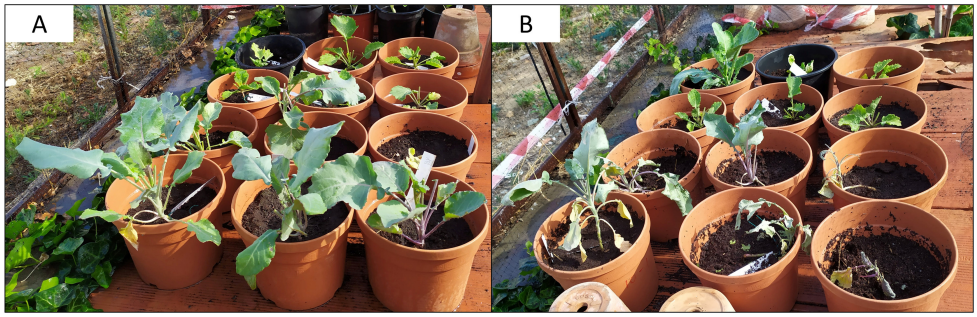

Figure 1.3: Control (A) and stressed (B) accession analyzed for the water deficit trial.

By taking into account both chemical and phenotypic characteristics we compared the different cultivars using a PCA. As it can be seen in Fig. 1.4 using these traits it has been possible to cluster the plants with their response characteristics

to the drought stress. Moreover, from Fig. 1.4A it is possible to notice how the tolerant plants cluster close to the control ones. On the other hand, in Fig. 1.4B we can observe that the variables "Leaf Area", "H_2O_2" and "Leaf Lamina Length" are those contributing more to this plants' classification.

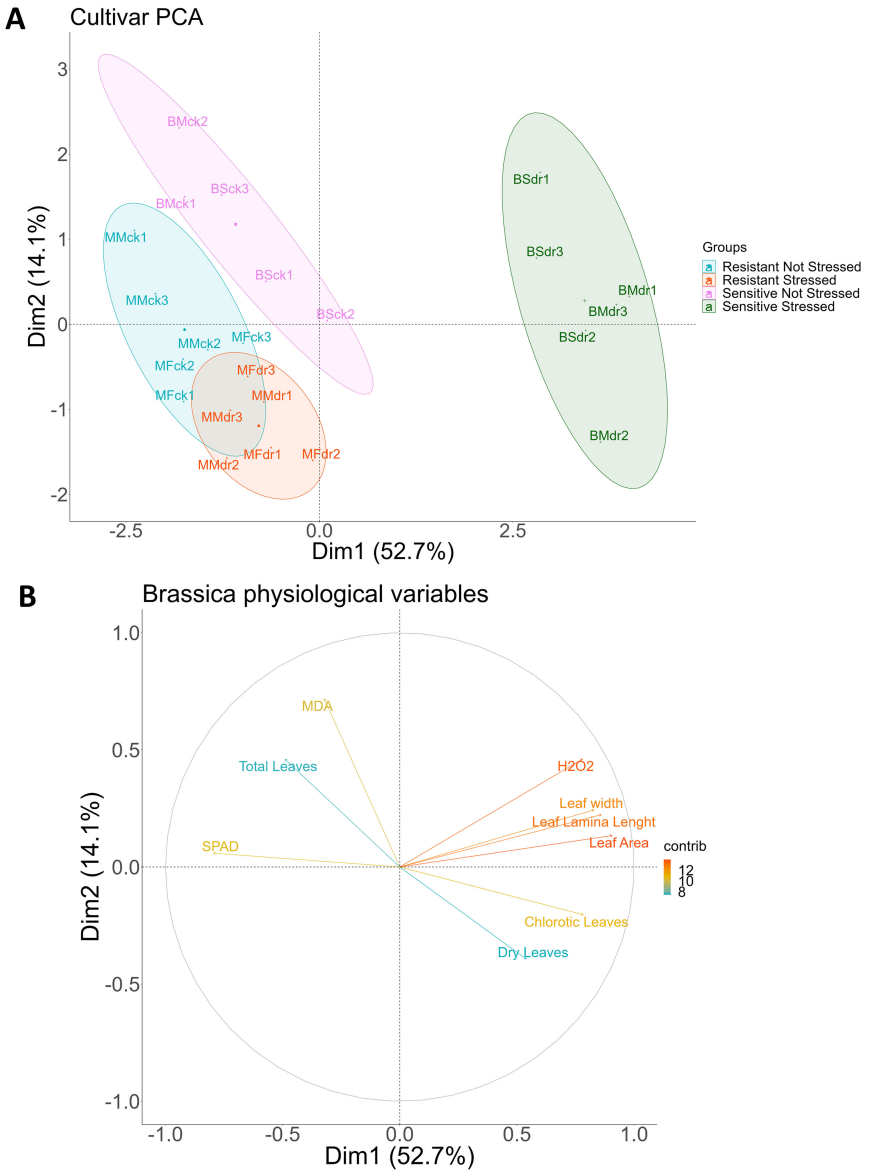

Figure 1.4: PCA on the basis of the cultivars characteristics. A) distribution of accessions in relation to drought stress; B) Contributions of the chemical and phenotypic characteristics.

Table 1.2: Variation of the morphometric and chemical parameters in relation to the water deficit application. Particularly, the traits reported were the number of the total, chlorotic and dry leaves (n), SPAD index, leaf area (cm^2), leaf lamina length and width (cm), as well the H_2O_2 (%) and MDA (ng/mg). ***, **, * indicate p value $\leq$ 0.001, 0.01, 0.05, respectively. The values in the table represent the mean and the standard deviation values of three biological replicates for each accession.

	NIR									IRR						
Accession	Total Leaves (n)	Chlorotic Leaves (n)	Dry Leaves (g)	SPAD	Leaf Area (cm^2)	Lamina Length (cm)	Width (cm)	H_2O_2 (%)	MDA (ng/mg)	Total Leaves (n)	Chlorotic Leaves (n)	Dry Leaves (g)	SPAD	Leaf Area (cm^2)	Lamina Length (cm)	Width (cm)
H_2O_2 (%)	MDA (ng/mg)															
MF	8 (1)	3 (1)	0.33 (0.58)	42.9 (5.11)	40.03 (1.92)	7.73 (0.62)	5.24 (0.26)	0.23 (0.14)	1.53 (0.6)	8 (1)	0 (0)	0.33 (0.58)	47.87 (4.07)	38.83 (2.16)	7.68 (0.64)	5.20 (0.22)
0.35 (0.32)	2.03 (0.67)															
MM	8 (1)	2 (0)	0.33 (0.58)	53.9 (3.17)	40.9 (0.61)	7.88 (0.18)	5.47 (0.36)	0.20 (0.12)	1.17 (0.39)	9.67 (1.15)	0 (0)	0 (0)	48.4 (9.08)	38.33 (1.96)	7.82 (0.17)	5.72 (0.46)
0.14 (0.06)	2.37 (1.32)															
BM	7 (1)	3.67 (0.58)	1.33 (0.58)	35.5 (3.54)	58.4 (2.65)	9.38 (0.69)	8.06 (0.73)	0.92 (0.51)	1.82 (0.71)	9 (0)	0.5 (0.7)	0 (0)	53.4 (0.99)	40.7 (0.71)	8.28 (0.10)	5.70 (0.34)
0.35 (0.13)	5.10 (1.16)															
BS	8.33 (0.58)	3.33 (0.58)	0.3 (0.58)	32.4 (2.00)	52.5 (3.67)	9.70 (0.19)	7.18 (0.26)	1.28 (0.33)	1.59 (0.71)	9.67 (0.58)	0.33 (0.58)	0.33 (0.58)	48.1 (7.04)	46.9 (5.47)	8.19 (0.35)	6.15 (0.99)
0.53 (0.15)	2.14 (0.55)															
MM vs MF	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
ns																
MM vs BM	ns	ns	ns	**	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns
ns																
MM vs BS	ns	ns	ns	**	ns	***	**	*	ns	ns	ns	ns	ns	ns	ns	ns
ns																
MF vs BM	ns	ns	ns	ns	ns	*	*	ns	ns	ns	ns	ns	ns	ns	ns	ns
ns																
MF vs BS	ns	ns	ns	ns	ns	*	***	*	ns	ns	ns	ns	ns	ns	ns	ns
ns																
BM vs BS	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
ns																

1.6.3 Transcriptomic analysis

In our molecular analysis, we conducted a transcriptomic study to identify Differentially Expressed Genes (DEGs) between pairs of plants subjected to drought stress and those without. Furthermore, BUSCO analysis resulted in about 70% of complete and single copy Unigenes, indicating a good reliability and quality of the de novo transcriptome. Comparing stressed plants of each accession with their respective controls, in reference genome analysis we observed a higher number of DEGs in the sensitive accessions BM and BS (912 and 1147, respectively) compared to the tolerant accessions MF and MM in the reference genome analysis (514 and 383, respectively). Interestingly, this trend reverses when considering the *De novo* analysis, where the highest number of DEGs was recorded in the tolerant accessions MF and MM (3184 and 4446, respectively)(Table 1.3). To identify signatures of tolerance and sensitivity among cultivars, we examined the genes common to each group. We found only 9 genes common among all four cultivars in the reference genome analysis and 133 genes in the *De novo* analysis. The sensitive signature comprised 320 genes in the reference analysis and 545 in the *De novo* analysis, while the tolerant signature contained 27 genes in the reference analysis and 498 in the *De novo* analysis. Additionally, the intersection signature included 356 genes in the reference genome analysis and 658 in the *De novo* analysis. Among the investigated genes, *Bo9g041010* (homologous to *Arabidopsis thaliana* Transcription factor bHLH112, SwissProt Q94JL3) was common to both tolerant and sensitive cultivars, displaying a clear expression trend (See Fig. 1.5). Specifically, it was upregulated in tolerant cultivars and downregulated in sensitive ones. However, this differential expression was only statistically significant for BS and MF, as indicated by the adjusted p-value.

Table 1.3: Number of Differentially Expressed Genes (DEGs) in control (CK) and stressed (DR) plants of the four accessions analysed.

Accessions	**Degree of Resistance**	**DEGs in reference genome analysis (n)**	**DEGs in de novo analysis (n)**
MF	Tolerant	514	3184
MM	Medium Tolerant	383	4446
BM	Medium Sensitive	912	1591
BS	Sensitive	1147	2890

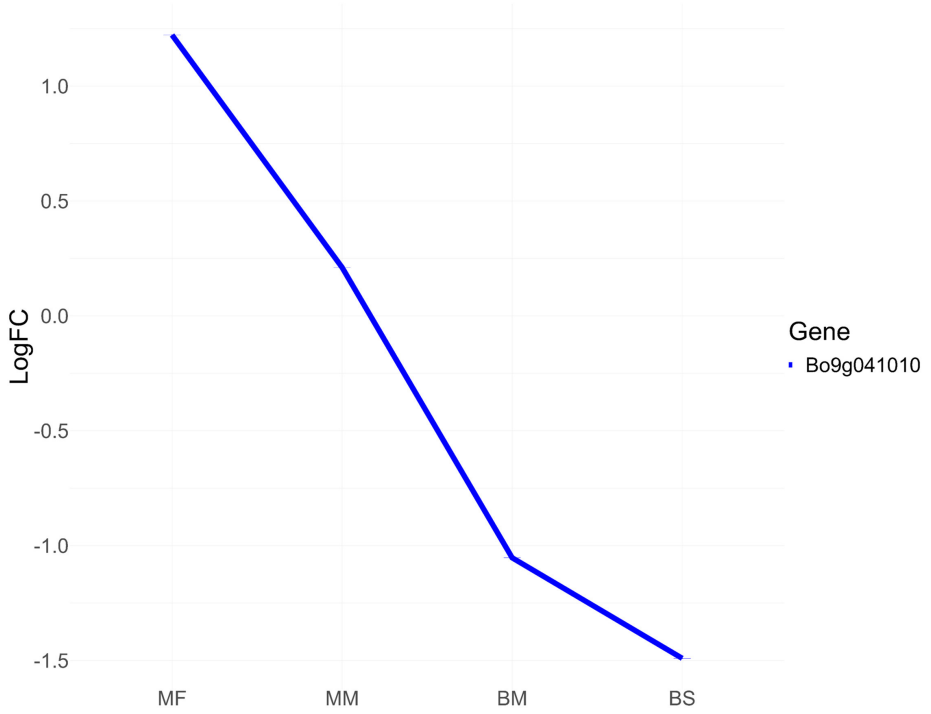

Figure 1.5: Line plot depicts the behavior of gene Bo9g041010 in the whole group of cultivars. The gene was upregulated in tolerant plants (MF and MM) and downregulated in sensitive ones (BM and BS).

3.5. Gene ontology analysis of tolerant and sensitive signature

Fig. 1.6 provides a comprehensive overview of the Gene Ontology (GO) results corresponding to each gene within the respective signatures. This figure shows GO annotations filtered to include only those terms containing at least 5 genes. Our GO analysis encompasses both the reference genome signatures and the de novo signatures, with a focus on Biological Process (BP). The analysis conducted on the sensitive signature resulted from the reference analysis returned 36 genes annotated in different GO terms.Fig. 1.6 shows the main annotated Biological Processes in the sensitive signature. They include Regulation of transcription, DNA-templated (GO:0006355) with Bo01141s010 involved also in auxin-activated signaling pathway (GO:0009734); Response to abscisic acid (GO:0009737) with the genes Bo1g117540, Bo3g001410, Bo3g132570, Bo3g134720, Bo4g014570, Bo8g091960, Bo9g004450; methylation (GO:0032259);

pectin catabolic process (GO:0045490); carbohydrate metabolic process (GO:0005975); cell wall modification (GO:0042545) and response to salt stress (GO:0009651). In our De novo analysis related to the sensitive signature in the context of Gene Ontology (GO), we identified 37 functionally annotated genes, with the majority (over 30) involved in the regulation of transcription, DNA templated (GO:0006355). Additionally, processes such as protein phosphorylation (GO:0006468) and sucrose metabolism process (GO:0005985) were strongly involved in the response of sensitive accessions to drought stress Fig. 1.6. These findings underscore the complexity and diversity of biological processes involved in the sensitive signature identified in our De Novo analysis, highlighting both known pathways and areas warranting further investigation. In Fig. 1.6, the functional gene annotations related to Biological Processes (BP) within the tolerant signature of the reference analysis highlights crucial functions characterizing tolerant plants, such as the auxin-activated signaling pathway (GO:0009738) (genes Bo1g016790 and Bo2g134160) and the abscisic acid-activated signaling pathway (GO:0009734) (genes Bo7g075740 and Bo4g190030). Turning to the de novo analysis presented in Fig. 1.6E, a notable gene count is observed in the regulation of transcription, DNA-templated (GO:0006355) followed by the protein phosphorylation (GO:0006468) and transmembrane transport (GO:0055085). In Fig. 1.6 C, the intersection signature reveals four significant terms in Biological Processes (BP), with protein phosphorylation (GO:0006468) having the highest gene count of 15, followed by protein ubiquitination (GO:0016567) and regulation of DNA-templated transcription (GO:0006355). Shifting to the de novo analysis signature in , the BP terms exhibit a different order of the terms compared to the reference genome analysis. The regulation of DNA-templated transcription (GO:0006355) is the richest (40 genes), followed by protein phosphorylation (GO:0006468)and transmembrane transport (GO:0055085).

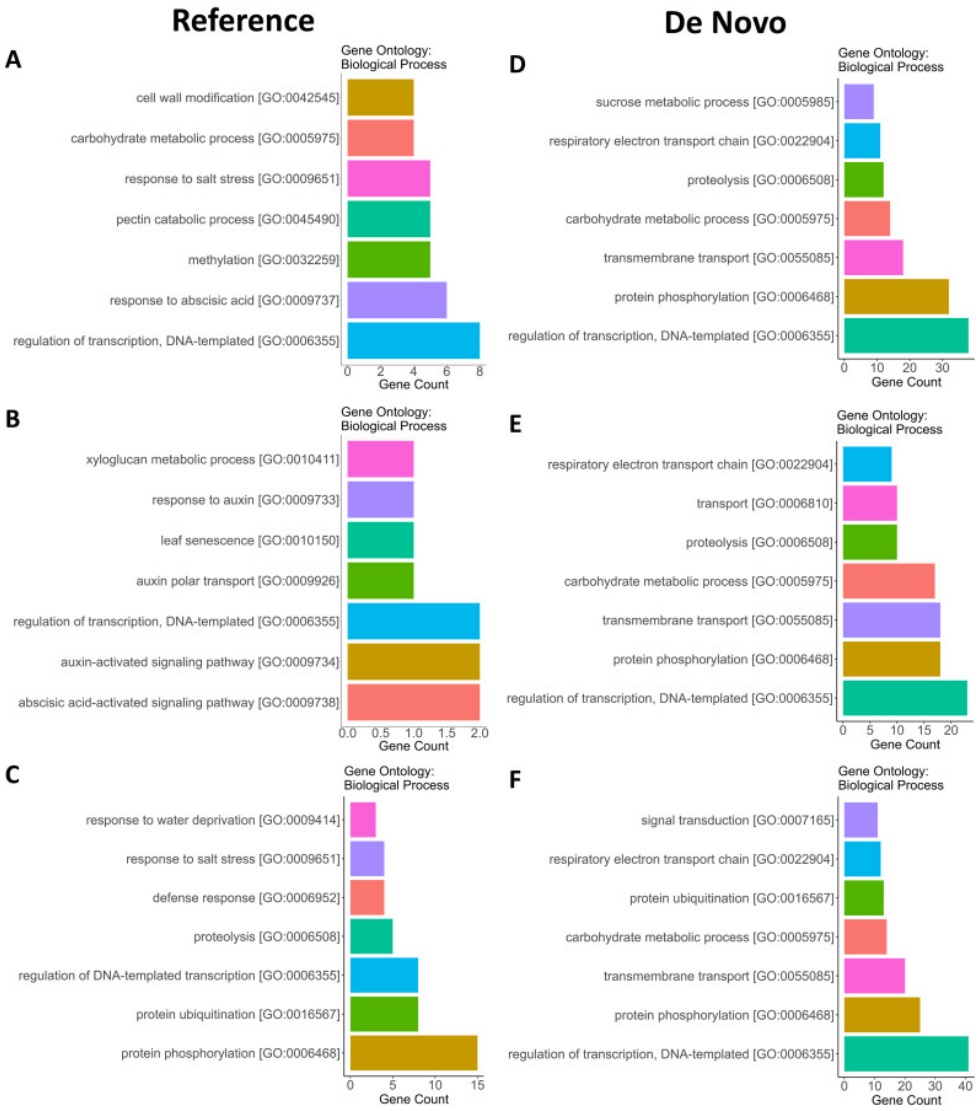

Figure 1.6: GO Barplot of Biological Process (BP) of both the reference genome (A,B,C) and de novo analysis (D,E,F) signatures, describing (A,D) sensitive signature, (B,E) tolerant signature, (C,F) intersection signature.

3.6. Pathways analysis of tolerant and sensitive signature

Pathway analysis using KEGG was conducted for both the reference genome and de novo analyses, as illustrated from Fig. 1.7 to Fig. 1.9. In 1.7, six KEGG path-

ways have been found perturbed in the reference genome analysis with the sensitive signature. Notable among these pathways: (i) ABC transporters, involving genes Bo4g186480, Bo2g047420, and Bog9g008680; (ii) Motor protein, with gene Bo5g126420, and gene Bo3g116270, which is also linked to (iii) Phagosome, involving genes Bo4g023960, Bo1g016970, and Bo1g006800; (iv) Propanoate metabolism, featuring genes Bo9g022510 and Bo4g125890, which is further connected to (v) Biosynthesis of unsaturated fatty acid, and (vi) Fatty acid metabolism, which also includes Bo7g026690 (Fig. 1.7).

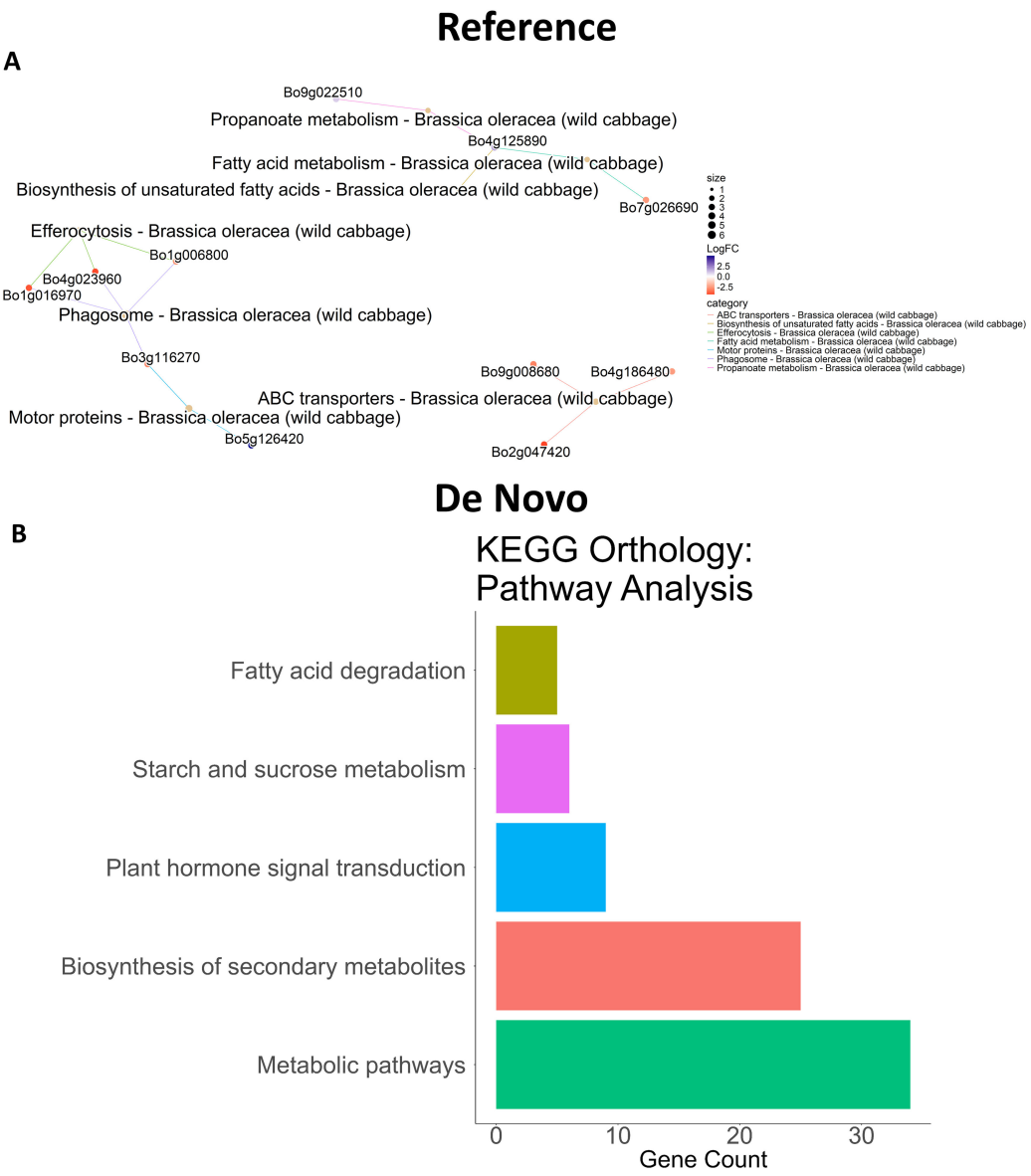

Figure 1.7: Pathways network of sensitive signature showing reference genome (panel A) and de novo analysis (panel B).

In the de novo analysis (Fig. 1.7), five perturbed pathways are observed. Among them, Fatty acid degradation, previously identified in the reference genome, is noteworthy. Additionally, the de novo analysis highlights other perturbed pathways, including: (i) Biosynthesis of secondary metabolites, (ii) Plant hor-

mone signal transduction, and (iii) Starch and sucrose metabolism. In Table 1.4 are listed genes belonging to these pathways involved in stress response. In particular, genes encoding for probable protein phosphatase 2C 78 and Indole-3-acetic acid-amido synthetase GH3.6 have been found up-regulated, while genes encoding for Cytochrome P450, Abscisic acid receptor PYR1, Auxin transporter-like protein 3, and Auxin-responsive protein SAUR50 resulted down-regulated in the sensitive signature (Table 1.4), suggesting their involvement in the sensitive response to drought stress.

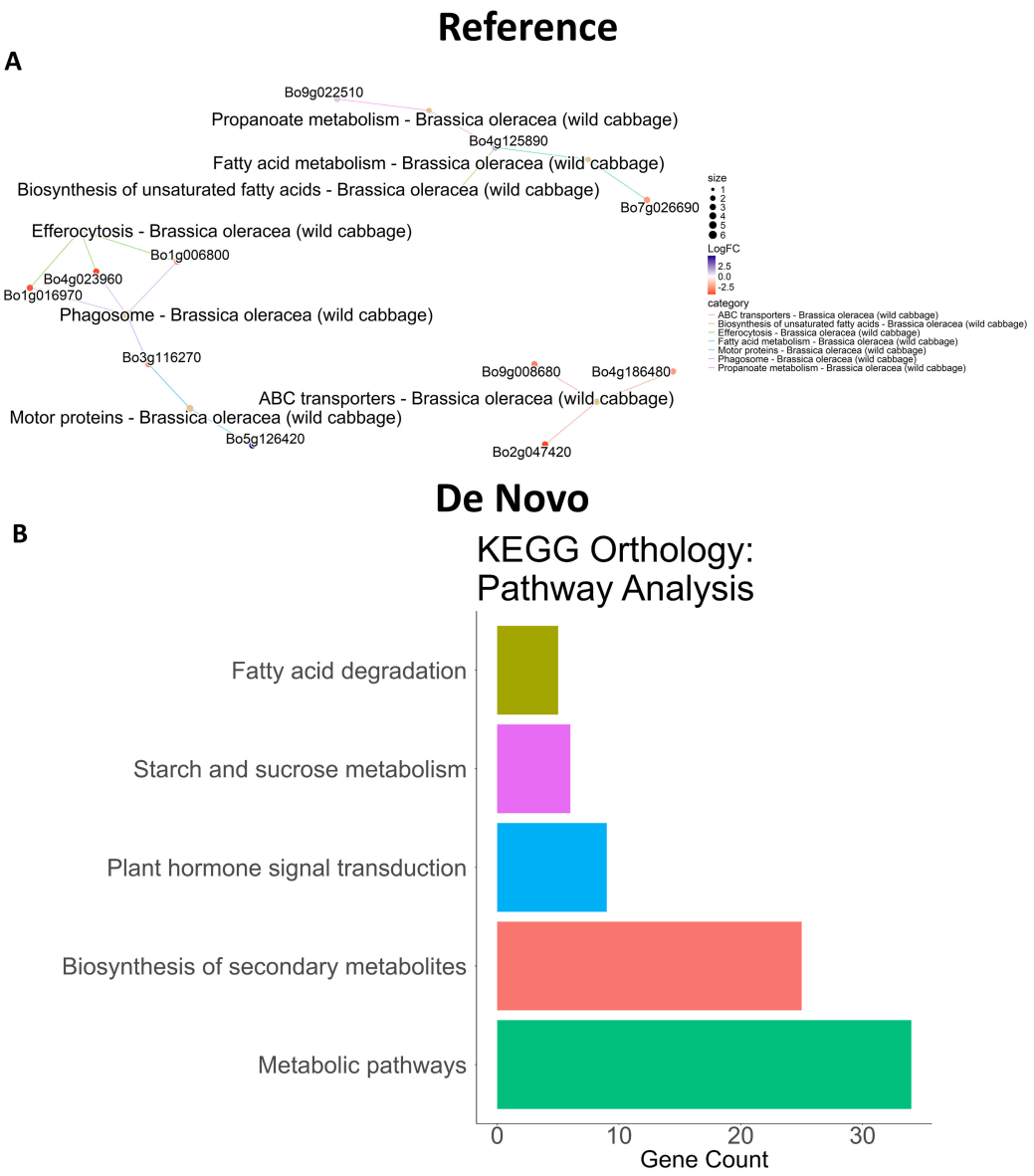

Figure 1.8: Pathways network of tolerant signature with reference genome (A) and de novo analysis (B).

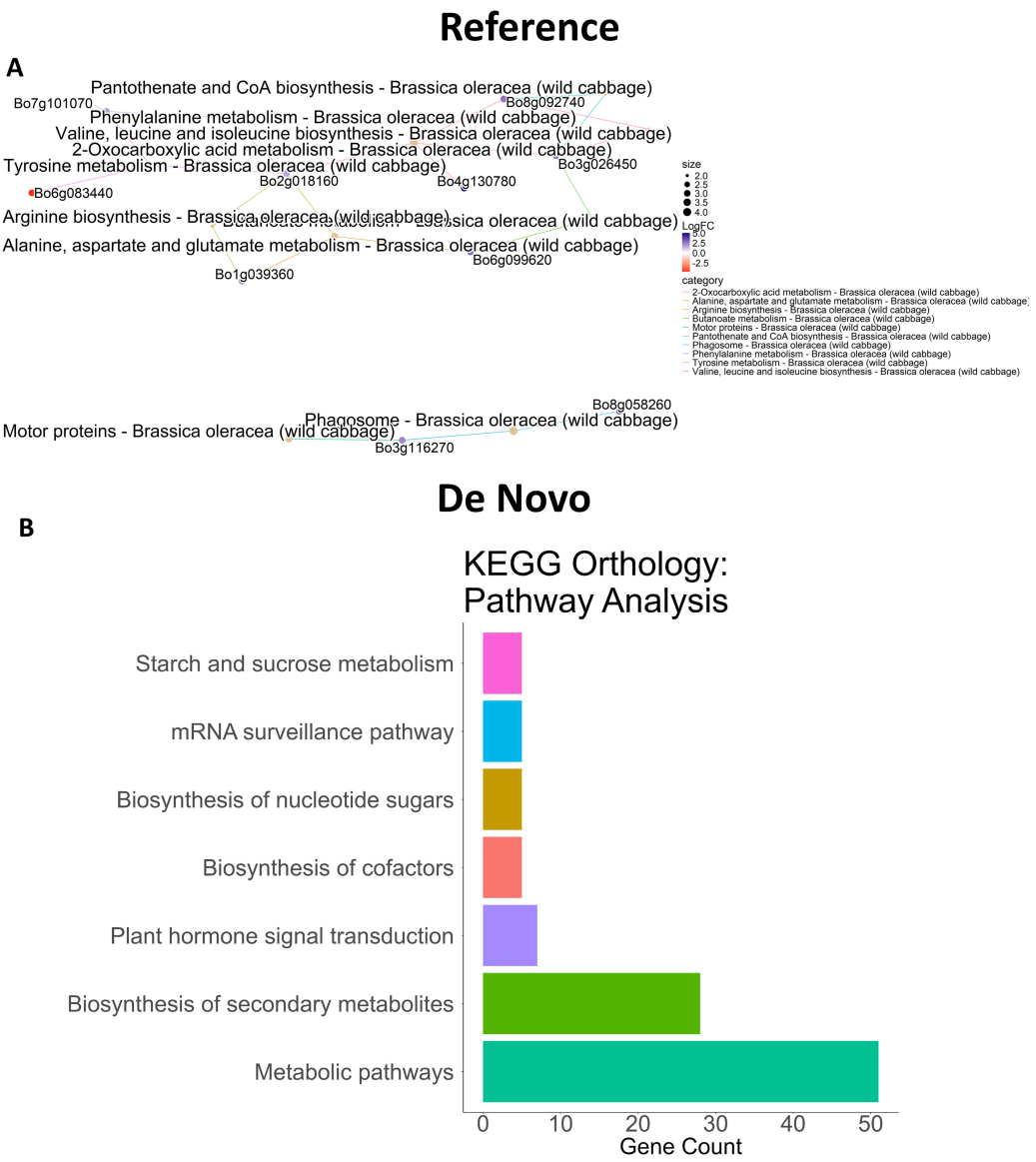

Figure 1.9: Pathways network of intersection signature with reference genome (A) and de novo analysis (B).

In Fig. 1.8, the reference genome analysis of tolerant signature shows only one significant pathway: the MAPK signaling pathways in which are involved two significant genes: Bo4g190030 and Bo7g075740 (Table 1.5). In de novo analysis shown in Fig. 1.8, several significant pathways associated with the tolerant

signature are observed. Among them, notable are the metabolic pathways and the biosynthesis of secondary metabolites, including genes such as ABSCISIC ACID-INSENSITIVE 5-like protein 6 and Protein phosphatase 2C (up-regulated), Magnesium dechelatase SGRL, chloroplastic, Transketolase-1, chloroplastic, and Auxin-responsive protein IAA28 (down-regulated) (Table 1.4). Finally, the Pathways network of intersection signature with reference genome, in Fig. 1.9, shows ten perturbed pathways. The pathways (i) Arginine biosynthesis, (ii) Tyrosine metabolism, (iii) Phenylalanine metabolism, (iv) Alanine, aspartate and glutamate metabolism, (v) Butanoate metabolism, (vi) Pantothenate and CoA biosynthesis, (vii) Valine, leucine and isoleucine biosynthesis, (viii) 2-Oxocarboxylic acid metabolism are all concatenated through at least one gene. While (ix) Motor Protein and (x) Phagosome are concatenated by the gene Bo3g116270. In the de novo analysis (1.9), the Kegg Orthology highlights the biosynthesis of secondary metabolites with about 50 genes. This pathway is followed by Plant hormone signal transduction, Biosynthesis of cofactors, mRNA surveillance pathway, and Starch and Sucrose metabolism. The main genes involved in these pathways are Cytochrome P450 83A1 (up-regulated) and 9-cis-epoxycarotenoid dioxygenase NCED3 chloroplastic (down-regulated) (Table 1.4).

Table 1.4: List of genes involved in stress response obtained by de novo assembly. In the regulation column is reported the Log_2FoldChange mean value of the comparisons considered in each signature.

Cluster ID	Description	Swissprot ID	Regulation (Log_2FoldChange)
Sensitive signature			
8815.8016	probable protein phosphatase 2C 78	Q9FIF5	6.92
8815.41697	Indole-3-acetic acid-amido synthetase GH3.6	Q9LSQ4	4.38
8815.44239	Cytochrome P450	P48421	-3.80
8815.26346	Abscisic acid receptor PYR1	Q04986	-3.88
8815.2881	Auxin transporter-like protein 3	Q9CA25	-5.61
8815.52826	Auxin-responsive protein SAUR50	O65695	-5.20
Tolerant signature			
8815.28341	ABSCISIC ACID-INSENSITIVE 5-like protein	Q9M7Q3	2.08
8815.26738	Protein phosphatase 2C	P49598	1.88
8815.28482	Magnesium dechelatase SRL, chloroplastic	Q94AQ9	-1.10
8815.32878	Transketolase-1, chloroplastic	Q8RWV0	-1.21
8815.44828	Auxin-responsive protein IAA28	Q9XFM0	-1.57
Intersection signature			
8815.31925	Cytochrome P450 83A1	P48421	3.65
8815.51356	9-cis-epoxycarotenoid dioxygenase NCED3, chloroplastic	Q9LRR7	-2.96

Table 1.5: List of genes involved in stress response obtained by reference assembly. The regulation column reports the Log_2FoldChange mean value of the comparisons considered in each signature.

Gene	Description	Gene Name	Regulation (Log_2FC)
Sensitive Signature			
Bo1g117540	Peptidase A1 domain-containing protein	106,315,440	-3.53
Bo3g001410	RING-type domain-containing protein	106,336,016	1.93
Bo3g132570	Abscisic acid-activated signaling pathway	106,336,161	-2.89
Bo3g134720	TCP domain-containing protein	106,335,966	-1.84
Bo4g014570	Remorin_C domain-containing protein	106,337,585	1.41
Bo8g091960	Remorin_C domain-containing protein	106,307,888	1.45
Bo9g004450	Response to abscisic acid	106,319,095	1.35
Bo5g126420	Dynein light chain	106,343,361	4.95
Bo01141s010	Auxin response factor	106,320,938	-3.52
Bo1g006950	Response to auxin	106,297,419	-5.32
Bo7g095650	Auxin-responsive protein	106,303,757	-1.65
Bo7g119560	Response to auxin	106,304,001	-6.30
Tolerant Signature			
Bo4g190030	Abscisic acid receptor	106,337,293	-7.96
Bo7g075740	Abscisic acid receptor	106,301,784	-1.72
Bo1g016790	Auxin-responsive protein	#N/D	-2.29
Bo2g134160	Auxin efflux carrier component	106,327,550	-1.67
Bo3g086850	Xyloglucan endotransglucosylase/hydrolase, EC 2.4.1.207	106,336,417	-4.19
Bo8g112780	LOB domain-containing protein	106,310,345	4.01
Intersection Signature			
Bo4g130780	Cytochrome P450 CYP83A1	106,342,466	5.01
Bo6g083440	Enoyl reductase (ER) domain-containing protein	106,298,631	-4.72
Bo2g052680	C2H2-type domain-containing protein	106,320,995	1.45
Bo3g001410	RING-type domain-containing protein	106,336,016	-1.54
Bo3g018500	RING-type domain-containing protein	106,332,045	-2.45
Bo3g132570	FAS1 domain-containing protein	106,336,161	2.33
Bo3g134720	TCP domain-containing protein	106,335,966	1.78
Bo4g038720	EID1-like F-box protein 3	#N/D	-4.97
Bo8g080510	Dehydrin	106,311,856	-7.37
Bo1g103470	Auxin-responsive protein	106,325,843	-2.66
Bo3g179630	Auxin efflux carrier family protein	#N/D	-1.58
Bo5g030850	Auxin efflux carrier family protein	106,293,889	-4.50
Bo7g084150	Protein LAZY 1	106,304,822	-4.89

4. Discussion

Abiotic stresses are widely considered as severe environmental factors that significantly impairs crop production worldwide. Plants generally experience a wide range of abiotic stressors, including high levels of salt (salinity), extreme temperatures (chilling, freezing or heat) and insufficient water availability (drought or dehydration). These stressors collectively represent the primary drivers behind the substantial reduction of the yield of crops [111]. Overall, the susceptibility or tolerance of plants to these stresses emerges because of the intricate interplay of multiple stress-responsive genes. Consequently, these genes concurrently interact through the cross-talk with other components within the stress signal transduction pathways [112]. Among the stressors cited above, water deficiency represents an increasingly urgent worldwide issue, particularly in areas where agriculture is a major economic activity. The reduction of water used in agriculture, the strategies for conserving water resources, improving agricultural productivity and enhancing drought stress resistance, are the new frontiers of agriculture, mostly in relation to the incoming climatic changes. Among the several plant species damaged by drought, *Brassica oleracea* L. crops (n = 9 chromosomes), valued for their high antioxidant capacity conferred by bioactive compounds such as glucosinolates (GLSs) and polyphenols (PPs), are particularly vulnerable to the adverse effects of water deficiency, with a substantial impact on both their yield and growth processes. For these reasons, our research has been centered on conducting transcriptomic analysis on four accessions within the *Brassica oleracea* complex species selection showing a different response to water deficit imposition (tolerance and sensitivity). The transcriptomic response of the tolerant and sensitive genotypes was thus compared with the aim of identifying specific genes and pathways associated with the plant's response to drought stress. A noteworthy aspect of our research regards the comprehensive comparison of two distinct bioinformatics approaches for transcriptome assembly: reference and de novo. As it emerged by phenotypic analysis, *B. macrocarpa* accessions exhibited a notable drought stress tolerance. This assertion finds reinforcement in its native habitat, which is characterized by rocky slopes along the coast of Egadi's Island, in which this particular genetic resource thrives an endemism. Conversely, the identified sensitive broccoli landraces belong to the "ciurietto" group, a category encompassing cultivars exhibiting intermediate traits between broccoli and cauliflowers. It is widely recognized that these cultivars were selected in Sicilian

local gardens due to their astonishing organoleptic properties and they were not selected for their resilience traits [113]. In our analysis of morphological traits, we did not detect substantial variations in the total and chlorotic leaf counts in both NIR and IRR conditions between sensitive and tolerant plants. The different response to water deficit imposition between sensitive and tolerant plants can be observed when considering SPAD, leaf area, leaf lamina length and leaf width.

As concerning the analysis of the two metabolites significantly involved in the oxidative stress (MDA and H_2O_2), no differences in MDA content were measured in both IR when comparing sensitive and tolerant plants. Meanwhile, in NIR condition the sensitive plants showed a significantly higher value of H_2O_2 with respect to the tolerant plants, indicating that a high level of secondary oxidative stress triggered by water stress has been achieved in sensitive plants. In fact under normal physiological activities, plants produce reactive oxygen species (ROS), such as superoxide anion radicals (O_2^-), singlet oxygen (O_2), hydroxyl radicals (·OH) and hydrogen peroxide (H_2O_2), as signal transmitters to regulate gene and protein expression in plant cells, and the production and elimination of ROS are always in a state of dynamic equilibrium [114]. When the plant is stressed, the balance will be broken, the physiological and biochemical functions of the plant cell membrane will be disturbed, and the production of reactive oxygen species will increase [115]. Concerning transcriptomic analysis, we have conducted a comprehensive analysis comparing de novo whole transcriptome sequencing with reference genome analysis employing TO1000 [116]. Remarkably, the de novo analysis resulted in an absolute highest number of DEGs with respect to the reference method when taking into account all the comparisons analyzed. The highest number of DEGs detected by the de novo approach can be explained considering the different approach of the two methods in gene expression quantification. A large number of paralogous sequence reads from members of the same gene family are often de novo assembled and quantified. Furthermore, the de novo approach allows to estimate the levels of genes that are lowly expressed, have long CDSs, or belong to large gene families, resulting in a higher number of genes that could be differentially expressed when comparing different samples [117]. This observation can provide substantial support for our findings regarding the greater number of differentially expressed genes (DEGs) when comparing the de novo method to the reference genome-based approach. In our specific context, although the reference genome employed was one of the most extensively annotated for the *B. oleracea* species, it still offered a limited set

of annotations. Notably, we employed as reference genome the TO1000 (BOL), derived from *Brassica oleracea* var. *oleracea*. Moreover, as it was highlighted [118], de novo assembly often uncovers a significant abundance of transcript isoforms. In order to highlight the transcriptomic response differences to drought stress we intersected the DEGs obtained by the cultivars with the same behavior resulting in three different signatures. The above mentioned DEGs were then enriched for Gene Ontology Biological Processes and KEGG pathways. Firstly, our analysis revealed that within the sensitive reference signature 7 genes play vital roles in abscisic acid (ABA) response and regulation. The phytohormone abscisic acid (ABA) controls various aspects of plant growth throughout development. During vegetative growth, its major role is to mediate adaptive responses to various adverse environmental conditions. Its critical role in the adaptation or acclimation to drought, freezing, and high salinity is well documented [119, 120]. In this work Bo1g117540 resulted downregulated, it encodes a peptidase with aspartic-type endopeptidase activity involved in responding to water deprivation and drought avoidance. Furthermore, the gene Bo3g134720, also downregulated, encodes the TCP14 transcription factor involved in various growth and development processes. Several studies by different authors have consistently associated TCP transcription factors primarily with ABA response and stress applications. Drought stress in plants involves various proteins, with dynein light chain (DLC) playing a crucial role as part of the dynein complex—a motor protein connected to myosin and kinesin. DLC facilitates ATP hydrolysis for mechanical motion, contributing to essential cellular processes. Among the upregulated genes in this signature we have found the Bo5g126420 gene which encodes a DLC. This finding aligns with previous research on cotton, rice, Arabidopsis, and tomato, where DLC and microtubule-based process genes were studied in relation to development, abiotic stresses, and phytohormone treatments [121, 122, 123, 124, 125]. Auxin plays an important role during abiotic stress-induced changes in the root and developmental modifications to root system architecture (RSA), is vital for tolerance to water deficiency [126]. We have found four genes implicated in auxin regulation, all downregulated. The downregulation of Auxin response factors (ARFs) such as Bo01141s010 and Aux/IAA Proteins such as Bo7g095650 show the interruption of auxin uptake and the braking of the plant growth due to the water absence. Consistently to what was found in the sensitive reference signature, genes involved in auxin transport and regulation have been found down-regulated in the de novo sensitive signature. In detail, auxin transporter-

like protein 3 involved in proton-driven auxin influx was down-regulated in de novo sensitive signature. It mediates the formation of auxin gradient in plants by contributing to the loading of auxin in vascular tissues and facilitating acropetal (base to tip) auxin transport within inner tissues of the root apex. The down regulation of auxin transporter-like protein 3 suggests a slowdown of auxin transport through the plant with a consequent negative effect on root development and architecture, thus leading to drought stress suffering in sensitive plants. The hypothesis is also supported by the down-regulation of auxin-responsive protein SAUR50 involved in plant growth by promoting cell elongation [127]. The abscisic acid (ABA) signaling, and regulation resulted strongly impaired by water deficiency in sensitive plants. In fact an up-regulation of protein phosphatase 2C 78, a negative regulator of ABA signaling for stomatal closure in leaves and a negative regulator of response to drought [128, 129], was measured. Furthermore, abscisic acid receptor PYR1 resulted down-regulated. PYR1 has been reported to promote drought tolerance [130]. Finally, the production of glucosinolates is also affected by drought stress since a down-regulation of cytochrome P450 83A1-like was measured. It is involved in the biosynthesis of both short-chain and long-chain aliphatic glucosinolates [131].

Secondly, in tolerant reference signature we have found two downregulated genes correlated to ABA signaling pathways with respect to control samples. In particular Bo4g190030 and Bo7g075740, identified with Uniprot as orthologues of respectively PYL6 and PYL1, are ABA receptors. Both of them are required for ABA-mediated responses such as stomatal closure and germination inhibition. Other three downregulated genes, instead, are involved in auxin regulation. In particular, Bo1g016790 is an Aux/IAA protein that functions as repressors of early auxin response genes at low auxin concentrations, its downregulation makes us think about a higher auxin intake of the plant. Bo2g134160 is an orthologues of PIN4, PIN3 and PIN7 of *Arabidopsis thaliana*. All of them are classified as auxin efflux carriers, this suggests that its downregulation can be involved in higher concentration of auxin in the tolerant plants. Furthermore, Bo3g086850 Catalyzes xyloglucan endohydrolysis (XEH) and/or endotransglycosylation (XET) [132], participating in cell wall construction. Moreover, we have found the gene Bo8g112780 upregulated, this gene encodes Lateral Organ Boundaries (LOB) domain-containing protein and it has two orthologues in *Arabidopsis thaliana* [133], LBD1 and LBD11. As seen by Ye et al. in 2021, LBD genes overexpression promotes cell growth and cell division enhancing secondary growth

suggesting a resistance to drought stress. As regards the de novo tolerant signature, a gene encoding ABSCISIC ACID-INSENSITIVE 5-like protein 6 (ABF3) resulted up-regulated. It binds to the ABA-responsive element (ABRE) mediating stress-responsive ABA signaling. In detail overexpression of ABF3 or ABF4 in *Arabidopsis* resulted in ABA hypersensitivity and enhanced drought tolerance with changes in the expression levels of a number of ABA- or stress-regulated genes [134]. Furthermore, a gene encoding for protein phosphatase 2C 37-like resulted also up-regulated. This protein prevents stomata closure by inactivating the S-type anion efflux channel SLAC1 and its activator SRK2E [135]. Interestingly, homologous of both ABSCISIC ACID-INSENSITIVE 5-like protein 6 and protein phosphatase 2C 37-like were found up-regulated in *Arundo donax* when subjected to high levels of salt stress [86], confirming the importance of ABA signaling in the tolerance of plants to stresses leading to osmotic imbalance. Finally, a gene encoding magnesium dechelatase SGRL was found among the downregulated genes in the tolerant signature. It is involved in chlorophyll degradation in the chlorophyll-protein complexes of photosystem I (PSI) and photosystem II (PSII) [136]. Contributes to abiotic stress-induced chlorophyll degradation and leaf yellowing during vegetative plant growth [137]. This result is consistent with the high level of SPAD index measured in tolerant plants when subjected to water deficit conditions suggesting that stress tolerance is related with regular photosynthetic processes.

Through the intersection of differentially expressed genes between tolerant and sensitive conditions we identified the intersection signature. In this signature we have found 7 genes involved in ABA regulation (4 down and 3 upregulated) and 4 in auxin regulation (all downregulated). In particular, the gene Bo2g052680 [10] encodes for a C2H2-type domain-containing protein; these proteins have been shown to respond to abiotic stress in plants. It is involved in negative abscisic acid regulation as its orthologues in *Arabidopsis thaliana* (ZFP4) [138]. The gene Bo3g13472, already seen downregulated in sensitive signatures, in intersection signature results upregulated. Its orthologue in *Arabidopsis*, called TCP14 [139], regulates germination. Bo5g030850 and Bo3g179630 are two auxin efflux carriers which result in being downregulated in tolerant versus sensitive plants, suggesting that drought stress tolerant plants retain more auxin than sensitive ones.

In our study, we observed significantly higher expression of the Bo4g130780 gene involved in GO such as glucosinolate biosynthetic process [GO:0019761];

response to insects [GO:0009625]. This gene, identified as cytochrome P450 83A1 [140] monooxygenase (CYP83A1), plays a pivotal role in glucosinolate biosynthesis suggesting a modulation of glucosinolate production, particularly in the biosynthesis of aliphatic glucosinolates, in response to drought stress. The cytochrome P450 superfamily [141] in fact brings to the synthesis of numerous secondary metabolites that function as growth and development signals to plants. Furthermore, our findings are corroborated by different studies [142, 143], where they observed reduced drought stress resistance in *Arabidopsis* as a consequence of the loss of function of the *CYP83A1* gene. The importance of cytochrome P450 83A1 is highlighted by the fact that it was found strongly up-regulated also in the de novo intersection signature. Another identified gene, Bo6g083440, orthologues of *ADH1* of *Arabidopsis thaliana*, encodes an alcohol dehydrogenase enzyme. We observed a significantly lower expression of this gene in tolerant plants. This finding aligns with a previous research [144], who detected the upregulation of genes in *B. napus* sensitive to drought stress, resulting in increased fatty acid degradation and a reduction in oil content.

Among the examined genes, Bo9g041010 (a homolog of *Arabidopsis thaliana* Transcription factor bHLH112, SwissProt Q94JL3) emerged as a common denominator in both tolerant and sensitive cultivars, exhibiting a discernible trend in expression. Specifically, it displayed upregulation in tolerant cultivars and downregulation in sensitive ones. AtbHLH112, a nuclear-localized protein, is known to undergo induction of nuclear localization in response to salt, drought, and abscisic acid (ABA). Gain- and loss-of-function analyses have highlighted a positive correlation between the transcript level of AtbHLH112 and salt and drought tolerance. Additionally, AtbHLH112 facilitates the upregulation of POD and SOD genes, enhancing the scavenging ability of reactive oxygen species (ROS) [10].

Chapter 2

Variation of soil microbiome and of yield of tomato crop growth

This study was conducted to evaluate the effect of sustainable agricultural practices on intensive horticultural production while minimizing ecosystem impact. Two commercial hybrid tomato rootstocks and two scion cultivars were used, comparing grafting combinations in both plots treated with a commercial microbial amendment and untreated control plots. The assessed parameters included yield components and rhizosphere microbiome composition, in order to investigate how grafting and microbial treatments may interact to enhance production in an eco-friendly manner.

2.7 Materials and Methods

2.7.1 Plant material and microorganism amendment

A protocol composed of two commercial products; composed mainly of mycorrhizal fungi and rhizosphere bacteria, Klappy (*Glomus mosseae, Glomus intraradices, Pochonia, Clamidosporia* and *Purpureocillium liliacinum*) and Klaster (*Glomus mosseae, Glomus intraradices, Bacillus firmus* and *Streptomyces avermitilis*) provided by B4Green S.r.l., Catania, Italy; was applied to different tomato grafting combinations to enhance root development and resistance to soil pathogens in horticultural crops.

Two commercial rootstocks: OptiFort (Bayer) and RS4 (NewBreed) were grafted

on one commercial variety (MVS101, F1) (Mondoverde emilla, Scicli, Italy) and on a Sicilian landrace 'Pizzutello medio' (PO267) provided by the genebank of the Department of Agriculture, Food and Environment (Di3A) of the University of Catania (UNICT). Four other controls were present: the auto-grafted (the scion is grafted on its own roots) and the non-grafted (self-rooted) scions Table 2.1).

Table 2.1: Grafting combination and treatment.

Code	**Description**
R1	OptiFort Rootstock
R2	RS4 Rootstock
S1	MVS101 Scion
S2	PO267 Scion
NG	Non grafted
TR	Treated with Klappy and Klaster
NT	Non treated

2.7.2 Field experiment and plant characterisation

Tomato rootstocks and scions were sown and manually grafted in a high-tech plant nursery. The 300 m^2 experimental greenhouse was adopting a Split-Plot design dividing it in three replicates, each replicate was divided into two main plots: treated (TR) and non-treated (NT). Each combination (rootstock/scion) was represented in one elementary sub plot of five plants.

In the beginning of October 2023, (50 days after sowing and 27 days after grafting), the combinations were transplanted with a distance of 0.40 m between plants and 1.2 m between rows, with a total of 8 elementary sub-plots per main plot. The microorganism amendments were applied (400 mL/1000 m^2 of Klappy and 300 mL/1000 m^2 Klaster) once every 2 weeks alternatively by fertirrigation; one product every week, as suggested by the supplier, from the second week after transplant until the first fruit truss maturation.

Plants were grown vertically on a single stem until the 8th fruit truss after that they were pruned. Fruit harvest was done by the collection of the entire truss. Yield components were calculated, harvested fruit per plant were weighted and the sum of the eight trusses formed the yield per plant (Y). Roots were

characterised by the end of the growing season and root weight and dimensions were registered.

2.7.3 Rhizosphere sampling, DNA extraction and microbiome sequencing

Microbiome sampling was done 120 days after the transplant. Fresh roots at 20 cm depth from two plants in each sub-plot were taken separately, shaken to eliminate excess soil then soaked in 25 mL of distilled water in a sterile falcon. A total of 6 repetitions of each combination (rootstock/scion + treatment) were sampled and conserved at -80°C. Genomic DNA extraction from the rhizosphere was done from the formation of a solid pellet formed through the centrifugation of the solvent in which the roots were soaked. The extraction was performed using the Soil DNA Isolation Plus Kit (Norgen Biotek, Thorol, Canada), following the manufacture instructions.

2.7.4 Library preparation, Quality control and Sequencing

The library preparation, quality control and sequencing were performed by Novogene (UK) company Limited (25 Cambridge Park, Milton Road, Cambridge, CB4 OFW, United Kingdom). The 16S rRNA gene region V3-V4 for the bacterial community and the ITS1 for the fungal community were amplified. The PCR products of proper size were selected through agarose gel electrophoresis. The same amount of PCR products from each sample was pooled, end-repaired, A-tailed, and further ligated with Illumina adapters. Libraries were sequenced on a paired-end Illumina platform (PE250). The library was checked with Qubit and real-time PCR for quantification, while a bioanalyzer was used for size distribution detection. Quantified libraries were pooled and sequenced on Illumina platforms according to the effective library concentration and data amount required.

2.7.5 Bioinformatic analysis

Raw FASTQ were trimmed using trim-galore [145] to eliminate sequencing adapters and to erase poor-quality reads. QIIME2 pipeline (v. qiime2-amplicon-2024.10) [146] was used to process the trimmed FASTQ files. Paired-end sequences were

first imported, and then quality-filtering was performed with the DADA2 denoise method cutting to a quality score of 30. Alpha (Shannon entropy and Chao1) and beta diversity (Jaccard, Weighted-Unifrac, Unweighted-Unifrac and Bray–Curtis) were estimated. Differences between groups in terms of alpha-diversity were calculated using Kruskal–Wallis and Wilcoxon tests or ANOVA and T-test depending on the Shapiro-Wilk test result. The former was used when the distribution was not normal, the latter when the distribution was normal. The significance for beta-diversity was calculated with a PERMANOVA analysis. Taxonomy was assigned to the amplicon sequence variant (ASV) using the UNITE (v.10) [147] database for fungi and the Greengenes (v.2022) [148] database for bacteria. Phylum, genus and species tables were also built, collapsing the feature table and the taxonomy. Differential abundance analyses were performed using the R (v.4.5.0) package DESeq2 (v.1.48.1). p-value < 0.05 was considered as statistically significant. All the graphics were computed using ggplot2 (v3.5.2) [110] and ggpubr (v.0.6.0) [109]. Correlation between morphometric traits and microbial abundance have been computed in R using Spearman correlation. Only correlations with a p-value < 0.05 have been considered statistically significative.

2.8 Results

2.8.1 Morphometric traits

Root Weight (RW) and Yield (Y) have been compared between all couples of TR and NT samples. R2/S2 RW comparing TR versus NT samples showed a significant difference (p-value = 0.04) with the TR samples having a median RW (m:133.8, sd:6) lower than NT samples (m:158, sd:11.5). All these results can be inspected in Figure 2.1. This result is confirmed by comparing all S2 TR samples with all S2 NT samples independently of the type of grafting. TR S2 RW samples have a lower RW (p-value = 0.04). Moreover, comparing S1 and S2 it can be seen that in both TR and NT samples S2 RW is significantly lower than S1 RW. RW and Y measurement can be found in from the study *Variation of Soil Microbiome and of Yield of Tomato Crop Grown in Mediterranean Cold Greenhouse Conditions by Grafted Plants and Microbial Consortia* by Al Achkar et al. [1].

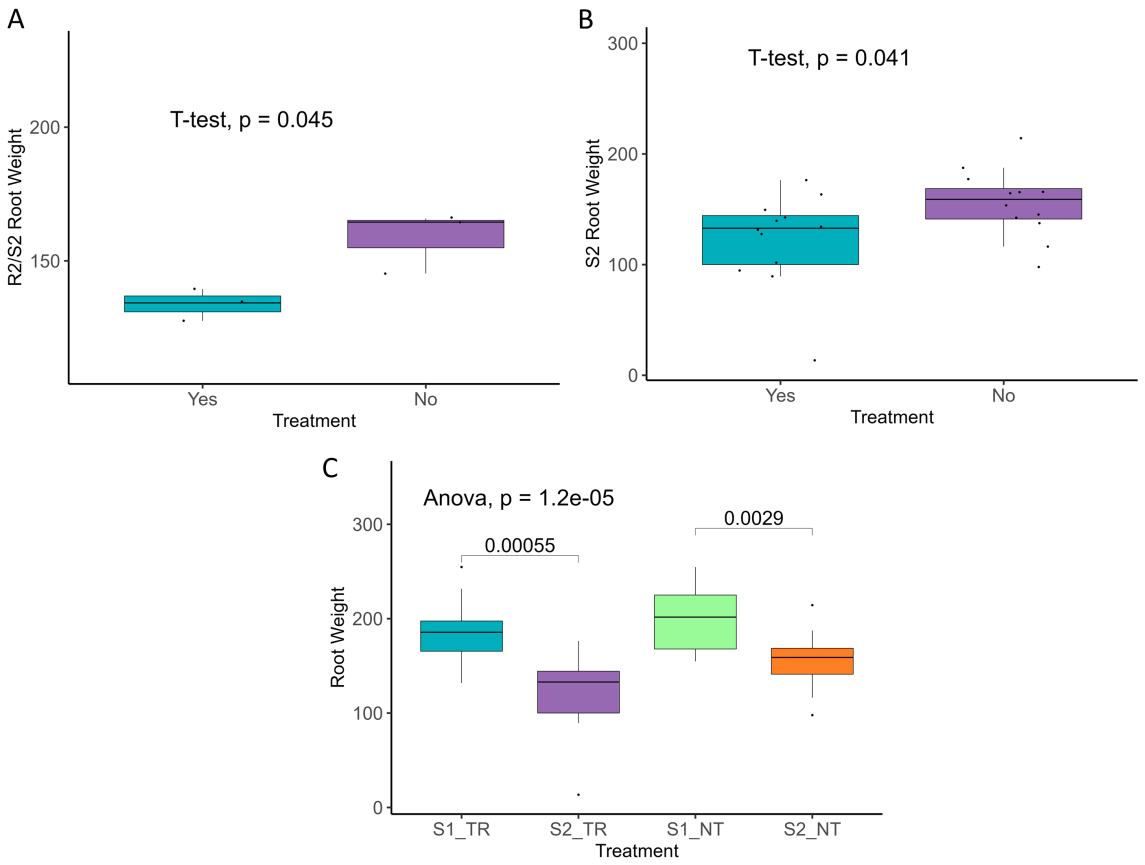

Figure 2.1: Comparison of Root Weight (RW) and Yield (Y) between TR and NT samples. A significant difference was observed in R2/S2 RW when comparing TR versus NT plants (p-value = 0.04), with TR samples showing a lower median RW (m = 133.8, sd = 6) compared to NT samples (m = 158, sd = 11.5).

2.8.2 Microbiome profile

Analysis of fungal percentages in our samples revealed that neither of the inoculated fungi were detected in TR samples (with the exception of Auto-grafted S2, where 0.08% of *Purpureocillium* was observed). Regarding bacterial inoculation composition, *Streptomyces* was found, without species classification, with a relative abundance between 1% and 4%, while the *Bacillus* genus accounted for less than 0.01%.

All the TR vs NT pairs have been tested for alpha and beta diversity. For ITS

only the couple R1/S1 TR versus R1/S1 NT showed a significant result with a p-value of 0.032 for Shannon entropy (Figure 2.2A). This result shows that in TR samples the entropy is increased suggesting a better state of plant health.

Regarding Bacteria, significant differences in alpha diversity have been shown between TR and NT S2 root. As seen in Figure 2.2B the Shannon entropy was increased in NT samples compared to treated ones. We have obtained the same result comparing the Rooted S2 (NGS + auto-grafted S2) TR with the Rooted S2 NT (Figure 2.2C).

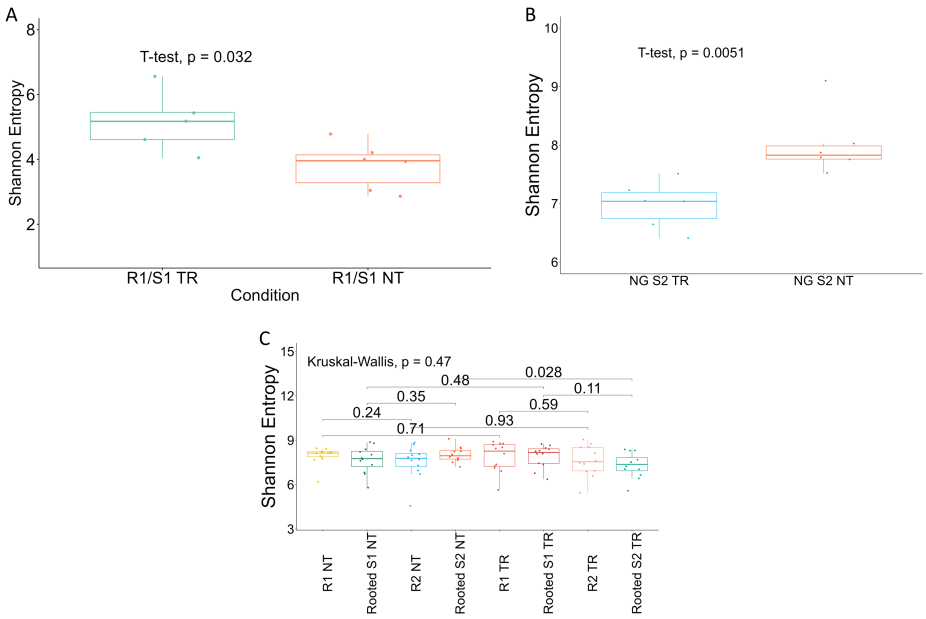

Figure 2.2: Shannon index of significant alpha diversity condition. (A) Comparison of treated and untreated R1/S1 in ITS. (B) Comparison of treated and untreated S2 root in bacteria. (C) Comparison among treated and untreated R1 and R2, and treated and untreated rooted S1 and S2.

As shown in Figure 2.3A, Ascomycota constituted the highest percentage in R2/S2 TR samples (80%), while R2/S1 NT samples exhibited the lowest (43.8%). R1/S2 TR samples, conversely, showed only 46% Ascomycota, indicating the rootstock's significant influence. Regarding Basidiomycota, the rootstock consistently increased their percentage in NT samples (S2 NT: 3%, R2/S2 NT: 7%, R1/S1 NT: 12.7%). Comparing TR and NT samples, the treatment decreased Ba-

sidiomycota percentages in R2 rootstocks but increased them in R1 rootstocks. Regarding Bacteria (Figure 2.3B), we can observe that Bacteroidota remain stable in both TR and NT samples. All TR samples (excluded R2/S1) show a decrease in Actinobacteriota, for instance R1/S1 TR has a percentage of 7.77% while the NT 9.73%. The other phyla do not seem to have treatment-dependent behavior. Additional percentages are reported in Table S3 of the supplementary materials from the study *Variation of Soil Microbiome and of Yield of Tomato Crop Grown in Mediterranean Cold Greenhouse Conditions by Grafted Plants and Microbial Consortia* by Al Achkar et al. [1].

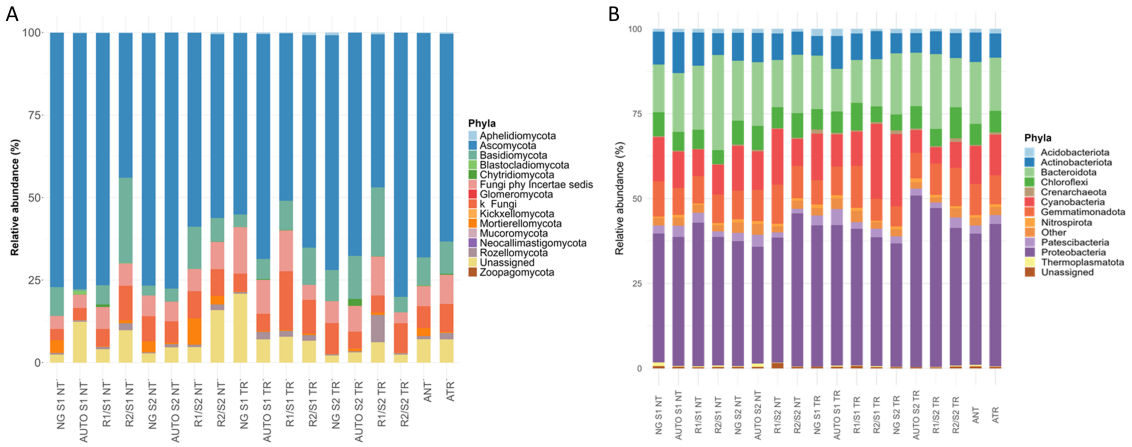

Figure 2.3: Percentage of Phyla in (A) ITS and (B) Bacteria for each group analyzed. ANT = All Not Treated. ATR = All Treated.

Differential abundance analysis

All possible pairs of TR vs NT samples have been compared at the phylum, genus and species level. Distinct differences were not observed at the Phylum level. Regarding genus, comparing the differential abundance for fungal ITS between R1/S1 TR and NT samples, a significant decrease in the genera *Alternaria*, *Arthrobotrys*, *Cephaliophora*, *Dothideomycetes*, *Melanocarpus*, *Nigrospora*, *Subramaniula*, *Thermoascus* and *Zopfiella* was observed. Conversely, *Malassezia* showed a significant increase. Under the same conditions for R2/S1, an increase in the abundance of *Bjerkandera*, *Microascaceae* and *Thermoascus* was noted, alongside a decrease in *Parasarocladium* and *Schizophyllum* (Figure 2.4A). Considering the

microbiome of rootstock R1 with S1 and rootstock R1 with S2 in TR versus NT samples, similar trends were observed for *Malassezia* (Figure 2.4B). When comparing the Auto S1 and the S1 NG root, TR versus NT, the sole common result was the upregulation of the *Malassezia* (Figure 2.4). These findings suggest that the treatment increases *Malassezia* in the S1 root regardless of the rootstock, and that other increases result from synergistic interactions between the rootstock and the S1 root.

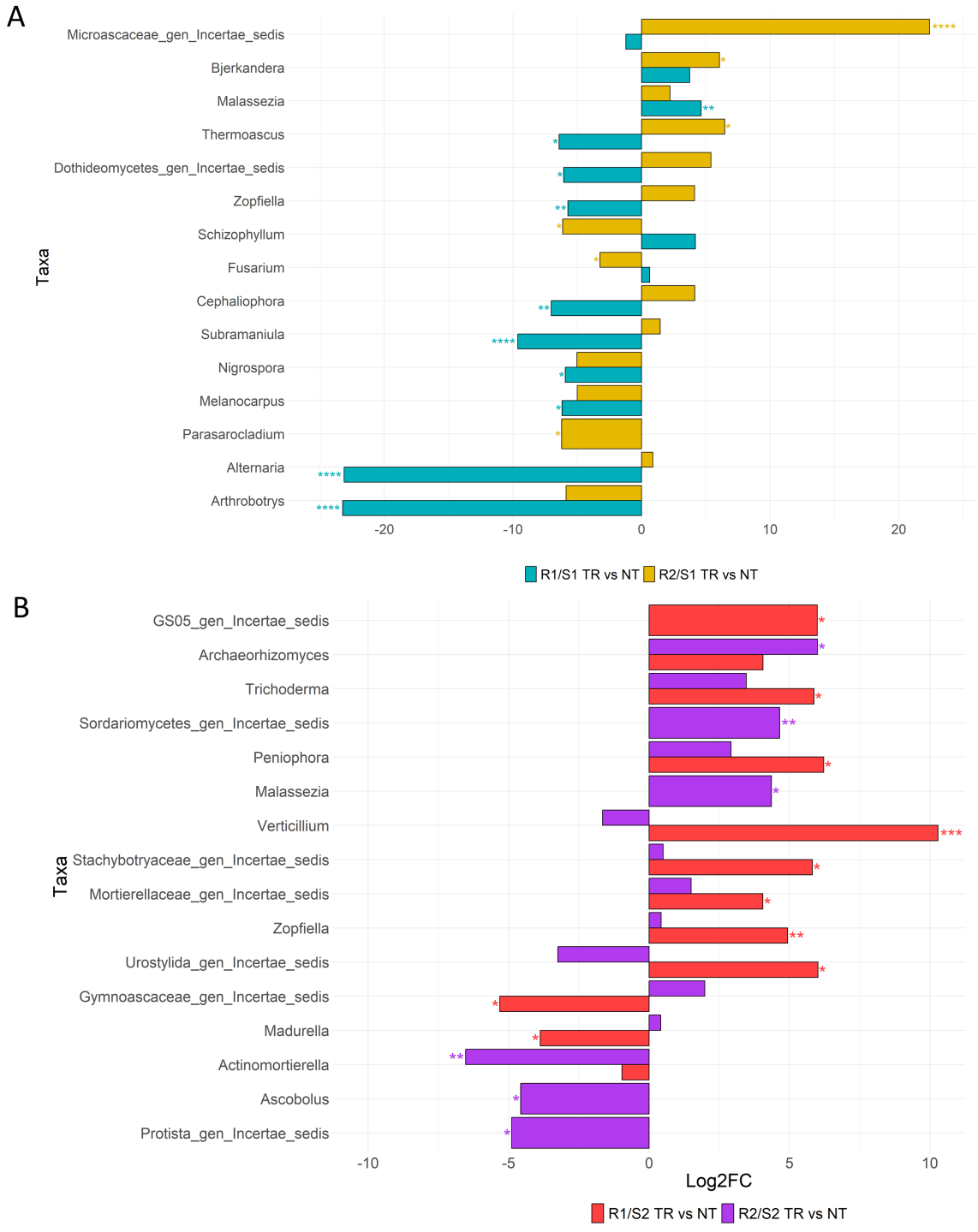

Figure 2.4: Genera differential abundance between (A) R1/S1 TR vs NT (blue bar) and R2/S1 TR vs NT (yellow bar), and (B) R1/S2 TR vs NT (red bar) and R2/S2 TR vs NT (violet bar). Positive $\log_2$FC values indicate an increase in condition one compared with condition two, while negative values indicate a decrease in condition one compared with condition two.

Comparing the differential abundance between Auto S1 TR vs NT (Figure 2.4A), a significant increase in the genera *Penicillium*, *Metarhizium*, *Paragibberella*, and *Malassezia* was observed. These genera include taxa with biocontrol or plant-associated potential. In addition, for Auto S2 TR vs NT, a strong increase was detected in *Alphoboma*, *Tolypocladium*, *Chaetosphaeria*, and *Purpureocillium* (Figure 2.4A). The comparison between S1 TR vs NT (Figure 2.4B) showed a notable decrease in the relative abundance of *Rhizoctonia*, *Stemphylium*, *Acremonium* and *Madurella*. The reduction of *Rhizoctonia*, a major soilborne pathogen causing root and stem rot in tomato, is particularly relevant and may suggest a beneficial effect of the treatment. Conversely, *Malassezia* displayed a significant increase. For S2 TR vs NT (Figure 2.4B), a significant increase in the abundance of *Rhizoctonia*, *Plectosphaerella*, *Chloridium* and *Malassezia* was detected. *Madurella* was instead reduced. Species differential abundance of ITS can be observed in Figure S2, S3, S4 and S5 from the study *Variation of Soil Microbiome and of Yield of Tomato Crop Grown in Mediterranean Cold Greenhouse Conditions by Grafted Plants and Microbial Consortia* by Al Achkar et al. [1].

Bacterial differential abundance

A comparison of bacterial communities in R2/S1 TR vs R2/S1 NT plants (Figure 2.6A) revealed a decrease in *Sphingobacterium nematocida* abundance in TR plants. This bacterium is known for its nematicidal properties. Its observed decrease in TR plants suggests that the treatment applied to the RS4 rootstock may directly or indirectly contribute to nematode suppression. This specific difference was not observed in the Optifort rootstock (R1/S1) [10].

In R1/S1 TR vs NT, we observed an increase in *Nitrosotenium*, a genus comprising ammonia-oxidizing archaea, playing a crucial role in the nitrogen cycle. Its increase suggests enhanced nitrogen availability for plant growth. This trend is further supported by Figure 2.8B, which also shows an increase in *Nitrosotenium* in TR plants [51].

Conversely, an inverse trend was observed for the abundance of *Rhizobium A 500098* when comparing Rootstock S1 TR plants to NT (Figure 2.7), versus Rootstock S2 TR plants to NT.

In Rootstock S1, *Rhizobium* abundance decreased, whereas in Rootstock S2, it increased, as also observed in S2 NGS TR vs NT [132]. Furthermore, *Rhizobium*. A 500098 increases in S1 TR vs NT plants, suggesting the role of the rootstocks in its decreases (Figure 2.8).

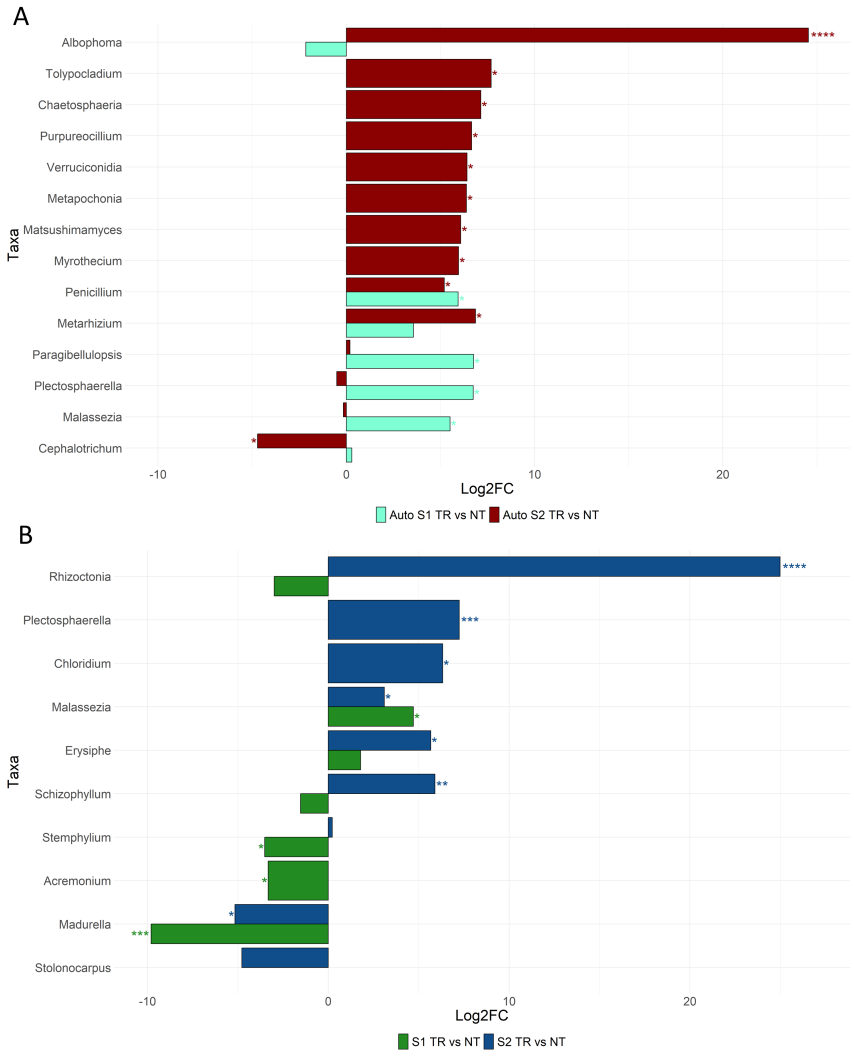

Figure 2.5: Genera differential abundance between (A) Auto S1 TR vs NT (light-blue bar) and Auto S2 TR vs NT (burgundy bar), and (B) S1 TR vs NT (green bar) and S2 TR vs NT (blue bar). Positive $\log_2$FC values indicate an increase in condition one compared with condition two, while negative values indicate a decrease in condition one compared with condition two.

This genus is well-known for its roles in nitrogen fixation, plant growth promotion, and yield enhancement [149]. The auto-grafted plants exhibited a different pattern of abundance, with the decrease of *Pseudoorhizobium* in Auto S1 TR vs NT and an increase of *Bryobacter* in Auto S2 TR vs NT, which is known to ameliorate plant health [125]. Genera differential abundance for R1/S1 TR vs NT and R2/S1 TR vs NT can be observed in Figure 2.1, while other species-level differential abundance of bacteria is shown in Figure S7, and S8, and S9 of the supplementary materials from the study *Variation of Soil Microbiome and of Yield of Tomato Crop Grown in Mediterranean Cold Greenhouse Conditions by Grafted Plants and Microbial Consortia* by Al Achkar et al. [1].

As previously stated in alpha diversity analysis, the RS4 rootstock exhibited a greater increase in species diversity compared to the Optifort rootstock in TR samples. This observation is further supported by Figure 2.6, which demonstrates a higher number of differentially abundant taxa in RS4 samples compared to R1 samples.

Morphometric traits and microorganism correlations

To investigate the overall role of microorganisms, we correlated morphometric parameters with microbial genus abundance. In particular, for fungi in the R1 TR samples, a negative correlation was observed between *Alternaria spp.* abundance and the Yield (Y), a correlation not present in NT samples. As previously stated, *Alternaria spp.* can cause diseases in tomato crops. Its decreased abundance in TR R1 plants, coupled with a negative correlation with tomato yield, indicates a healthier state in these treated plants [150]. Additionally, a positive correlation was identified between *Aspergillus* abundance and Root Weight (RW). While certain *Aspergillus* species are known to cause root diseases and produce ochratoxin A, potentially facilitating nematode colonization, the specific correlation observed here warrants further investigation. Specifically, RW was positively correlated with *Aspergillus ochraceus*. Conversely, a positive correlation was observed between *Aspergillus niger* and Y. It is well-established that *A. niger* produces oxalic acid (OA), a nematicidal compound effective against root-knot nematodes, and also promotes plant growth [151]. As previously noted, *Zopfiella* abundance decreased in TR R1/S1 samples compared to NT R1/S1 samples. Furthermore, *Zopfiella* exhibited a negative correlation with Y in TR R1 samples and a positive correlation in NT R1 samples. This contrasting behaviour may stem from different *Zopfiella* species, which our experimental design could not differentiate. For

sure it will need a more detailed analysis [152]. In R2 TR samples, *Pseudoidium neolycopersic* showed a negative correlation with Y, indicating that a decrease in its abundance corresponded to an increase in yield [153]. This specific correlation was not observed in the corresponding NT samples. *P. neolycopersic* is a known fungal pathogen responsible for tomato powdery mildew. Overall, a decrease in the relative abundance of this species was observed in treated plants compared to NT ones, specifically, 0.08% in R2 TR plants versus 0.5% in R2 NT plants, Table S2 of the supplementary materials from the study *Variation of Soil Microbiome and of Yield of Tomato Crop Grown in Mediterranean Cold Greenhouse Conditions by Grafted Plants and Microbial Consortia* by Al Achkar et al. [1]. . Regarding bacterial communities, a negative correlation was observed between *Rhodococcus spp.* and RW in both S1 TR and R2 TR samples. *Rhodococcus spp.* is a known biocontrol agent, which suggests a role in disease control within the treated samples [154]. Furthermore, its abundance was negatively correlated with Y in S1 NT samples. Conversely, in S2 TR samples, a positive correlation was identified between RW and the genera *Flavobacterium*, *Pseudoxanthomonas*, and *Achromobacter* [67].

2.9 Discussion

In this study, two agricultural eco-friendly practices were investigated for their effect on the profiles of microbial communities in tomato, an important horticultural crop grown worldwide. Both the application of exogenous microbial consortia and grafting on vegetables have previously shown efficiency in yield increase and quality support, especially in infected areas and monoculture regions such as Mediterranean traditional greenhouse areas [155, 156, 157]. A commercial microbial consortium was applied in this study. Interestingly, none of the microbial components of the products, mainly *Glomus mosseae*, *Glomus intraradices*, in addition to *Pochonia chlamydosporia* and *Purpureocillium lilacinum*, increased in the treated plots compared to NT ones. On the other hand, the addition of such consortia had a remarkable effect on other fungal species, ameliorating the abundance of some taxa, such as *Bjerkandera*, *Microascaceae*, and *Thermoascus*, while decreasing the abundance of others, such as *Alternaria*, *Arthrobotrys*, *Cephaliophora*, *Dothideomycetes*, *Melanocarpus*, *Nigrospora*, *Subramaniula*, *Thermoascus*, and *Zopfiella* [151, 158]. This manipulation of microbial abundance in fungi was also noted in different studies, where the applied microbial consortium tended to modulate the rhizosphere fungal community, involving taxa not present in the

inoculant, such as enrichment of *Mortierella* and *Leptodontidium* [159, 160]. The capacity to increase the abundance of symbiotic microorganisms and beneficial species is a key feature for the success of commercial microbial consortia [23]. The results shed light on the intricate interplay between grafting, microbial consortia treatments, and soil and root microbiome in tomato crops, ultimately impacting plant health and potentially yield. Considering fungal diversity (ITS), the observation that TR R1/S1 samples showed a significantly higher Shannon entropy ($p=0.032$) suggests that the applied microbial treatment fostered a more diverse and potentially more resilient fungal community in this specific graft combination [161]. Higher diversity often correlates with improved ecosystem stability, resource utilization, and enhanced resistance to disturbances, which could directly translate to better plant health. This aligns with literature supporting the role of diverse fungal communities in promoting plant growth and disease suppression [28]. Regarding bacterial diversity, for specific conditions like the S2 root (both TR vs NT S2 root and Rooted S2 TR vs Rooted S2 NT), the NT samples exhibited higher bacterial Shannon entropy. While a decrease in overall diversity might sometimes be seen as negative, it can indicate a more specialized and beneficial microbial community, where certain key beneficial bacteria become dominant due to the treatment, outcompeting less beneficial ones [162]. Both high diversity and the dominance of specific beneficial taxa can contribute to plant health and performance.By considering taxonomic composition, shifts in the relative abundance of major fungal phyla like Ascomycota and Basidiomycota indicate a restructuring of the fungal community in response to treatment and rootstock. Ascomycota, including many plant pathogens, saprophytes, and endophytes, showed the highest percentage in treated R2/S2 samples, suggesting that the treatment is promoting specific beneficial or commensal Ascomycetes, or outcompeting other groups. Conversely, the rootstock's role in increasing Basidiomycota in NT samples, and the differential impact of treatment on Basidiomycota percentages (decrease in R2, increase in R1), points to a complex genotype-by-microbe interaction [163]. The significant decrease in genera like *Alternaria*, *Arthrobotrys*, *Cephaliophora*, *Dothideomycetes*, *Melanocarpus*, *Nigrospora*, *Subramaniula*, *Thermoascus*, and *Zopfiella* in treated R1/S1 samples is a highly significant finding. Many of these genera (e.g., *Alternaria*, *Nigrospora*) are well-known plant pathogens or producers of mycotoxins [150, 164]. The reduction of *Alternaria spp.* suggests the usefulness of the microbial treatment in S1 roots with R1, exerting a biocontrol effect and contributing to a healthier root environment. Moreover,

Alternaria spp. was negatively correlated with tomato Y, suggesting that its decrease improves plant production.Conversely, *Arthrobotrys* plays a beneficial role in tomato roots as a control agent against nematodes; in particular, *Arthrobotrys oligospora* has been extensively studied for biological control of tomato root-knot nematodes. In the presence of nematodes, *A. oligospora* forms specialized predation organs to capture nematodes for digestion and absorption [165]. The decrease of nematode-controlling *Arthrobotrys* may also explain the consistent increase in *Malassezia* across different treated samples (R1/S1, S1 NG, and general trend in S1 root). *Malassezia* species are predominantly associated with animal hosts (e.g., skin microbiota) [166], and previous research has demonstrated a role of soil nematodes in their dispersal, potentially impacting distribution and interactions with other organisms [167].

Considering bacterial phyla composition, we do not observe any significant change. This indicates that the treatment does not have a strong role in reshaping bacterial phyla composition. Nevertheless, several differences can be observed in genus and species differential abundance. The provided results highlight dynamic shifts in the microbial communities associated with TR versus NT plants, underscoring the genotype-specific interactions between rootstocks and applied treatments [23]. The observed decrease in *Sphingobacterium nematocida* abundance in TR plants grown on RS4 rootstock is particularly noteworthy. Given that *S. nematocida* possesses well-documented nematicidal properties, its reduction in TR plants suggests that the applied treatment might directly or indirectly interfere with its proliferation or activity. This could imply that the treatment itself, or a cascade of microbial community changes induced by the treatment on the RS4 rootstock, may influence nematode suppression mechanisms. The absence of a similar decrease in the Optifort rootstock with S1 root (R1/S1) indicates a differential response, highlighting that the effectiveness of treatment in modulating nematicidal bacteria is rootstock-dependent [168]. Conversely, the increase in *Nitrosotenium*, an ammonia-oxidizing archaeon (AOA), in R1/S1 TR plants is significant. As a key player in the nitrogen cycle, the enhanced presence of *Nitrosotenium* suggests improved nitrification processes within the rhizosphere, leading to increased nitrogen availability for plant uptake. This microbial enrichment likely contributes to enhanced plant growth, as nitrogen is a critical macronutrient [169]. This positive trend, consistently observed in TR plants and further supported by Figures 5B and 6B, indicates that the treatment positively influences nitrogen cycling, potentially leading to more efficient nutrient utilization by the

plant [170]. The *Rhizobium* abundance exhibits a complex and inverse trend between Rootstock + S1 and Rootstock + S2 in TR plants. *Rhizobium* is a genus known for its symbiotic relationship with plants, primarily through nitrogen fixation and broader roles in plant growth promotion and yield enhancement. The decrease in *Rhizobium* in Rootstock S1 TR plants, despite its known benefits, while increasing in Rootstock S2 TR plants, suggests distinct rootstock-microbe interactions or varying responses to the applied treatment. This dichotomy could be attributed to differences in root exudates, nutrient demands, or competitive microbial dynamics unique to each rootstock type under the given treatment conditions [171]. Among the bacterial genera correlated with morphometric traits, *Rhodococcus* deserves attention. It is widely recognized for its beneficial roles, including pollutant degradation [172] and functioning as a biocontrol agent against various plant pathogens [173, 174]. The negative correlation between *Rhodococcus* abundance and RW in TR samples is particularly interesting. In a healthy plant environment where pathogens are under control due to beneficial microbes like *Rhodococcus*, the plant may not need to invest heavily in developing large root systems for nutrient absorption or defense. Thus, a lower root weight could be a marker of an efficient and healthy plant. The negative correlation between *Rhodococcus* and Y in NT samples further supports its role as a key player in plant health. In addition, the positive correlation between *Flavobacterium*, *Pseudoxanthomonas*, and *Achromobacter* abundance and RW in S2 TR samples strongly suggests a cooperative and beneficial relationship within the treated rhizosphere. *Flavobacterium* is known for promoting plant growth, degrading complex organic matter, and facilitating nutrient uptake, critical for robust root development [175]. *Achromobacter* is versatile in biodegradation and nitrogen cycling, with certain species acting as plant-growth-promoting bacteria (PGPB), enhancing nutrient availability and root growth [176]. *Pseudoxanthomonas* contributes to the microbial community's function in a healthy rhizosphere. Its positive correlation with RW, alongside other beneficial genera, suggests it is part of a healthy and diverse microbial community promoted by treatment.

Collectively, these results underscore the intricate and often rootstock-specific effects of treatments on the plant microbiome. The modulation of key microbial players like *S. nematocida*, *Nitrosotenium*, and *Rhizobium* suggests that targeted interventions can influence critical ecosystem functions such as pest control and nutrient cycling. Future research should focus on clarifying the precise molecular mechanisms underlying these rootstock-specific microbial shifts and their direct

correlation with plant physiological outcomes, which is crucial for developing tailored microbiome engineering strategies in agriculture [177].

Earlier agricultural microbiome studies primarily focused on how particular management strategies such as organic versus conventional farming [178, 179] or tillage practices [180] influence soil microbial communities and rarely incorporate host genotypic effects and their interaction with agricultural practices. Using tomato as a grafting model, this study provides a new perspective on the effects of host genotype on microbial communities [181]. The results for microbial diversity and composition suggest that grafting with specific rootstocks influences microbiome assembly. This study demonstrates that rootstock genotype plays a significant role in structuring the tomato rhizobiome, influencing both microbial diversity and community composition in a compartment-specific manner. Previous findings suggested that interspecific rootstocks (crosses of wild tomato relatives or other *Solanum* species), specifically bred for disease resistance, genetically differ from the tomato scion (*Solanum lycopersicum L.*), leading to observed differences in microbial communities between grafted and non-grafted treatments. Genotype-specific traits—including root architecture and exudation profiles—modulate the assembly of associated microbial taxa [182].

These observations are in line with our findings where the interspecific rootstock OptiFort was associated with an increased number of differentially abundant ASVs. Furthermore, the diverse response of specific grafting combinations with microorganism amendment on microbiome profiling highlights the strong genetic effect on the efficiency and response to exogenous amendment [183, 184]. The role of genotype in the effectiveness of microbial consortia was previously highlighted in [185]. In our study, we underlined the effect of scion and rootstocks independently and their combination on microbial amendment efficiency.

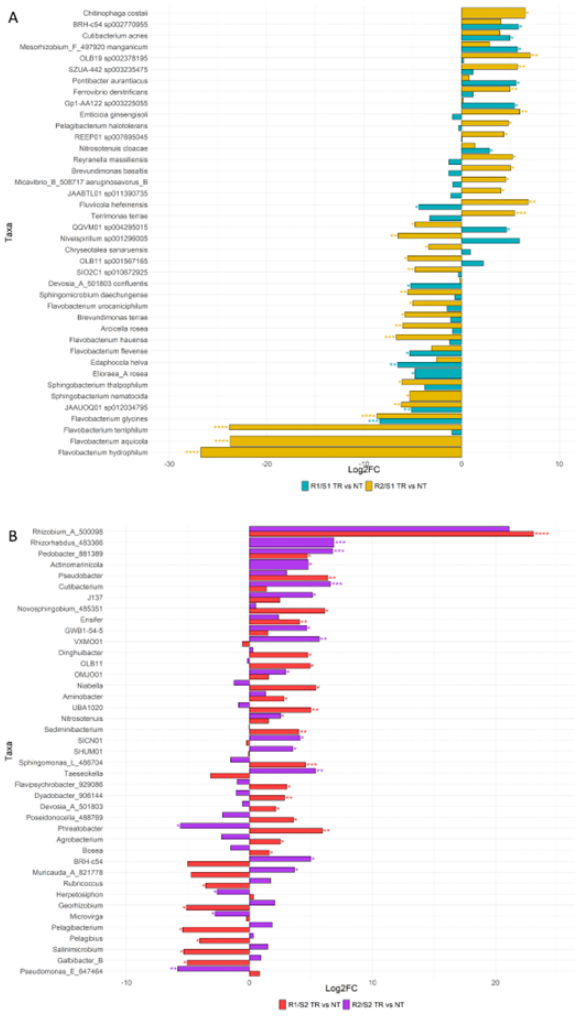

Figure 2.6: Differential abundance in bacteria. (A) Species between R1/S1 TR vs NT (blue bar) and R2/S1 T vs NT (yellow bar). (B) Genera between R1/S2 TR vs NT (red bar) and R2/S2 T vs NT (violet bar). Positive $\log_2$FC values indicate an increase in condition one compared with condition two, while negative values indicate a decrease in condition one compared with condition two.

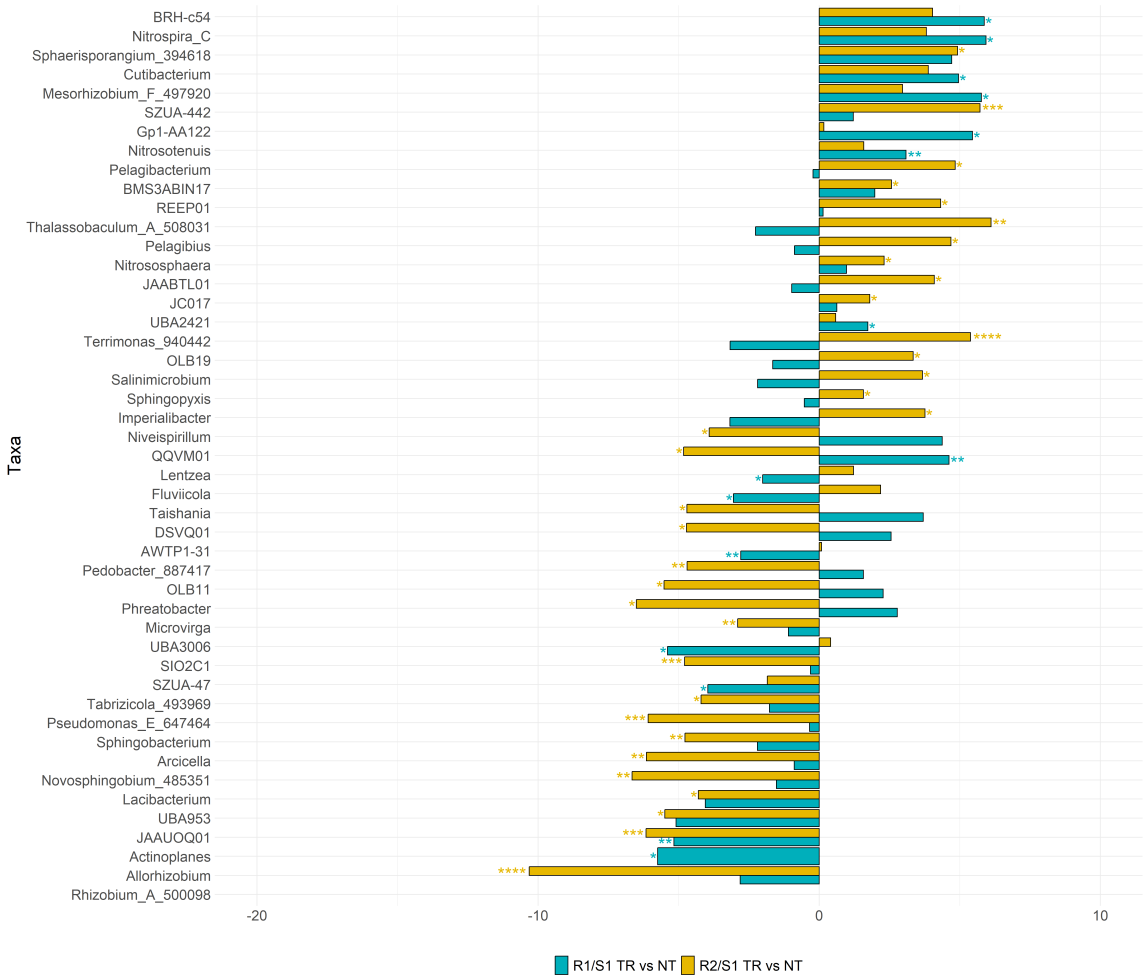

Figure 2.7: Differential abundance of *Rhizobium A 500098*. Rootstock S1 TR plants compared to NT show an opposite trend in relative abundance when contrasted with Rootstock S2 TR plants versus NT. Positive $\log_2$FC values indicate enrichment in TR compared with NT, whereas negative values indicate reduction in TR compared with NT.

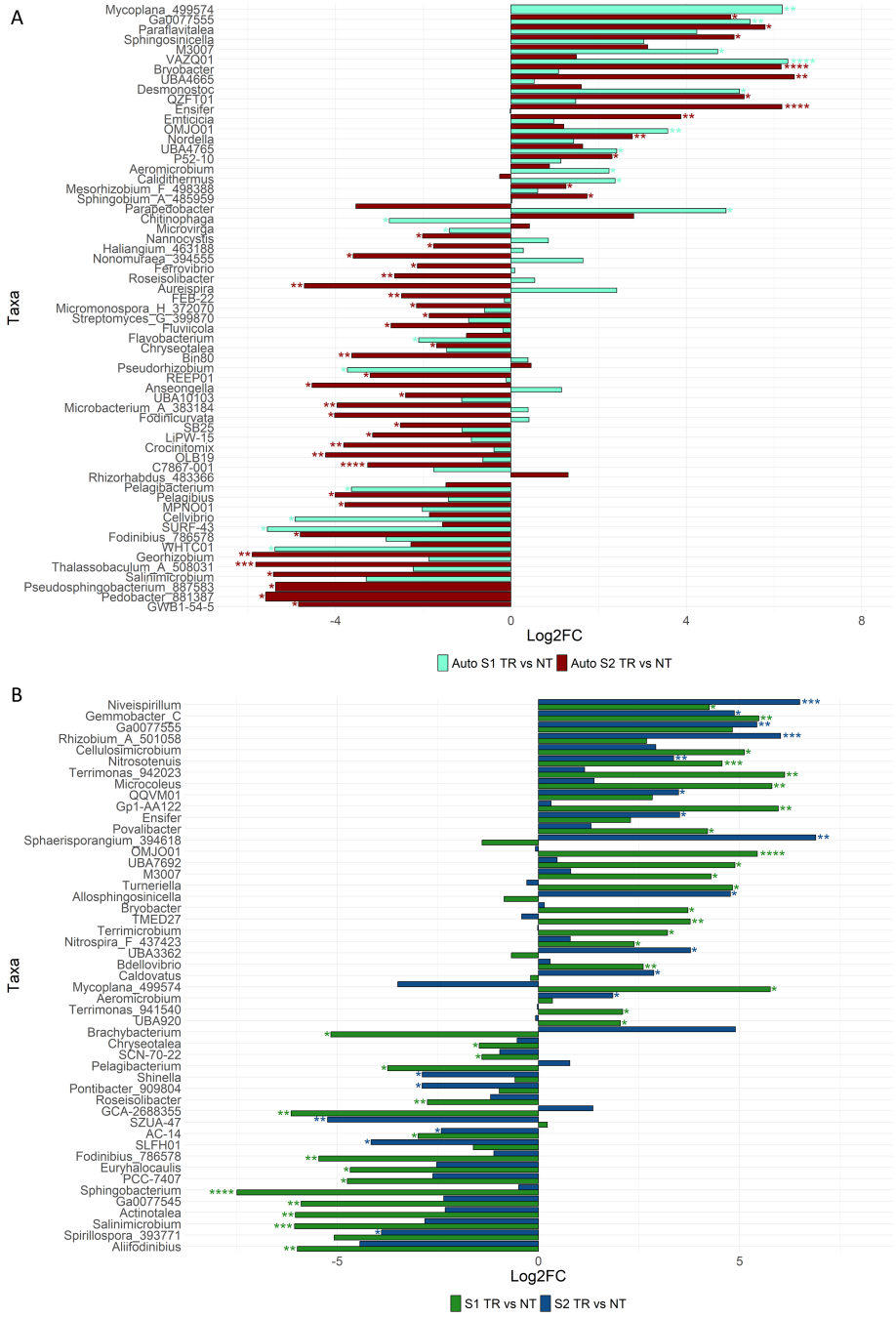

Figure 2.8: Genere differential abundance in bacteria. Positive log_2FC values suggest an increase in condition one compared with condition two, while negative values suggest a decrease in condition one compared with condition two.

Conclusions

The results obtained in this study, together with those reported in recent works [186, 117], underscore the importance of investigating microbial communities to better understand plant–soil interactions under diverse management practices. Both studies demonstrate that changes in microbial diversity, at the level of fungal ITS regions or bacterial taxa, are closely associated with plant health and response to treatments. This suggests that the careful monitoring and manipulation of soil microbiomes can serve as a valuable tool to enhance crop resilience and productivity [187].

Modern agriculture faces unprecedented challenges. The global population is continuously increasing [188], natural resources are increasingly limited, and climate change is imposing extreme conditions on food production [189]. These factors demand the adoption of innovative strategies to ensure food security while maintaining environmental sustainability. Advanced technologies such as high-throughput sequencing, precision agriculture, and the application of biostimulants or microbial inoculants provide practical approaches to optimize crop yield and reduce the ecological footprint of farming [67, 190].

In this context, studies like the present one contribute both to fundamental scientific knowledge and to the development of actionable strategies for modern agriculture. By integrating microbiome analyses with agronomic practices, it is possible to design more resilient cropping systems that are better equipped to cope with biotic and abiotic stresses. The ongoing combination of molecular techniques and sustainable farming practices is therefore essential to address the dual challenges of increasing food demand and environmental preservation [191, 192].

Transcriptomic analysis stands as a cornerstone in unraveling plant stress re-

sponses, offering a pivotal avenue for advancing stress-tolerant crop development—an imperative for sustainable agriculture amidst global environmental shifts [85, 87]. The vast genetic diversity inherent in the *B. oleracea* complex species ($n = 9$) provided a rich resource for identifying genotypes pivotal in elucidating genes and pathways crucial for drought tolerance in *B. oleracea* crops.

By employing two transcriptomic methodologies—reference and *de novo*—we delved into the intricate realm of drought stress in *B. oleracea* crops, garnering nuanced insights and broadening our understanding of genes pivotal to water stress [88, 89, 90]. In particular, the scarcity of robust annotations for *B. oleracea* in the reference analysis underscored the challenge posed by limited functional annotations, profoundly impacting our analysis. Conversely, the *de novo* approach unveiled an extensive gene repertoire, shedding light on crucial metabolic pathways pivotal in stress adaptation, particularly in plant hormone signal transduction mechanisms [96, 105]. Here, the roles of auxin and abscisic acid emerged as central in stress perception and adaptation.

A noteworthy discovery surfaced in the identification of a potential candidate gene for water stress tolerance—transcription factor bHLH112—significantly associated with drought tolerance [100]. The adoption of a dual reference/*de novo* transcriptome assembly approach yielded a more comprehensive dataset compared to a singular approach, enabling an unbiased interpretation of outcomes.

Lastly, leveraging the *ex situ* conserved genetic diversity by the Di3A of the University of Catania in investigating drought stress responses across various *B. oleracea* complex species ($n = 9$) proved instrumental. This approach not only facilitated the exploration of latent traits but also unearthed forgotten alleles, shedding light on the domestication processes of *B. oleracea* crops and guiding future endeavors in crop improvement [86].

The study regarding tomatoes highlighted the effect of grafting and microorganism application, as two agricultural practices, on the rhizobiome and their potential for microbial community modification. Despite the limited or undetectable presence of the inoculated fungi in treated samples, the observed shifts and modifications within the overall microbiome strongly suggest their influence. These indirect changes in microbial community structure indicate that the treatment, even without persistent colonization by the inoculated fungi, elicited significant ecological responses within the plant-associated microbial environment.

In addition, the interaction between grafting combinations and the efficacy of

microorganism amendment highlighted the genotype effect on microbial consortia activity. Future work should aim to resolve the functional roles of unclassified species and clarify the role of environmental conditions on exogenous rootstocks, particularly under biotic stress conditions, to inform the rational design of microbial consortia for enhanced plant performance and resilience [193].

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