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# *Pre-breeding of Brassica oleracea and Responses to Organic Agriculture Needs*

*Ph.D. Thesis*

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## ACKNOWLEDGMENT

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***"Perseverance fuels hard work, turning obstacles into stepping stones on the path to success."***

***Hajer BEN AMMAR***

# Index

# Abstract

*Brassica oleracea*, a fundamental crop in global agriculture, boasts an astonishing array of biodiversity and plays a pivotal role in human nutrition, thanks to its rich glucosinolate content. However, the looming challenge of water stress, exacerbated by the specter of global warming, poses a threat to both crop growth and the accumulation of these valuable compounds. Innovative breeding strategies have become imperative to bolster water stress tolerance while safeguarding glucosinolate levels, thereby contributing to the creation of climate-resilient agriculture.

Our research delves deeply into the hidden genetic diversity concealed within the Brassica oleracea germplasm. This serves as the bedrock for developing resilient varieties that can adapt to our changing climate. Leveraging SSR (Simple Sequence Repeat) markers, renowned for their dependable polymorphic nature, we meticulously dissect the genetic landscape of this invaluable crop. Through cutting-edge Qiaxl techniques, our genetic analysis reveals distinct genotypes and provides profound insights into the intricate tapestry of population structure and genetic relationships among the diverse accessions. Additionally, we conducted comprehensive biochemical analyses via HPLC and carried out transcriptomic analyses to delve even deeper.

Drought tolerance, an urgent concern in the face of climate change, takes the spotlight in our investigation. Our goal is to identify potential drought-tolerant genotypes within the *Brassica oleracea* species suitable for organic cultivation. Through rigorous experimentation, we subjected 20 *Brassica oleracea* accessions to varying water treatments, ranging from optimal irrigation to severe water stress conditions. A wide range of agronomic and biochemical parameters is encompassed by comprehensive assessments, providing insight into the multifaceted responses to drought stress. Significant variations in genotypic and treatment responses are unveiled, underscoring the critical role of antioxidant compounds as stress biomarkers. Furthermore, in response to the escalating global challenge of drought, research hones in on a curated selection of Brassica accessions, including *Brassica oleracea* and its complex species (n=9). Under conditions of water deficit, GLSs compounds within both root and leaf tissues are meticulously examined. This investigation encompasses plant morphometric traits, GLSs profiles, and environmental stressors. Findings reveal significant qualitative and quantitative differences in GLSs across various Brassica accessions, with a specific focus on highlighting the differences between leaf and root tissues. Significantly, a substantial rise in the accumulation of GLSs in reaction to water stress is discovered, especially in specific accessions like broccoli and cauliflower

In the relentless pursuit of genotypes abundant in antioxidant compounds and distinctive GLS profiles, this research enhances the efficacy of breeding programs. By leveraging the potential of various chemotypes and their singular GLS profiles, a strategic path is delineated to expedite breeding programs, facilitating the cultivation of resilient Brassica oleracea varieties capable of flourishing under water stress conditions, all the while safeguarding their nutritional and bioactive traits.



**Key words:** *Brassica oleracea*- Biodiversity- SSR markers- Water Stress- Glucosinolates – Antioxidant compounds- Pre-breeding.

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# Abbreviation list

**DNA:** Deoxyribonucleic Acid
**RNA:** Ribonucleic Acid
**AsA:** Ascorbic Acid
**CAEs:** The cis-acting element
**CTAB:** Cetyltrimethylammonium Bromide
**CWR:** Crop Wild Relatives
**Ct:** Cycle threshold
**DNase:** Deoxyribonuclease
**ETc :** Evapotranspiration
**GAE:** Gallic Acid Equivalence
**GLSs:** Glucosinolates
**GSH:** reduced **G**lutathione
**GSSG:** Oxidized Glutathione
**GAE:** Gallic Acide Equivalence
**HPLC:** High Pressure Liquid Chromatography
**MAS:** Marker-assisted Selection
**MeOH**: Methanole
**MGL:** Multilocus genotypes
**QTL:** Quantitative Trait Loci
**ROS:** Reactive Oxygen Species
**rpm:** Rotation per minute
**RT-PCR**: Reverse Transcription Polymerase Chain Reaction
**SNPs:** Single Nucleotide Polymorphisms
**TPC:** Total Polyphenols Content

# General Introduction

# Introduction &Purposes

Global warming, caused by human activities, was a highly debated topic two decades ago but has now become an undeniable reality, with its global impacts evident since the early 20th century (Mahalingam. 2015). This phenomenon is primarily characterized by a continuous increase in carbon dioxide (CO2) emissions and global temperatures (Olivier et al., 2012). Rising global temperatures contribute to increased evapotranspiration, which exacerbates drought conditions and leads to soil salinization (Zhao and Running. 2010). In response to these environmental changes, plants have evolved specific mechanisms to detect and adapt to stressors, minimize damage, and optimize resource allocation for growth (Atkinson et al., 2013). Understanding the intricacies of plant responses to environmental stress is crucial for targeted varietal improvement. Unlike animals, plants are immobile and must withstand various stressors such as drought, salinity, and extreme temperatures. These stressors significantly affect plant distribution, growth, development, and ultimately crop productivity. Recent advances in our understanding of the molecular mechanisms underlying plant responses to abiotic stresses have shed light on their multifaceted nature, involving processes such as sensing, signaling, transcription, transcript processing, translation, and post-translational modifications of proteins.

Historically, unintentional plant selection and subsequent crop domestication coupled with the need and desire to obtain more food and feed products have resulted in the continuous development of plant breeding and genetic efforts. Plants are susceptible to a range of environmental challenges during growth and development in both natural and agricultural environments. Biotic and abiotic stresses pose severe risks to the sustainability of plant production and global food security under current environmental changes (Luo et al.,2022). Drought stress is an abiotic stress that is gaining attention because it has a negative impact on plant growth and development and significantly reduces plant biomass and production, thereby contributing to global food insecurity. *B. oleracea* crops and related wild species (n=9) have received special attention among leafy vegetables because of their high phytochemical content, which includes high levels of vitamins, minerals, dietary fiber, glucosinolates (GLS), and phenolic compounds. The leaves are characterized by a typical taste owing to the presence of a wide array of sulfur compounds and are one of the ingredients of the Mediterranean diet. The easy detection of numerous secondary metabolites makes them an optimal model trait for investigating complex quantitative genetics and the pressures that maintain this variation.

Cultivation under stressful conditions can promote the production of bioactive molecules associated with the antioxidant system and plant defense mechanisms. The abundance of bioactive compounds can differ significantly among species and genotypes, as they exhibit distinct responses to stress conditions. Investigating the physiological and biochemical adaptations related to drought resistance in plants can serve as valuable criteria for selecting and developing drought-tolerant cultivars. Plant breeding aims to create novel varieties by combining specific traits determined by breeders to meet the demands of both the breeders and consumers. The process of genetic enhancement commences with the introduction of genetic diversity, and the subsequent selection of superior genotypes occurs within this diverse pool. To facilitate early and efficient selection of desired traits, molecular markers can be employed to identify and isolate genetic material carrying the target gene.

Overall, this study aims to contribute to the development of sustainable agriculture by selecting elite *Brassica oleracea* materials with improved resistance to water stress. By incorporating the principles of genetics and plant physiology, we strive to enhance the adaptation and resilience of *Brassica oleracea* crops to water-limited environments, ensuring their viability and productivity in the face of changing climatic conditions.

**Chapter I. Morphological and genetic diversity of *Brassica oleracea* L. complex species (n=9) core collection:** In this chapter, our main objectives are threefold: First, we aimed to assess the morphological diversity within the core collection of *Brassica oleracea* complex species, carefully observing and meticulously documenting variations in observable traits. Second, we will employ state-of-the-art molecular techniques to evaluate the genetic diversity among accessions, shedding light on the underlying genetic relationships and patterns. These insights will be invaluable for targeted trait mapping and breeding programs, with the ultimate aim of developing improved *Brassica oleracea* cultivars that boast resilience, high yield, and enhanced nutritional content. By achieving these objectives, we hope to advance our understanding of *Brassica oleracea* and contribute to the sustainable improvement of agriculture and conservation efforts.

**Chapter II. Biochemical responses involved in water stress tolerance:** By conducting a comprehensive evaluation, we aim to gain crucial insights into the plant's adaptability and resilience under water-limited conditions. Understanding the adaptability of *Brassica oleracea* accessions to water deficiency can pave the way for the development of more resilient and drought-tolerant crop varieties. By identifying specific biochemical markers associated with water stress tolerance, we can pinpoint genotypes that exhibit exceptional performance under

limited water availability. These superior accessions can then serve as valuable genetic resources for breeding programs, promoting the development of water-efficient and stress-resistant cultivars These findings will contribute to the development of sustainable agricultural practices that promote organic cultivation and enhance the health-promoting characteristics of Brassica oleracea crops, while minimizing their reliance on pesticides and reducing water consumption.

**Chapter III. Evaluation of *Brassica oleracea* Based on Agronomic Trait: Glucosinolates:** In this chapter, we will conduct a comprehensive evaluation of *Brassica oleracea*, focusing specifically on GLSs, a group of secondary metabolites with significant health-promoting properties. By studying both roots and leaves, we aim to gain valuable insights into the GLSs profiles and quantities present across different plant tissues. Our investigation will involve subjecting *Brassica oleracea* plants to controlled water stress conditions, simulating the impact of abiotic stress on GLSs accumulation. Through this approach, we seek to deepen our understanding of the plant's adaptive responses to water scarcity, shedding light on how these compounds function as part of the plant's defense mechanisms against environmental challenges. By delving into the intricate relationship between *Brassica oleracea* and GLSs under water stress, this chapter seeks to advance our knowledge of plant stress responses, contributing to the development of drought-tolerant and nutrient-rich crop varieties. These efforts align with the overarching goal of sustainable agriculture and bolstering global food security in the face of an ever-changing climate.

# Bibliography

### 1. *Brassica oleracea* L crops and complex species (n=9)

*B. oleracea* L. (2n = 2 = 18) is a species of flowering plants in the family Brassicaceae, commonly known as the cabbage family. It is a highly diverse group of crops that includes several popular and widely cultivated vegetables. The species is known for its remarkable phenotypic variation, with different varieties exhibiting distinct morphological traits. *Brassica oleracea* crops are considered complex species due to their genetic complexity and the extensive range of cultivated forms. This plant species stands out as an exceptional example of structural evolution among cultivated plants (Babula et al., 2007). Landraces and synthetic varieties clearly differ from hybrid types in terms of important agronomic traits. Various morpho-physiological characteristics such as harvest time, head size, color, and leaf count have been observed to differentiate landraces and synthetics in previous studies (Branca et al., 2008). The taxonomy of *Brassica oleracea* is as follows:

**Kingdom**: Plantae

**Subkingdom**: Tracheobionta

**Subdivison**: Spermatophyta

**Division**: Magnoliophyta

**Class**: Magnoliopsida

**Order**: Capparales

**Family**: Brassicaceae

**Genus**: Brassica L.

**species**: *Brassica oleracea* L.

Phenotypic variations observed in different cultivated forms of *B. oleracea* are a result of underlying genetic factors. Genetic studies have provided insights into the involvement of key regulatory genes that control important traits such as leaf morphology, inflorescence development, and GLS biosynthesis. These genes play a crucial role in shaping the diverse phenotypes observed within the cultivated forms of *B. oleracea* (Guo et al., 2021).

In addition to genetic variations, cultivated *B. oleracea* crops have also adapted to diverse environmental conditions. They exhibit adaptability to variations in temperature, photoperiod, and soil types. This adaptability is a result of local adaptation and selection, which have played a significant role in shaping the crop's response to different agroecological niches. Varieties

have been selected for specific temperature tolerances, photoperiod requirements, and adaptations to different soil types, allowing them to thrive under varying environmental conditions (Lema et al., 2019).

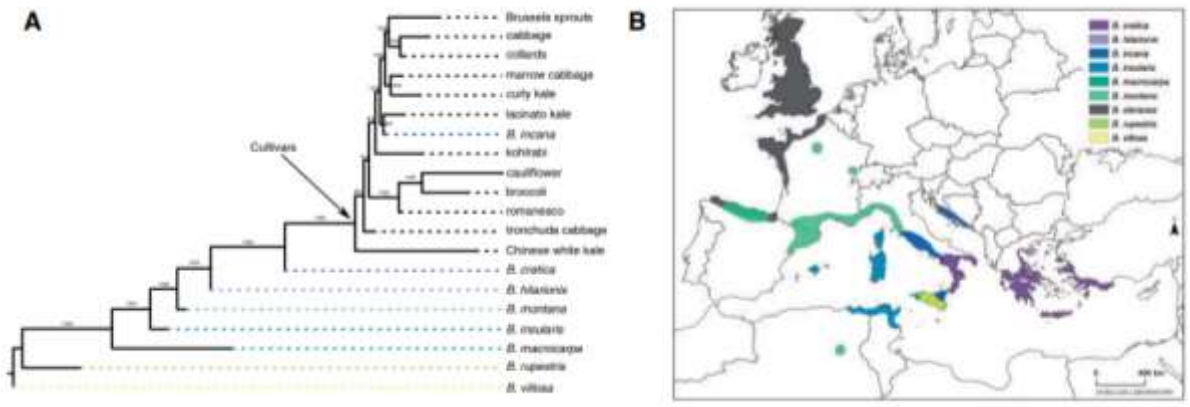



**Figure 1.** (A) Species tree of wild and cultivar samples (B) current species distribution of wild relatives (by Mabry et al.,2021).

### 1.1 Domestication and Diversification

The domestication and diversification of *Brassica oleracea* provide a fascinating example of human-driven selection and genetic adaptation. Through intentional breeding and selection, a diverse array of cultivars with distinct morphotypes and traits have been developed. The Cole crops (*B. oleracea*) are native to Europe and the Mediterranean and consist of a diverse array of domesticated and wild varieties (Allender et al., 2007) (Figure 1-B). Each crop type possesses distinct domestication traits that are not commonly found in their wild counterparts (Maggioni et al., 2015). These traits include dwarfism with compressed internodes in the main stem (cabbages), elongated main stems with highly compressed lateral branches (Brussels sprouts), proliferation of floral meristems (broccoli), proliferation of aborted floral meristems (cauliflower), swollen stems (kohlrabi, Marrow-stem kale), and ornate leaf patterns (kales) (Lan and Paterson. 2000) (Figure 2). Defining the specific domestication traits shared by all these crop types, as distinct from the wild species, is not a straightforward task. During the early stages of domestication, selection pressures likely favored traits such as reduced bitterness, decreased fibrousness, thicker stems, and more succulent storage organs (Maggioni et al., 2010). Continuous selection over generations resulted in the development of distinct morphotypes.

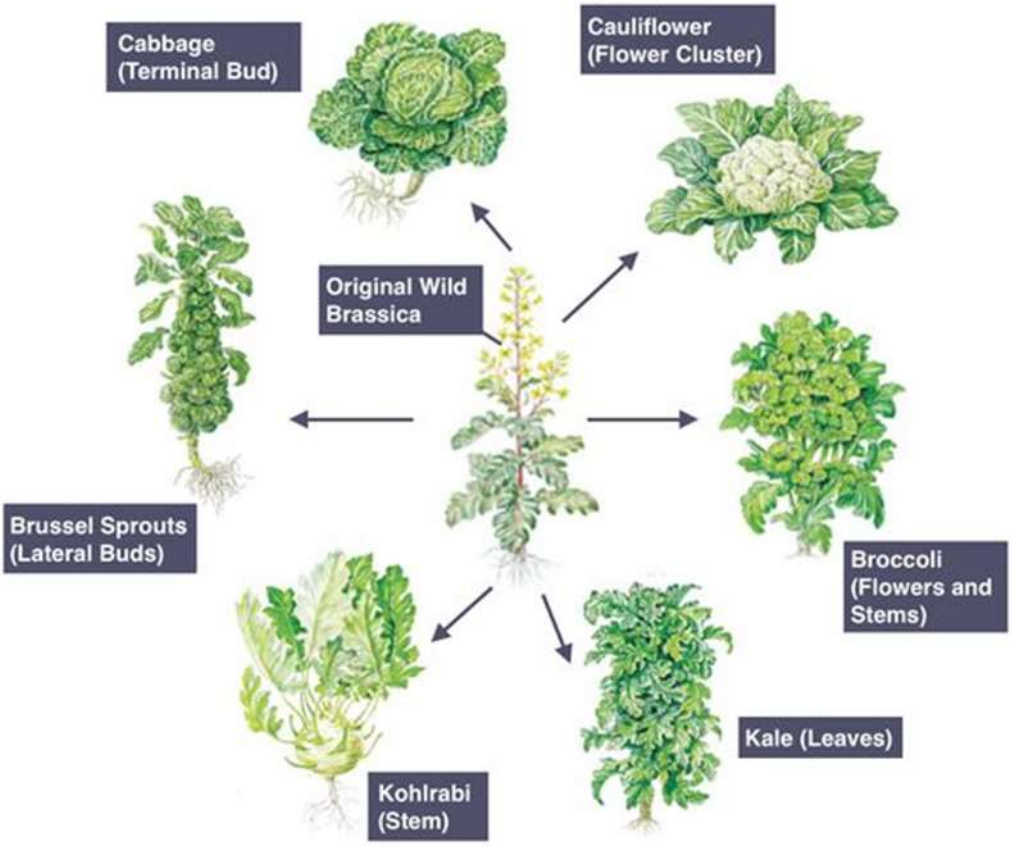



**Figure 2.** Evaluation of wild mustard through domestication

The domestication and diversification of *Brassica oleracea* provide a fascinating example of human-driven selection and genetic adaptation. Through intentional breeding and selection, a diverse array of cultivars with distinct morphotypes and traits have been developed (Glémin et al., 2009). The genus Brassica encompasses a wide range of agricultural crops, with two major species, *B. oleracea* and *B. rapa*, playing a significant role in this diversity (Figure 3). *B. oleracea* exhibits the highest genetic and phenotypic variability in Europe (Branca et al.,2012), while *B. rapa* finds its primary area of diversification in Asia. These two species are responsible for many agricultural crops that are distributed worldwide. *B. oleracea* includes a variety of horticultural and forage forms, making it one of the most widely distributed species globally (Mittell et al.,2020). Examples of crops within this species are kale, cabbage, broccoli, Brussels sprouts, and cauliflower, among others (Stansell et al., 2020). These vegetables are staples in many diets and play a crucial role in global agriculture. On the other hand, *B. rapa* comprises horticultural forms such as turnips, Chinese cabbage, and pak choi, along with some forage and oilseed crops. These crops are essential for various culinary purposes and as sources of oil and forage. *B. napus* is another species within the Brassica genus, primarily known for varieties used in oil production from its seeds, such as rapeseed. However, it also includes other leafy

and forage horticultural crops like rutabaga and kohlrabi, respectively. Lastly, the mustard group consists of *B. carinata*, *B. nigra*, and *B. juncea*, primarily cultivated for their seeds used as condiments. *B. juncea*'s leaves and heads are also consumed for horticultural purposes, particularly in Asian countries (Cartea et al., 2010).

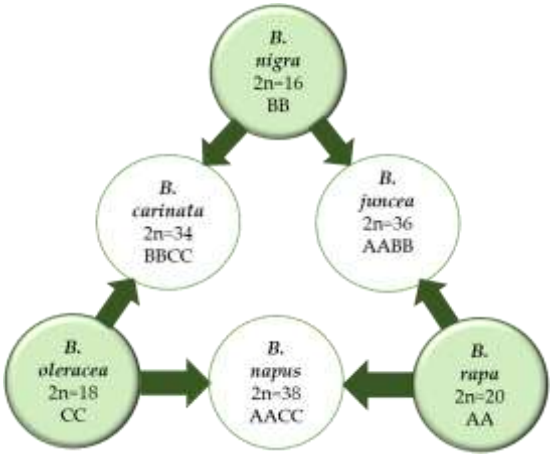



**Figure 3.** The Systematic triangle of cross-breeding among economically important six Brassica species comprising three diploids (green) and three allotetraploids (white).

Understanding the domestication and diversification processes of *B. oleracea* has significant implications for plant breeding and crop improvement. The knowledge gained can be utilized to develop improved cultivars with enhanced nutritional value, disease resistance, and adaptability to changing environmental conditions (Meyer et al., 2013).

### 1.2 Plant Description

*Brassica oleracea* is a species of flowering plant in the family Brassicaceae. It is a biennial or perennial herbaceous plant that is primarily grown as an annual crop for its edible parts. *B. oleracea* is known for its remarkable morphological diversity, with several distinct cultivated forms or varieties. Each variety of *Brassica oleracea* has undergone selective breeding to emphasize specific traits, such as leaf shape, flower structure, and the development of specific edible parts. This breeding has resulted in the wide range of cultivated forms we see today. For example, kale is known for its curly or flat leaves, while broccoli is recognized for its large flower heads.

The morphological diversity within *Brassica oleracea* not only offers a variety of culinary options but also provides an opportunity for plant breeders to continue developing new varieties with desired traits. This diversity and adaptability have contributed to the widespread cultivation and popularity of *Brassica oleracea* vegetables in various cuisines worldwide (Han et al.,2021).

- **Leaves**

The leaves of *Brassica oleracea* are large, lobed, and usually arranged in a rosette pattern at the base of the plant. The shape and texture of the leaves can vary depending on the variety. For example, kale has curly or flat leaves, while cabbage has smooth, round or elongated leaves.

- **Flowers**

Flowers in the genus Brassica are hypogynous, mostly actinomorphic. Sepals 4, in 2 decussate pairs and free. Petals 4, alternate with sepals, arranged in the form of a cross. Stamens 6, in 2 whorls, tetradynamous (lateral (outer) pair shorter than median(inner) 2 pairs). There are four nectar glands which are median and lateral. Anthers are dithecal, dehiscing by longitudinal slits. Pollen grains 3-colpate, trinucleate. Nectar glands receptacle and dispose around base of filaments, always present opposite bases of lateral filaments, median glands present or absent. Pistil 2-carpelled; ovary superior, sessile or borne on a distinct gynophore, mostly 2-locular and with a false septum connecting 2 placentae (Erbar and Leins, 1997). The flowering stage is the most temperature-sensitive phase of development.

- **Seeds**

The seeds are 1.5 to 3 mm in length and 1.3 to 2.8 mm in width, with color ranging from bronze, brown, or grayish-black to reddish. Surface characteristics include a fine, narrow reticulum that sometimes displays a waxy appearance. The interspaces formed by the reticulum are very small in comparison to other Brassica species, with very small stipples that are more often visible in the interspaces than on the reticulum

### 1.4 Development and Growth Staging of *Brassica oleracea*

*Brassica oleracea,* is a biennial plant that undergoes various developmental and growth stages throughout its life cycle. The stages can be broadly categorized as vegetative growth, reproductive growth, and senescence (Leijten et al., 2018). Here is a general overview of the development and growth staging of *Brassica oleracea:*

**Seed Germination:** The life cycle of *Brassica oleracea* begins with the germination of seeds. Under favorable conditions of moisture and temperature, the seeds sprout, and a primary root emerges, followed by the development of cotyledons and true leaves Finch-Savage & Bassel, 2016).

**Vegetative Growth:** During the vegetative growth stage, the plant focuses on leaf and root development. The stem elongates, and more leaves are produced in a rosette arrangement. The plant establishes a strong root system to support its growth.

**Bolting:** Bolting is a critical transition stage in the life cycle of *Brassica oleracea*. It is characterized by the elongation of the stem and the initiation of reproductive structures. The plant undergoes a shift from vegetative growth to reproductive growth (Seepaul et al.,2021).

**Flowering and Reproduction:** At this stage, the stem continues to elongate, and flower buds form at the apical meristem. The buds develop into flowers, which consist of petals, sepals, stamens, and pistils. Pollination occurs, leading to fertilization and the formation of seeds (Alvarez-Buylla et al.,2010).

**Seed Maturation:** After successful fertilization, the flowers wither, and the seeds start to develop within seed pods. The pods gradually mature and turn brown, indicating the readiness of the seeds for dispersal.

**Senescence:** Senescence refers to the natural aging and deterioration of the plant. The leaves and other plant parts start to wither and turn yellow or brown. This stage marks the end of the life cycle of *Brassica oleracea*.

It is important to note that the exact timing and duration of each growth stage can vary depending on environmental factors, cultivar, and growing conditions (Nelson et al.,2022). The growth and development of *Brassica oleracea* can also be influenced by cultural practices, such as pruning, fertilization, and pest management (Valenzuela, 2023)

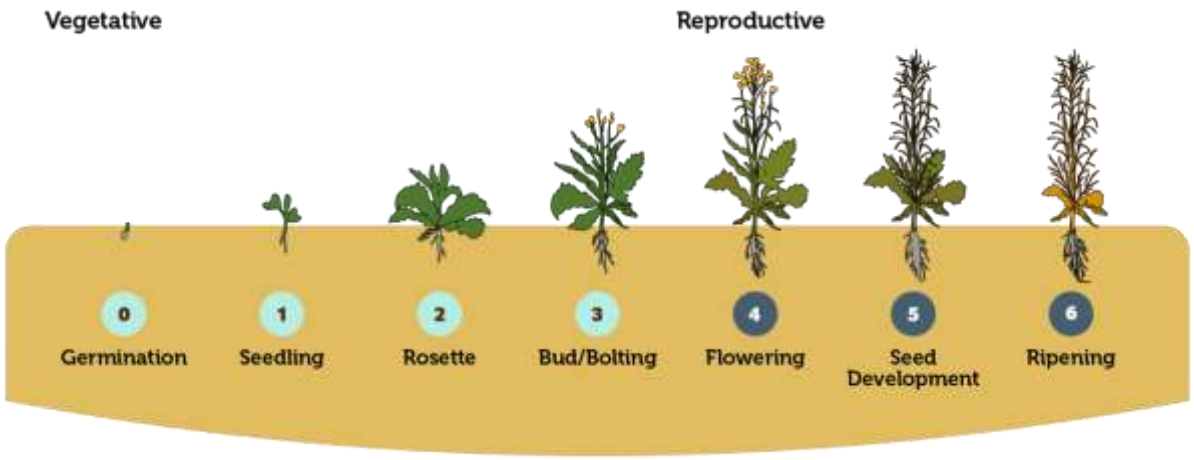



**Figure 2.** Development and Growth Staging of *Brassica oleracea*

During the vegetative growth stage, breeders may focus on traits such as leaf shape, size, color, and overall plant architecture. This stage is important for the development of a strong and healthy plant structure. Reproductive growth is a critical phase for breeders, as it involves flower formation, pollination, and seed set. Selection during this stage may target traits related to flower size, fertility, self-pollination ability, and seed quality (Poethig. 2013).

### 1.3 Agronomic and economic importance of Brassica

*Brassica oleracea* is a highly significant crop species that plays a crucial role in both agronomy and economics. It possesses various traits that contribute to its economic importance, such as adaptability to diverse climatic conditions and soil types, high yields, and resistance to pests and diseases. The nutritional value and distinct taste of *Brassica oleracea* make it a popular and staple food worldwide (Abiya et al., 2022).

The global market for *Brassica oleracea* vegetables, including cabbage, cauliflower, and broccoli, is expanding rapidly due to the increasing demand for healthy foods and convenience products. These vegetables are cultivated in numerous countries, and their production areas and volumes are substantial. For instance, cabbage has the largest harvested area and production, followed by cauliflowers and broccoli (Han et al.,2021).

One of the notable features of *Brassica oleracea* is its ability to thrive in areas unsuitable for other crops, making it valuable for crop diversification. Additionally, it exhibits relatively high yields per hectare, making it economically significant for growers. Its resistance to pests and diseases reduces the reliance on pesticides and contributes to a more sustainable agricultural system (Greer et al., 2023). *Brassica oleracea* also plays a crucial role in crop rotation systems, breaking the life cycle of soil-borne pests and diseases and promoting soil health. Moreover, it has applications in the food processing industry, where it is used to produce various products such as sauerkraut, kimchi, frozen vegetables, and vegetable purees (Carmody.2017).

The growing interest in healthy foods and plant-based diets has further propelled the demand for *Brassica oleracea*. The cultivation of novel forms, including sprouts, baby leaf, and microgreens, has gained popularity due to their convenience, versatility, and high nutritional value. These new variations offer consumers innovative ways to incorporate *B.oleracea* into their diets (Ebert. 2022).

Geneticists have also found *Brassica oleracea* to be an excellent model for plant genetics studies due to its remarkable morphological diversity. This diversity has sparked interest in

genetic improvement programs and the exploration of traditional and new phenotypes for use in food processing and transformation processes (Singh et al., 2023).

These distinctive traits likely developed over time through adaptation to local environmental conditions and selection by farmers who favored particular characteristics such as maturity date, shape of the flowering cluster, or flavor. Detailed characterization of these varieties is valuable for promoting products derived from local variations, a practice already underway for numerous crops (Santos et al., 2020). Additionally, utilizing the diversity present in local populations is crucial for breeding programs. Developing new varieties from these local sources can help preserve their genetic diversity in agricultural fields. Recent advancements in our understanding of the *B. oleracea* crop group include deciphering the fundamental genomic architecture (Cheng et al., 2016), generating high-quality reference genomes (Belser et al., 2018), investigating diversity and domestication processes (Lazaro et al., 1998; King et al., 2003; Mabry et al., 2021), and identifying genomic regions or candidate genes associated with horticultural quality as well as resistance to biotic and abiotic stresses (Stansell et al., 2018).

Overall, *Brassica oleracea* holds significant agronomic and economic importance, driven by its adaptability, high yields, nutritional value, versatility, and growing market demand. Its cultivation contributes to sustainable agriculture, food security, and economic growth in various regions around the world.

### 1.4 Biochemical Composition

Plants produce diverse metabolites to cope with the challenges presented by complex and ever-changing environments. Brassica vegetables have garnered significant attention due to their abundance of phytochemicals, which are known for their beneficial functions in the human body, including disease risk reduction. These crops serve as important sources of fiber, vitamins, and minerals (Borges et al., 2018). Notably, they contain a variety of anti-carcinogenic and antioxidant compounds, such as Vitamin C, phenolic acids, flavanols, anthocyanidins, carotenoids, and amino acids.  research focus has primarily centered around secondary metabolites, particularly GLSs, in Brassica (Neugart et al., 2018). Exploring the intricate polyphenolic profiles in different cultivars will open doors for future advancements in breeding strategies and targeted selection of Brassica varieties, thereby enhancing their nutritional value and functional properties (Di Bella et al., 2020).

### *1.4.1 Polyphenols and their health benefits*

Brassica vegetables, known for their diverse array of phytochemicals, offer a rich and varied composition of bioactive compounds. These vegetables are particularly notable for their polyphenol content, which has been extensively studied. The polyphenols in Brassica vegetables exhibit a wide range of structures and classes, including flavonols, flavones, flavan-3-ols, anthocyanidins, flavanones, isoflavones, phenolic acids, hydroxycinnamates, stilbenes, and others (Ayadi et al., 2022). One of the key groups of polyphenols found in Brassica vegetables is flavonoids, comprising flavonols, kaempferol, quercetin, and isorhamnetin. These compounds are commonly present as O-glycosides, conjugated to sugars and organic acids. Additionally, Brassica vegetables contain a diverse range of non-flavonoid polyphenols, such as phenolic acids and hydroxycinnamates (Favela el al., 2020). Interestingly, the composition of polyphenols can vary significantly not only between different species of Brassica but also among cultivars within the same species. This variation highlights the influence of genetic factors and environmental conditions on the biosynthesis and accumulation of polyphenols in these vegetables. It is noteworthy that certain cultivars of Brassica vegetables, like violet cauliflower, kale, and kohlrabi, have gained popularity due to their anthocyanin content, which imparts vibrant red and purple hues (Mageney et al., 2017). To differentiate between cultivars, researchers have focused on analyzing anthocyanin concentrations as potential markers. For instance, red and pink radish cultivars exhibit higher levels of pelargonidin and delphinidin derivatives, while purple cultivars show a prevalence of cyanidin derivatives (Hanlon and Barnes. 2011). This differentiation further emphasizes the intricate diversity of polyphenols within Brassica vegetables

### *1.4.2 Carotenoids and their role as antioxidants*

Carotenoids play a pivotal role in the nutritional composition of Brassica oleracea. The Brassica genus, which encompasses B. oleracea, is renowned for its wide array of carotenoid pigments, which contribute to the vibrant colors observed in various cultivars. Carotenoids not only enhance visual appeal but also offer significant health benefits owing to their antioxidant properties and provitamin A activities (Eroglu et al., 2022). Numerous studies have explored the carotenoid content and composition in diverse B. oleracea cultivars. For instance, a study conducted by Bang et al. (2007) investigated the carotenoid profiles of different B. oleracea vegetables, including broccoli, cabbage, and kale. Their findings revealed that lutein and β-carotene were the predominant carotenoids in these vegetables. Similarly, Rosa et al. (2012) analyzed the carotenoid content of various B. oleracea cultivars and documented variations in

the levels of β-carotene, lutein, and zeaxanthin among them. Furthermore, the impact of environmental factors and agricultural practices on carotenoid accumulation in *B. oleracea* has also been investigated. A study by Kopsell and Kopsell (2006) evaluated the effects of light intensity and nitrogen fertilization on the carotenoid content of broccoli. They found that increasing light intensity and nitrogen levels led to enhanced accumulation of carotenoids, including β-carotene and lutein. Overall, the carotenoid content and composition in *Brassica oleracea* vary among cultivars and can be influenced by environmental factors and cultivation practices (Baek et al.,2016).

### *1.4.3 Vitamins and their nutritional significance*

*Brassica oleracea* is widely recognized as a valuable source of vitamins, making it a beneficial addition to a healthy and balanced diet. These vegetables are renowned for their rich vitamin content, which contributes to their overall nutritional value. Among the vitamins found in *Brassica oleracea,* vitamin C (ascorbic acid) is particularly notable (Nath et al.,2015). Vitamin C is an essential water-soluble vitamin known for its antioxidant properties. It helps neutralize free radicals, reduces oxidative stress, supports immune function, and plays a role in the regeneration of other antioxidants, such as vitamin E (Traber et al.,2011). Vitamins C is accounting for 10-12% of the total antioxidant capacity in broccoli and cabbage (Domínguez-Perles et al.,2014). Studies have also shown that the content of vitamin C in broccoli sprouts can be influenced by factors such as light/dark cycles, with higher levels observed when grown under a 16/8-hour light/dark cycle (Pérez-Balibrea et al.,2008). The vitamin content in cruciferous vegetables, including *Brassica oleracea*, can vary depending on factors such as cultivar, growing conditions, and cooking methods. The stage of maturity is an important factor that influences the macronutrient, vitamin, and mineral content in cruciferous vegetables (Šamec et al.,2011). Additionally, abiotic stresses like salinity in irrigation water can affect the vitamin C content in edible parts of Brassicas, such as broccoli, leading to a decrease in vitamin C levels proportional to the extent of water deficiency or hydric stress (Toscano et al., 2019).

### *1.4.4 Glucosinolates*

The GLSs are among the metabolic compounds that have frequently been used as chemical markers in chemotaxonomy. Their distribution in Brassica is complex and varies across species and within crops from the same species (Velasco et *al*.,2007). GLSs are sulfur-based molecules generated during secondary metabolism of which nearly 200 types having different substituents have been identified. Variations in the GLSs content between and within

the same species due to different biosynthetic pathways led to the theory that this content is modulated by both genetic and environmental factors (Chhajed et *al*.,2020).

#### 1.4.4.1 The biological effect of the Glucosinolates

In addition to their role in the plant's defensive system, GSLs are potentially involved in the survival mechanism of the Brassicaceae family. GSLs have long been suggested to serve as sulfur storage components, alongside their roles as chemical defenses, owing to their unique structure, which includes a sulfate group and a thioglucosidic linkage, and may contain additional sulfur in the variable side chain (Blažević et al.,2020). The biological activity of GSLs is primarily attributed to the products of their hydrolysis (Malhotra and Bisht. 2020), which can break down into various compounds with distinct biological activities. Young leaves and reproductive tissues, such as siliques and seeds, often contain the highest concentrations of GSLs (Touw et al.,2020). However, the quantity of GSLs decreases in mature leaves. The established role of the GLSs in plant defense against herbivorous insects is well-documented. Following tissue damage, myrosinase activity catalyzes the hydrolysis of GLSs, producing reactive compounds effective against insects and pathogens (Czerniawski et al., 2021). It has been observed that moderate salt stress can lead to increased levels of GLSs in Brassica crops. This finding suggests that GLSs may serve as an adaptive component of salt tolerance in these plants, especially under conditions of low water potential. Salt stress has the potential to disrupt the water balance within plant cells, resulting in osmotic stress and possible dehydration (Martínez-Ballesta et al.,2015). In response to salt stress, plants activate various adaptive mechanisms to mitigate its detrimental effects. One of these mechanisms involves the accumulation of compatible solutes, which act as osmo-protectants, helping to maintain cellular water balance and protect against osmotic stress (EL Sabagh et al.,2019). The observed increase in GLS levels under salt stress suggests that these compounds may play a role in salt tolerance and adaptation in Brassica crops. They could contribute to osmotic adjustment and cellular protection by serving as osmo-protectants and antioxidants (Guo et al., 2014). GLSs may play a pivotal role in maintaining cell integrity, protecting against oxidative damage, and regulating ion balance in the presence of high salt concentrations. Additionally, GLSs and their breakdown products have been associated with plant defense against pests and pathogens (Buxdorf et al., 2013). Salt stress can weaken plants' defense mechanisms, making them more susceptible to various stresses, including biotic stressors. The upregulation of GLSs under salt stress may offer a dual benefit by enhancing both salt tolerance and defense against potential herbivores and pathogens.

### 1.4.4.2 Structure and Diversity

The GLSs are a type of β-thioglucosides-N-hydroxysulfates, characterized by their chemical structure consisting of a glucose molecule linked to a sulfur-containing group (Figure5). The specific side chain varies depending on the amino acid precursor from which they are derived. Based on their amino acid precursors, it can be classified into three main classes: aliphatic, indole, and aromatic (Cavaiuolo et al., 2014). The aliphatic class is derived from amino acids such as methionine, leucine, valine, and alanine. These compounds have side chains composed of straight or branched carbon chains and are commonly found in cruciferous vegetables like broccoli, cabbage, and kale.  indole class is derived from the amino acid tryptophan (Kitaindac and Jez. 2021). GLSs in this class have indole-derived side chains and are commonly found in Brussels sprouts and cauliflower. The aromatic class of GLSs is derived from the amino acids phenylalanine and tyrosine, it contains aromatic side chains and are present in watercress and rocket (Nguyen et al.,2020).

By classifying the GLSs into these three classes based on their amino acid precursors, we can understand the structural diversity and variations in these compounds. This structural diversity contributes to the wide range of biological activities and potential health benefits associated with GLSs and their breakdown products (Sikorska and Beneduce. 2021).

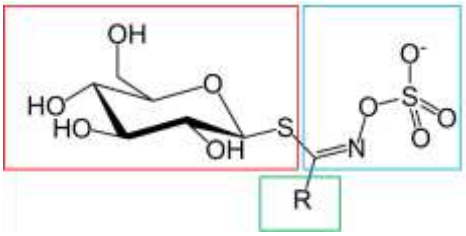



**Figure 3.** General structure of a GSL molecule.

The sulfonated oxime group is marked in blue, the β-thioglucose group in red, and the amino acid-derived side chain in green. GSLs can be classified according to which amino acid the side chain is derived from.

The aliphatic and indole clades act antagonistically toward each other, exerting a type of reciprocal negative control to maintain homeostasis either in the form of repression when one type of GLS accumulates, or as compensation when the accumulation of one decrease (Textor and Gershenzon. 2009). The enzyme SUR1 is shared by the aliphatic and indole pathways. This sharing generates the possibility that, if there is considerable competition among the two groups precursors for access to SUR1, changing the concentration of enzymes in the aliphatic pathway could impact the flux through the indole one (Mitreiter et al.,2021). The biosynthesis pathway

of GLS, consists of three fundamental phases as described in figure 4. These phases are essential for the synthesis and accumulation of GLSs. The steps of biosynthesis are described as follows:

**Primary Metabolite Formation:** The first phase involves the formation of primary metabolites that serve as precursors for GLSs synthesis. These primary metabolites include amino acids, such as methionine and phenylalanine, and sugars, such as glucose. These precursor molecules are derived from various metabolic pathways within the plant cell (Kitainda & Jez, 2021).

**Core Structure Formation:** In the second phase, the core structure of GLSs is formed through a series of enzymatic reactions. This process involves the conversion of precursor molecules into intermediates, which undergo subsequent modifications. Key enzymatic steps in this phase include chain elongation, amino acid side chain modification, and sulfur incorporation. These reactions are catalyzed by specific enzymes, such as methyltransferases, cytochrome P450 monooxygenases, and sulfotransferases (Radojčić et al.,2008).

**Side Chain Elaboration**: The final phase of the biosynthesis is the side chain elaboration, where the final modifications and diversification of GLSs structures occur. This phase involves the introduction of different functional groups, such as hydroxyl, methyl, and acetyl groups, into the side chain of GLSs. These modifications are catalyzed by specific enzymes, including side chain modifying enzymes (Agerbirk and Olsen. 2012).

Overall, these three phases of GLS biosynthesis are tightly regulated and coordinated to ensure the production of a diverse array of compounds in response to various environmental cues and developmental stages (Gigolashvili et al., 2009).

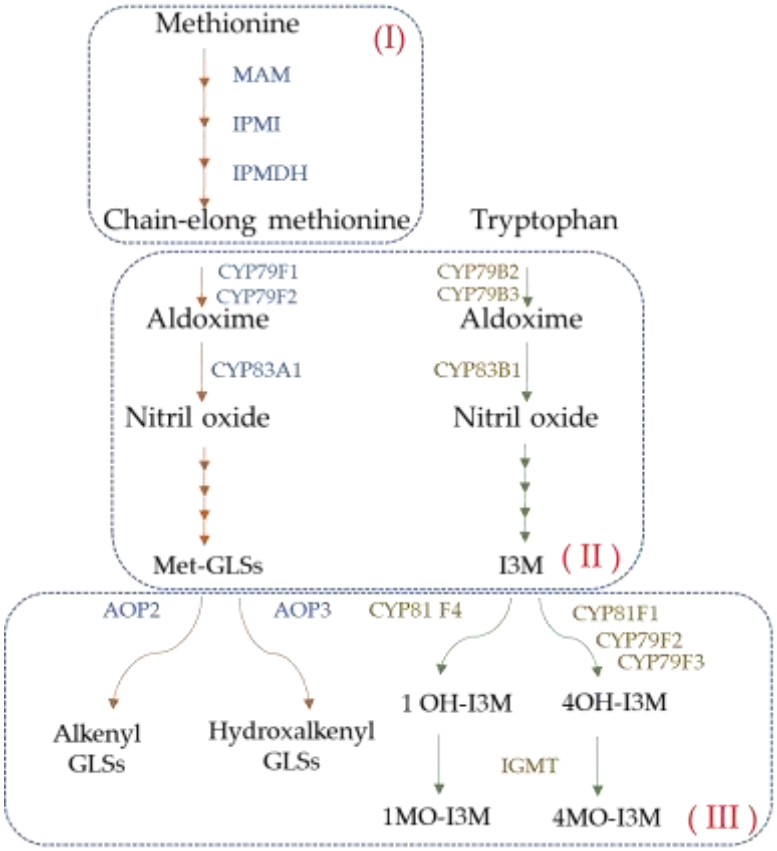



**Figure 4. Scheme for the biosynthesis of GSLs generated by methionine and tryptophan.**

Each line indicates an enzyme reaction, with red arrows representing the pathway generated from aliphatic methionine and green ones signifying the pathway obtained from indolic tryptophan. Some of the unidentified enzymes are shared by the two pathways, while a few intermediates, enzymes, or enzyme families are given names. The three steps of GSL synthesis are indicated by numbers: (I), elongation of the amino acid chain, (II) ,creation of the core structure, and (III), secondary side-chain changes.

The predominant aliphatic glucosinolates in kale are reported to be sinigrin, glucoiberin, and glucoraphanin (Table 1). For the glucobrassicin content was comparatively analyzed in different Brassica vegetables, kale contained significantly lower amounts than broccoli and Brussels sprouts (Charron et al .,2005).

**Table 1. Classification of Glucosinolates by Groups**

| | Type | Name | Abbreviation | Lateral systematics |
|---|---|---|---|---|
| C3 | Aliphatic | Glucoputranjivin | GJV | 1-Methylethyl |
| | | Sinigrin | SIN | 2-Propenyl |
| | | Glucoiberverin | | 3-Methylthiopropyl |
| | | Glucoiberin | | 3-Methylsulfinylpropyl |

| | | | | |
|---|---|---|---|---|
| C4 | Aliphatic | Gluconapin | GNA | 3-Butenyl |
| | | Glucoerucin | GER | 4-Methylthiobutyl |
| | | Dehydroerucin | | 4-Methylthio-3-butenyl |
| | | Progoitrine | PRO | (2R)-2-Hydroxy-3-bytenyl |
| | | Glucoraphanin | GRA | 4-Methylsulfinulbutyl |
| | | Glucoraphenin | GRE | 4-Methulsulfinyl-3-butenyl |
| C5 | Aliphatic | Glucobrassicanapin | GBN | Pent-4-enyl |
| | | Glucoberteroin | | 5-Methylthiopentyl |
| | | Glucoalyssin | GAL | 5-Methylsulfinylpentyl |
| | | Gluconapoleiferin | GNL | 2-Hydroxy-pent-4-enyl |
| | Indole | Glucobrassicin | GBS | 3-Indolylmethyl |
| | | 4-Hydroxyglucobrassicin | 4-OHGBS | 4-Hydroxy-3-indolylmethyl |
| | | 4-Methoxyglucobrassicin | 4-OMeGBS | 4-Methoxy-3-indolylmethyl |
| | | Neoglucobrassicin | Neo-GBS | N-Methoxy-3-indolylmethyl |
| | Aromatic | Gluconasturtiin | GST | 2-Phenylethyl |

The regulation of Glucosinolates metabolism at different levels and the diverse physiological function of their hydrolytic products indicate a complex metabolic network.

#### 1.4.4.3 Biosynthetic Genes Controlling Glucosinolates Variability

It is evident that distinct species within the same genus and even different cultivars of the same species exhibit significant variations in GLS concentrations, as highlighted by Pan et al. (2023). This variability offers a compelling avenue for research, offering insights into the genetic underpinnings of GLS diversity and its potential manipulation in the realms of plant breeding and biotechnology. A fundamental aspect to grasp is the intricate network of biosynthetic genes governing GLS production. This complex process involves a cascade of enzymatic reactions, each orchestrated by specific genes and enzymes, a fact well-supported by the work of Sønderby et al. (2010).

There have been attempts to explore the genetic system controlling GLSs synthesis in Brassicaceae plants, mostly using syntenic data with the model plant *Arabidopsis thaliana*. Following the evolutionary divergence between Brassica and Arabidopsis, genome duplications and chromosomal rearrangements contributed to the presence of more genes in Brassica species than in Arabidopsis at each stage of GLS biosynthesis (Qin et al.,2023). However, Brassica has a greater number of homologous genes associated with GLS biosynthesis than those identified

in Arabidopsis. It is crucial to determine the extent of epistatic interactions between loci which may play an important role in determining variability for GLS content (Wang et al., 2011).

The plant ‘s genetic background is the major factor determining GLS amount and profile. Different species of the same genus and different cultivars of the same species have highly variable GLS concentrations (Pan et al., 2023). Understanding the biosynthetic genes involved in controlling GLS variability can provide insights into the genetic basis of their diversity and potential for manipulation in plant breeding and biotechnology. The biosynthetic pathway of GLS involves a series of enzymatic reactions, with each step catalyzed by specific genes and enzymes (Sønderby et al.,2010).

The identification of the genomic data underlying this GLS diversity may offer an opportunity to identify potential phytochemical and nutraceutical sources that might be used to Brassica crops. Additionally, knowledge of the connection between Brassicaceae GLS genes and abiotic stress tolerance is useful in breeding to get crops able to resist the effects of global climate change (Essoh et al., 2020). The GLS biosynthetic pathway depends on upstream genes essentially involved in initial elongation and side-chain modification reactions, Additionally, the molecular activity of genes shifts according to the species of plant, accession, allelic condition, and diversity of the regulatory network that controls it while genes involved in the synthesis of aliphatic and indole GLS, apparently specific to Brassicaceae, may depend on two set of gene clusters, known to be important for aliphatic and indole specific pathway ,CYP97 F1-F2 and CYP81F1-F4, respectively (Bischoff. 2021).

A pivotal player in this intricate genetic network is the locus GLS-ELONG, which governs the chain length variation of aliphatic GLSs. Remarkably, this locus comprises two distinct genes, MAM1 and MAM2, responsible for orchestrating the side chain extension reaction of aliphatic GLS originating from methionine. Notably, MAM1 and MAM2 are never found together within the same accession, a genetic divergence that highlights the role of gene duplication and neo-functionalization in the diversification of GLS profiles within the Brassicaceae family (Kroymann et al., 2001).

Another crucial discovery in the realm of GLS modulation is the identification of flavin monooxygenase FMOGS-OX1, mapped to the GS-OX locus. This enzyme plays a pivotal role in catalyzing the conversion of methylthioalkyl glucosinolates to methylsulfinylalkyl GLSs. This finding, rooted in both biochemical knowledge and transcriptome co-regulation database

analysis in Arabidopsis, sheds light on the biochemical mechanisms governing GLS transformations.

The QTL GS-AOP has emerged as another significant genetic locus within the complex network of GLS variability. GSL-AOP collectively refers to two closely linked loci, GS-ALK and GS-OHP, with fine-scale mapping revealing the presence of three AOP genes localized within these loci—AOP1, AOP2, and AOP3. Interestingly, AOP2 is localized within the GS-ALK locus, while AOP3 resides within the GS-OHP locus. Both of these loci stem from the ancestral gene AOP1 through gene duplication events. They encode a 2-oxoglutarate-dependent dioxygenase responsible for the conversion of methylsulfinylalkyl glucosinolate to methylalkenyl glucosinolate and hydroxylalkyl glucosinolate, respectively (Augustine et al., 2017). This genetic framework elucidates the intricate processes underpinning GLS variation. Within the realm of gene regulation, MYB and bHLH transcription factors (TFs) emerge as key players. These TFs serve as pivotal regulators that integrate diverse regulatory signals through their mutual interactions and binding to gene promoters. MYB28, MYB29, and MYB76 have been identified as positive regulators responsible for the production of aliphatic GLS with varying chain lengths. These factors reciprocally transactivate each other, as demonstrated by Møldrup et al. (2012), further underscoring the complexity of GLS regulation at the transcriptional level.

There is, however, a lack of understanding at the molecular level on the functional aspects such as signaling transduction pathways, control at transcriptional, translational, and post-translational levels, subcellular compartmentation, and interaction with many other metabolic pathways. According to the study of Sotelo et al 2014, the epistatic interactions of indolic GSLs were clearer than those of aliphatic GSLs. 49% of the detected epistatic interactions were between QTLs, indicating that variability in GSL content is determined directly by QTLs and indirectly by interacting with other loci.

#### 1.4.4.4 Epigenetic Regulation of Glucosinolates Biosynthesis

Beyond the classical transcriptional control mediated by cis- and trans-elements, the epigenetic code adds another level to the regulatory machinery of plants. Epigenetic regulation plays a crucial role in the control of GLS biosynthesis in plants (Huang et al.,2022a).

**DNA Methylation:** DNA methylation, the addition of a methyl group to DNA molecules, is an important epigenetic modification involved in the regulation of GLS biosynthesis. DNA methylation patterns can directly affect the expression of genes involved in GLS synthesis.

Changes in DNA methylation status, such as DNA demethylation or hypermethylation, can influence the transcriptional activity of these genes (Zhang et al.,2023a).

**Histone Modifications:** Histone proteins, which form the core of nucleosomes, can undergo various post-translational modifications, including acetylation, methylation, phosphorylation, and ubiquitination. These modifications can alter chromatin structure and gene accessibility (Bowman & Poirier. 2014). Specific histone modifications have been associated with the regulation of GLS biosynthetic genes, indicating their involvement in the epigenetic control of GLS synthesis.

**Chromatin Remodeling:** Chromatin remodeling refers to the dynamic changes in chromatin structure that influence gene expression. The remodeling of chromatin architecture can be mediated by ATP-dependent chromatin remodeling complexes, which can either promote or inhibit the accessibility of transcription factors to GLS biosynthetic genes. This process plays a critical role in the regulation of GLS synthesis.

**miRNAs**: Small RNA molecules, including microRNAs (miRNAs) and small interfering RNAs (siRNAs), are involved in post-transcriptional gene regulation. Several studies have implicated miRNAs in the control of GLS biosynthesis by targeting specific mRNAs encoding enzymes involved in GLS metabolism. The binding of small RNAs to their target mRNAs can lead to mRNA degradation or translational repression (Kumar et al., 2017).

**Environmental Influence:** Environmental factors, including biotic and abiotic stresses, can induce epigenetic changes that modulate GLS biosynthesis. Stress-responsive signaling pathways can trigger alterations in DNA methylation patterns and histone modifications, thereby influencing the expression of genes involved in GLS synthesis. This epigenetic plasticity allows plants to dynamically respond to changing environmental conditions (Akhter et al., 2021).

#### 1.4.4.5 Physiological Regulation of Glucosinolates Biosynthesis

The levels of GLSs in Brassicaceae vary widely from species to species and are mostly affected by genetic and environmental factors (such as light circumstances, circadian rhythm, temperature, salt, fertilization, hormones, and drought). The regulation of GSL metabolism at different levels and the diverse physiological function of their metabolites indicate a complex metabolic network (Del Carmen et al., 2013).

In addition to this modulation, plant hormones such as jasmonates, SA, and ET associated with specific and broad-spectrum defense responses can also affect GLS content. Jasmonates known to be involved in responses to insect attack and necrotrophic pathogens have shown increased indolyl and specific aliphatic GLSs (Brader et al., 2001; Mikkelsen and Halkier, 2003), possibly via multiple signaling pathways (Kliebenstein et al., 2002). Studies have demonstrated that wounds, pathogens, and hormones like JA and ET induced the expression of transcriptional factor genes (Schenk et al., 2000) and that nuclear proteins regulate GLSs metabolism (Yan and Chen, 2007).

It is interesting to note that the expression levels of many broccoli Glucosinolate related genes were expressed higher in sprouts than in seeds. Some previous studies had indicated the GLS concentration decreased exponentially after germination (Vanegas Torres & Rodov, 2022). The contrast between the lower levels of GLS and the increased presence of genes associated with biosynthesis might be attributed to the extensive consumption of GLS. This degradation process likely played a vital role during the germination and sprout development stages of broccoli seeds (Gao et al.,2014).

#### 1.4.4.6 The impact of abiotic stress on Glucosinolates biosynthesis

Abiotic stress, such as drought, salinity, high temperature, and nutrient deficiency, can significantly impact GLS biosynthesis in plants. Abiotic stress may increase the delivery of g GLSs from the vacuole to the cytosol in leaf cells or enhance the activity of myrosinase or its substrate affinity, in such a way that the hydrolysis products of the GLS (isothiocyanates) could lead to the inhibition of K+ in channels, to avoid water loss (Nicolas-Espinosa et al., 2023). In roots, it must be considered that the GLSs can be released into the rhizosphere and, due to their low volatility (Sarwar et al. 1998) and strong hydrophobicity (Laegdsmand et al., 2007), they could be adsorbed onto the periderm cells in the rhizosphere (Martínez-Ballesta et al.,2014). Here are some key impacts of abiotic stress on GLSs biosynthesis:

**Altered GLS Content**: Abiotic stress can lead to changes in the total GLS content of plants. In some cases, stress can result an increase in GLS accumulation, while in others, it may lead to a decrease. The response depends on the specific stress type, duration, and intensity, as well as the plant species and genotype **(**Zandalinas et al.,2022).

**Shifts in GLS Profile**: Abiotic stress can also affect the profile of GLSs in plants, altering the relative proportions of different compounds. This shift in profile may result from changes in the expression and activity of genes involved in GLS biosynthesis (Buckleyet al., 2019).

**Modulation of Biosynthetic Genes**: Abiotic stress can influence the expression of genes involved in GLS biosynthesis. Several studies have shown that the expression levels of key biosynthetic genes, such as MYB transcription factors and enzymes involved in side-chain elongation and modification, are regulated by abiotic stress signals (Tang et al.,2023).

**Induction of Stress-Responsive Pathways**: Abiotic stress can activate various stress-responsive signaling pathways in plants, such as the abscisic acid (ABA) signaling pathway (Tuteja, 2007). These pathways can intersect with GLS biosynthesis, leading to the upregulation of specific biosynthetic genes and subsequent changes in GLSs content.

**Crosstalk with Phytohormones:** Abiotic stress can also influence the interplay between GLSs biosynthesis and phytohormones. For example, jasmonic acid (JA), a phytohormone involved in plant defense, has been shown to regulate GLS biosynthesis in response to abiotic stress (Wang et al.,2020).

Understanding the impact of abiotic stress on GLS biosynthesis is important for crop improvement and breeding strategies (Table 2). By elucidating the underlying molecular mechanisms, researchers can develop stress-tolerant varieties with desirable GLS profiles, enhancing both plant resilience and the nutritional quality of crops (Hirayama & Shinozaki, 2010).

**Table 2**. Impact of Common Abiotic Stress Conditions on Glucosinolate Levels in *Brassica oleracea*

| Abiotic stress conditions | Plant cultivar | Glucosinolates content | References |
|---|---|---|---|
| **Drought** | | | |
| Severe stress two weeks | *Brassica oleracea* L. var. *capitata* | Increase | Radovich et *al*., 2005 |
| Severe stress two weeks | *Brassica oleracea* L. var. *italica* | Increase | Champolivier and Merrien 1996 |
| Mild stress (30% of available water) | *Brassica oleracea* L. var. *gemmifera* | No effect | Gutbrodt et al., 2012 |
| **Saline stress** | | | |
| NaCl (40, 80 mM), during two weeks | *Brassica oleracea* L. var. *italica* | Increase | López-Berenguer et *al*., 2008 |
| **Temperature** | | | |
| Elevated temperature (32 °C) | *Brassica oleracea* L. | Increase | Charron et *al.*, 2004, 2005 |
| **Light cycling** | | | |

| | | | |
|---|---|---|---|
| 16 h/8 h d/n or continuous darkness | *Brassica oleracea* L. var. *italica* | Increase upon light | Pérez-Balibrea et al.,2008 |
| **Nutrient availability** | | | |
| S-supply (150 kg/ha) | *Brassica oleracea* L. var. *italica* | No effect | Vallejo et *al.,* 2003 |
| S-limitation (15 kg/ha) | *Brassica oleracea* L. var. *italica* | No effect | Vallejo et *al.,* 2003 |
| Se-supply(5.2mM Na2SeO4) | *Brassica oleracea* L. var. *italica* | Increase | Kim et *al.,* 2011 |

### 1.5 Produce quality

Brassica crop production has been in continual expansion worldwide. In the past, agriculture strategy has been assessed on the basis of a narrow range of criteria, such as yields or profitability. Nowadays, increasing awareness among consumers for a constant supply of plants nutrients for getting optimal health benefits has led to demand for quality products with a higher added value. depending on consumer habits of different countries, Brassica vegetables can provide the 50% of the daily recommended dietary intake of vitamin C, leading to the sources of natural vitamin C for human populations (Röös et al.,2018).

One of the most crucial agriculture policy strategies for the revival of the Italian agriculture economy is the promotion and development of regional products, especially in the south where agriculture frequently lacks the economic and technical infrastructure required to compete with more advanced agriculture systems or to withstand competition from countries are producing at discounted rates (Hammer et al., 2018). Organic farming has specific breeding needs and it is essential for providing organic farmers with modern varieties suited to serve the present organic food sector. Organic plant breeding is still a small sector and the varieties used in organic farming are mainly derived from conventional plant breeding (Van Bueren, 2002). There are still considerable gaps in the assortment of suitable cultivars for organic and low-input farming (Lammerts van Bueren et *al.,* 2011). There is a need to better understand the relationship between yield, resilience, and product quality and how to combine these different traits in new cultivars for organic production.

Available genetic diversity still constitutes the foundation of all breeding efforts. Elite varieties, landraces, and crop wild species are important resources of useful variation that can be introgressed, re-introduced, or manipulated to obtain the required biotic and abiotic resilience in Brassica crops. The identification and exploitation of suitable variation are crucial for crop improvement and can be elucidated at the genome scale (Hu et *al*., 2018). Genomics can, in addition, contribute toward unraveling the genetic origin and molecular pathways

involved in biotic and abiotic stress tolerance traits (Paliwal et al., 2023). A complete and accurate understanding of the ancestry of the Brassica species will assist in the tracking and exploitation of the genetic inheritance of useful traits (Bancroft et *al*., 2011).

**2. Abiotic stress**

Plants encounter numerous challenges during their growth phase, which can significantly impact the regulation of biosynthetic pathways responsible for producing bioactive compounds. These challenges encompass both biotic factors, such as pests and diseases, as well as abiotic factors like drought, soil salinity, and extreme temperatures (Sharma et al.,2019).

Among these factors, abiotic stressors play a particularly prominent role in crop loss. Drought, for instance, can lead to water scarcity, depriving plants of essential hydration and impeding their physiological processes. Soil salinity, on the other hand, refers to high levels of salt in the soil, which can adversely affect plant growth and disrupt nutrient uptake. Additionally, extreme temperatures, both cold and hot, can disrupt the delicate balance required for optimal plant development (Kumari et al.,2022).

Abiotic stresses play a significant role in diminishing plant growth and yield, and plants have evolved mechanisms to respond and adapt to these challenging conditions in order to survive. One crucial aspect of the plant's response to abiotic stress is the activation of signaling pathways triggered by environmental cues (Zhang et al.,2023b). When plants encounter abiotic stresses such as drought, extreme temperatures, or salinity, they initiate specific signaling cascades to cope with these adverse conditions. These signaling pathways enable plants to perceive the stress signals and elicit appropriate defense responses. Through these responses, plants strive to mitigate the negative effects of abiotic stress and maintain their productivity (Paes de Melo et al., 2022). In the case of salt stress, which is a major abiotic stress affecting crop productivity worldwide, plants activate specific signaling pathways to combat the detrimental impacts of high soil salinity. Salt stress disrupts the water balance within plant cells, leading to osmotic damage and interfering with various physiological processes (Balasubramaniam et al.,2023).

**2.1 Water stress**

Water scarcity has emerged as a significant impediment to global development, affecting numerous regions and exacerbating socio-economic challenges. The consequences of water scarcity extend beyond its immediate impact on agriculture, encompassing broader aspects of life, from public health to environmental sustainability. Particularly, areas characterized by

semi-arid climates face the dual challenge of water shortage and high-salt concentrations in available irrigation water, impacting agricultural practices and nutritional quality of crops such as Brassica (Seleiman et al., 2021). In arid and semi-arid regions, Brassica plants are predominantly exposed to drought stress, which has profound effects on their physiology, morphology, and molecular structure. Stress-induced disturbances in plant homeostasis can disrupt vital physiological and metabolic processes, reduce energy production, and compromise cellular integrity (Ahluwalia et al., 2021).

### 2.2 Response of plants to water stress

In the world of plant biology, abiotic stress responses stand as a testament to the intricate genetic networks and regulatory pathways that govern the survival and growth of organisms in challenging environmental conditions. Among these stressors, drought is a particularly formidable foe, impacting various aspects of plant life, from growth and productivity to overall quality. However, the response to drought stress is far from uniform, with outcomes varying depending on factors such as phenological stages, cultivar types, and the intensity and severity of the drought (Shabbir et al., 2022). One of the key determinants of how Brassica crops respond to drought stress is the phenological stage at which they encounter it. Seedlings, for instance, may display different reactions compared to mature plants. Understanding these stage-specific responses is critical for optimizing agricultural practices in water-scarce regions. The genetic diversity within Brassica crops introduces another layer of complexity. Different cultivar types may exhibit varying degrees of tolerance to drought stress. Researchers are delving into the genetic makeup of these crops to identify genes associated with drought resilience, opening doors to crop breeding for improved stress tolerance (Manavalan & Nguyen, 2017).

The primary signs of waterlogging stress in leaves are curling, yellowing, wilting, falling off, decaying, etc. When exposed to waterlogging stress, leaves can respond in two different ways: thicken up or thin out. In the former, the increase of palisade tissue and spongy tissue as well as the reduction of leaf and stomata size, reduce water loss and enhance the water-holding capacity of plants. Photosynthesis decreases, which is correlated with reduced growth and a higher incidence of early senescence in plants (Lee et al., 2014).

In the face of abiotic stress, plants are not passive victims; they are dynamic and adaptive organisms (Figure 5). They rapidly adjust their physiological and metabolic responses to limit the stress and its detrimental effects. This remarkable process of adaptation is known as acclimatization (Pan et al., 2021). Understanding the intricate responses of plants to abiotic

stressors like drought and waterlogging is a pressing challenge in modern agriculture. As the world grapples with climate change and growing food demands, the need to decipher these complex interactions becomes even more urgent.

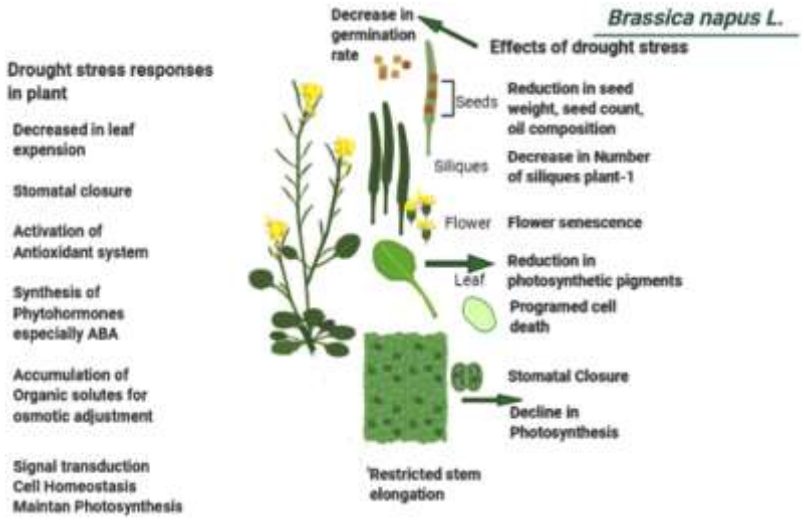



**Figure 5. Drought stress responses in Plant**

### 2.3. Strategies to develop tolerance

In the development of the population, identifying the patterns and mechanisms of adaptation to ecological gradients has been of the highest priority. Since crop production and food security depend on the management of limiting factors, it is necessary to develop efficient strategies that allow for the improvement of crop yield under water-deficit stress (Yu et al.,2020). To adapt to the environment under water stress, plants require an immediate morphological variation. It has been demonstrated that plants have developed a variety of interrelated systems that resist stress, such as osmotic adjustment, osmoprotection, antioxidation, and scavenging defense mechanisms (Yang et al., 2021).

Breeding programs have identified donor lines with tolerance to specific stresses, and these lines are utilized to develop improved varieties that exhibit reduced yield reduction under stress conditions compared to high-yielding varieties (Mahmood et.,2019). Enhancing water productivity potential can be achieved through three main approaches. Firstly, selecting drought-tolerant varieties helps increase crop yield potential while minimizing water consumption (Burridge et al., 2022). Secondly, adopting suitable agronomic practices, irrigation technologies, and management techniques enables higher yields with efficient water use (Farooq et al., 2012). Lastly, developing the capacity to monitor and predict regional water resources, improving water delivery efficiency, and coordinating water allocation at a regional

scale contribute to higher utilization efficiency and reduced water loss (Passioura, 2006). There are several strategies employed to develop tolerance to water stress in *Brassica oleracea:*

**Genetic selection and breeding:** In the quest to bolster plant resilience against water stress, a foundational strategy revolves around the identification and selection of plant lines or varieties with heightened tolerance. This pivotal approach, as outlined by Osakabe et al. (2014), entails a multifaceted process involving the scrutiny of diverse genetic resources to pinpoint traits associated with water stress tolerance. These traits typically encompass reduced water requirements, improved water utilization efficiency, or enhanced water retention capabilities. The initial phase of this undertaking involves the meticulous screening and evaluation of an extensive array of genetic resources. Researchers seek to uncover the hidden gems among plant varieties that exhibit promising traits related to water stress tolerance. Breeding programs focus on developing new varieties with enhanced water stress tolerance by incorporating these desirable traits through crossbreeding or genetic modification (Tuberosa and Salvi. 2006). Crossbreeding, a traditional and widely practiced method, involves mating plants with desirable traits to produce offspring that inherit these characteristics. Through controlled breeding, varieties with improved water stress tolerance can be developed over successive generations. Thus, in the era of biotechnology, genetic modification offers a more targeted approach. Researchers can introduce specific genes associated with water stress tolerance into plant genomes. This precise method allows for the rapid development of varieties with enhanced resilience (Hamdan et al.,2022).

**Physiological and molecular studies:** Understanding the physiological and molecular mechanisms underlying water stress tolerance in *Brassica oleracea* is crucial for targeted improvement. Research efforts aim to uncover the genes, proteins, and metabolic pathways involved in response to water stress (Chevilly et al., 2021). This knowledge can guide breeding strategies and facilitate the identification of genetic markers associated with water stress tolerance, allowing for more efficient selection and breeding processes.

**Agronomic practices:** Implementing appropriate agronomic practices can help mitigate the effects of water stress. This includes optimizing irrigation management techniques, such as deficit irrigation or precision irrigation, to ensure efficient water use and minimize losses. Mulching, crop rotation, and intercropping techniques can also contribute to conserving soil moisture and reducing water stress in *Brassica oleracea* crops (Chai et al., 2016).

**Hormonal and chemical treatments**: Exogenous application of plant hormones, such as abscisic acid (ABA), can enhance water stress tolerance *in Brassica oleracea*. ABA regulates various physiological processes, including stomatal closure and osmotic adjustment, which help plants conserve water and maintain cellular hydration. Additionally, certain chemical treatments, such as osmoprotectants or antioxidants, can be applied to mitigate the negative effects of water stress and improve plant performance (Yoon et al., 2020).

**Molecular breeding techniques:** Advances in molecular breeding techniques, such as marker-assisted selection (MAS) and genomic selection, can expedite the development of water stress-tolerant *Brassica oleracea* varieties (Shaw et al.,2021). These techniques allow for the identification and selection of plants with desired traits at the molecular level, reducing the time and resources required for traditional breeding methods.

## 3.Pre-Breeding Strategies for *Brassica oleracea*

### 3.1 Objectives and importance of pre-breeding

Pre-breeding is an essential step in plant breeding programs, aimed at broadening the genetic base of cultivated crops and improving their traits. The primary objectives of pre-breeding for *Brassica oleracea*, a species that includes vegetables like cabbage, broccoli, cauliflower, and kale, are as follows:

**Genetic Diversity:** Enhancing genetic diversity is crucial to counteract the genetic erosion that can occur due to intensive selection and cultivation of a limited number of varieties. Pre-breeding aims to introduce new genetic material into breeding populations, which can provide novel traits, disease resistance, and resilience to environmental stresses (Fu.2015).

**Trait Introgression:** Pre-breeding involves the transfer of desirable traits from wild or unadapted relatives of Brassica oleracea into cultivated varieties. These traits can include resistance to pests and diseases, tolerance to abiotic stresses (such as drought or salinity), improved nutritional content, or agronomically valuable characteristics (Bohra et al., 2022).

**Germplasm Conservation**: Pre-breeding efforts also contribute to the conservation and preservation of genetic resources by identifying and utilizing diverse germplasm sources, including landraces, wild relatives, and heirloom varieties. This helps maintain the genetic variability necessary for future breeding programs (Salgotra et al., 2023).

### 3.2 Methods and approaches in pre-breeding

**Germplasm Exploration and Evaluation**: Germplasm exploration involves the collection and evaluation of diverse *Brassica oleracea* genetic resources, including wild relatives and landraces. This approach aims to identify novel genetic traits and donor parents that can be used in breeding programs. Evaluation involves characterizing the collected germplasm for various traits of interest, such as disease resistance, yield potential, nutritional quality, and stress tolerance (Nanjundan, 2015).

**Phenotypic Selection:** Phenotypic selection involves visually evaluating and selecting plants based on their observable characteristics. In pre-breeding of *Brassica oleracea*, this approach is used to identify individuals with desirable traits such as disease resistance, plant architecture, leaf shape, flowering time, or other agronomically important traits. Selected plants can serve as parents for further breeding efforts (Mohd Saad et al., 2021).

**Molecular Markers and Genotyping:** Molecular markers are powerful tools used in pre-breeding to assess genetic diversity, identify trait-associated markers, and assist in selection decisions (Salgotra & Stewart ,2020). Techniques such as SSR (Simple Sequence Repeat) markers, SNP (Single Nucleotide Polymorphism) markers, and genotyping-by-sequencing (GBS) are employed to genotype Brassica oleracea populations and identify markers linked to desired traits. Marker-assisted selection (MAS) can then be used to make more informed breeding decisions based on marker data (Amiteye, 2021).

**Interspecific and Inter-generic Hybridization:** Interspecific and inter-generic hybridization involve crossing *Brassica oleracea* with other related species or genera to introduce new genetic diversity and desirable traits. By crossing with wild relatives or other Brassica species (e.g., *Brassica rapa*, *Brassica nigra*), breeders can transfer traits such as disease resistance, stress tolerance, or improved nutritional content into *Brassica oleracea* (Kaneko & Bang, 2014).

**Doubled Haploid (DH) Technology:** Doubled haploid production is a technique used to develop pure, homozygous lines in a single generation. DH technology can expedite the development of genetically stable lines and facilitate the fixation of desirable traits. It involves inducing haploid embryos through methods like microspore culture or embryo rescue, followed by chromosome doubling to obtain homozygous plants (Pires et al., 2020).

**Genetic Engineering and Gene Editing:** Biotechnological approaches such as genetic engineering and gene editing provide opportunities to directly introduce or modify specific

genes in *Brassica oleracea*. Techniques like Agrobacterium-mediated transformation or CRISPR-Cas9 gene editing can be used to enhance traits such as disease resistance, herbicide tolerance, or nutritional quality.

**Participatory Approaches and Farmer Engagement:** In pre-breeding, involving farmers and stakeholders in the selection and evaluation of germplasm can help ensure the relevance and practicality of breeding goals. Participatory approaches, such as on-farm trials and farmer-led selection, allow farmers to contribute their knowledge and preferences, leading to the development of varieties that meet local needs and preferences (Sperling et al., 2001).

These methods and approaches in pre-breeding of *Brassica oleracea* contribute to the development of improved varieties with enhanced traits, adaptability, and productivity. Specific techniques and approaches may vary depending on the objectives, available resources, and target traits of the breeding program (Ceccarelli et al.,2007).

### 3.3. Challenges and opportunities in pre-breeding of *Brassica oleracea*

- Challenges:

**Genetic Diversity:** *Brassica oleracea* has limited genetic diversity compared to its wild relatives. This can pose challenges in terms of introducing novel traits and improving the genetic base of cultivated varieties.

**Complex Genome:** *Brassica oleracea* has a complex genome, with multiple sub-genomes and a high degree of heterozygosity. This complexity makes it challenging to identify and manipulate specific genes or traits of interest (Ahmad et al.,2023).

**Trait Complexity:** Many important agronomic traits in *Brassica oleracea*, such as yield, quality, and disease resistance, are controlled by multiple genes and influenced by environmental factors. Understanding and manipulating these complex traits can be a significant challenge in pre-breeding (Walley et al.,2012). Comprehensive phenotypic and genotypic characterization of *Brassica oleracea* populations is essential for understanding the genetic basis of complex traits. This involves collecting detailed data on trait performance across diverse environments, conducting field trials, and implementing rigorous statistical analyses. Genomic tools, such as high-throughput sequencing and marker analysis, can be used to identify candidate genes associated with the target traits (Mohd Saad et al.,2021).

- Opportunities:

**Wild Relatives:** Wild relatives of *Brassica oleracea*, such as *Brassica incana* and *Brassica macrocarpa*, harbor valuable genetic traits that can be introgressed into cultivated varieties through breeding (Jesske et al., 2013). Exploring and utilizing the genetic diversity present in wild relatives presents opportunities for trait improvement. For example, wild relatives may possess genes or traits that confer tolerance to abiotic stresses such as drought, heat, or salinity. Incorporating these traits into cultivated varieties can help enhance their resilience under adverse environmental conditions, ensuring more stable yields and reducing yield losses due to abiotic stresses (Quezada-Martinez et al.,2021).

**Molecular Tools:** Advances in molecular biology and genomics have provided powerful tools for pre-breeding in *Brassica oleracea*. Techniques such as marker-assisted selection (MAS), genomic selection, and high-throughput sequencing can facilitate the identification and introgression of desirable traits (Shaw et al.,2021).

**Biotechnology:** Biotechnological approaches, including genetic engineering and gene editing, offer opportunities for targeted trait manipulation in *Brassica oleracea*. These techniques can accelerate the development of improved varieties by introducing specific genes or modifying existing ones (Marone et al.,2023).

**Emerging Technologies:** Emerging technologies, such as CRISPR-Cas9, RNA interference (RNAi), and high-throughput phenotyping, hold promise for accelerating pre-breeding efforts in *Brassica oleracea*. These technologies can enhance the efficiency and precision of trait selection and evaluation (Verma et al., 2023).

Addressing the challenges and leveraging the opportunities in pre-breeding *Brassica oleracea* requires collaborative efforts between breeders, geneticists, and biotechnologists. By combining traditional breeding techniques with modern tools and approaches, the genetic improvement of *Brassica oleracea* can be enhanced to meet the demands of sustainable agriculture and consumer preferences.

## 4. Organic Breeding Ethics and Methods

### 4.1 Organic farming principles and practices

Organic farming for brassica crops, such as *Brassica oleracea*, involves applying the principles and practices of organic agriculture to promote sustainable and environmentally friendly production.

**Soil Management:** Organic brassica farming prioritizes soil health and fertility. Practices such as crop rotation, cover cropping, and the use of organic matter (compost, green manure) help improve soil structure, increase organic matter content, and enhance nutrient availability (Scavo et al.,2022). These practices ensure a healthy soil ecosystem for optimal growth and yield of brassica crops.

**Organic Inputs:** Organic farming of brassica crops avoids the use of synthetic fertilizers, pesticides, and genetically modified organisms (GMOs). Instead, organic farmers utilize natural and organic inputs that are approved for organic production (Oliver,2014). Organic-approved fertilizers, such as compost, well-rotted manure, and organic-based fertilizers, are used to provide essential nutrients to the brassica plants.

**Pest and Disease Management:** Organic brassica farmers employ a range of techniques to manage pests and diseases without synthetic chemical pesticides. This includes crop rotation, which helps break pest and disease cycles, and the use of biological controls, such as beneficial insects or microbial agents, to manage pests naturally. Additionally, practices like maintaining proper plant spacing, promoting biodiversity, and monitoring crops for early signs of pests or diseases are essential for effective pest and disease management (Mpumi et al.,2020).

**Weed Control:** Organic farmers utilize various strategies to control weeds in brassica crops without relying on synthetic herbicides. This includes practices such as mechanical weed control (hand weeding, hoeing), mulching, and using cover crops to suppress weed growth. Proper crop rotation and maintaining healthy soil can also help minimize weed competition (Pannacci et al.,2017).

**Biodiversity and Habitat Conservation:** Organic farming for brassica crops promotes biodiversity and the conservation of natural habitats. Creating habitats for beneficial insects, preserving field margins, and planting flowering plants can attract pollinators and natural enemies of pests, contributing to improved pest control and crop pollination (Cloyd,2020).

**Certification and Compliance:** Organic brassica farmers adhere to organic farming standards and undergo certification processes to ensure compliance. Certification requires following specific organic practices, maintaining proper documentation, and undergoing periodic inspections by authorized certifying bodies (Chander et al.,2011).

**Quality and Traceability**: Organic farming for brassica crops emphasizes producing high-quality and traceable products. Organic certification provides assurance to consumers that the produce has been grown using organic practices and meets organic standards.

By applying these principles and practices, organic farming for brassica crops aims to produce healthy, nutrient-rich, and environmentally sustainable brassica vegetables while minimizing the use of synthetic inputs and reducing negative environmental impacts (Roberts & Mattoo. 2018).

### 4.2 Traits and characteristics desired in organic *Brassica oleracea* crops

Organic farming systems require crop varieties that possess specific traits and characteristics to thrive in the absence of synthetic chemical inputs. Regarding breeding for organic *Brassica oleracea* crops, several traits are desirable to enhance their performance and sustainability within organic production. These traits contribute to disease resistance, nutrient efficiency, and overall resilience in organic farming systems (Reda et al.,2021).

One key trait desired in organic *Brassica oleracea* crops is disease resistance Han et al.,2021). Organic farmers face challenges in managing diseases without relying on chemical pesticides (Pearce et al., 2012). Breeding for disease-resistant varieties can help mitigate disease pressures in organic production. Traits such as resistance to common diseases like clubroot (*Plasmodiophora brassicae*) and downy mildew (*Hyaloperonospora parasitica*) can significantly contribute to the success of organic Brassica crops (Buczacki et al., 2000). Enhanced nutrient-use efficiency is crucial in organic farming, where nutrient availability may be limited compared to conventional systems. Breeding for traits that optimize nutrient uptake, utilization, and assimilation can improve the organic performance of *Brassica oleracea* crops (Dawson et al.,2008). Traits related to root system architecture, nutrient transporters, and nutrient-use efficiency pathways are of particular interest in breeding for organic nutrient management (Malorgio et al., 2019). Furthermore, traits associated with stress tolerance are highly desirable in organic Brassica crops. Organic farming systems can present challenges such as water stress (Parkash et al.,2020), temperature fluctuations (Bhat et al.,2022), and soil nutrient imbalances. Breeding for stress tolerance traits, such as drought tolerance, heat tolerance, and nutrient-use efficiency under stress conditions, can enhance the resilience of *Brassica oleracea* crops in organic environments (Kim et al., 2020).

Moreover, organic farming practices often require reduced reliance on synthetic pesticides. GLSs also play a role in plant defense against pests and diseases, acting as natural allelochemicals and contributing to pest resistance (Divekar et al.,2022). Breeding for *Brassica oleracea* varieties with optimized GLSs profiles can enhance natural pest and disease resistance, reducing the need for chemical interventions in organic production (Bellostas et al.,

2007). By incorporating breeding strategies that prioritize specific glucosinolates profiles, organic *Brassica oleracea* crops can be developed to have enhanced nutritional benefits, distinct flavors, and increased resistance to pests and diseases. These efforts contribute to the overall quality, health-promoting properties, and sustainability of organic agriculture (Ishida et al.,2014).

### 4.3 Breeding strategies for enhancing organic performance

Organic agriculture places specific demands on crop varieties, requiring them to possess traits that promote resilience and productivity within organic farming systems. Breeding strategies aimed at enhancing organic performance focus on developing varieties that exhibit traits such as disease resistance, weed competitiveness, nutrient-use efficiency, and stress tolerance. These traits contribute to reduced reliance on synthetic inputs and promote sustainable organic farming practices (Lammerts van Bueren, et al., 2011).

One approach in breeding for organic performance is the use of participatory plant breeding (PPB) methods. PPB involves collaboration between breeders, farmers, and other stakeholders to select and develop crop varieties specifically suited to organic farming systems. This participatory approach ensures that the needs and preferences of organic farmers are considered, resulting in varieties that are better adapted to local organic conditions (Magrath & Amarowicz, 2011).

Another strategy is the incorporation of genetic diversity from landraces and wild relatives into breeding programs (Allier et al., 2020). These genetic resources often possess valuable traits, such as disease resistance or tolerance to environmental stresses, which can be transferred to cultivated varieties through breeding. This approach helps to improve the adaptability and resilience of organic crops (Smýkal et al., 2015). Additionally, breeding for improved nutrient-use efficiency is crucial for organic agriculture. Varieties that efficiently acquire and utilize nutrients from organic sources, such as compost or organic fertilizers, can thrive in nutrient-limited organic systems. Breeding programs can target traits related to nutrient uptake, nutrient-use efficiency, and nutrient partitioning to enhance the organic performance of crops (Malorgio et al., 2019).

Marker-assisted selection (MAS) is another valuable tool in breeding for organic performance. MAS allows breeders to select plants with desired traits based on molecular markers associated with those traits. This accelerates the breeding process, enabling the development of varieties with improved organic performance more efficiently (Willer &

Lernoud, 2020). The use of SSR markers in MAS offers several advantages. Firstly, SSR markers provide a robust and reliable means of genotyping due to their reproducibility and specificity to target genomic regions (Li et al.,2017). They can be easily scored and amplified using polymerase chain reaction (PCR) techniques, allowing for high-throughput genotyping of large populations. Secondly, the high level of polymorphism displayed by SSR markers enhances the resolution and accuracy of selection, enabling breeders to differentiate between closely related individuals and select those carrying the desired traits (Manzoor et al.,2023). Lastly, SSR markers are transferable across different genetic backgrounds, making them applicable in diverse breeding programs. The successful implementation of SSR markers in MAS has been demonstrated in various crop improvement programs (Sinha et al.,2023). The SSR markers have been utilized to facilitate the selection of traits such as disease resistance, abiotic stress tolerance, yield components, quality traits, and many other agronomically important traits (Hasan et al.,2021). Additionally, SSR markers have played a crucial role in identifying quantitative trait loci (QTL) associated with complex traits, enabling a better understanding of the genetic basis underlying these traits (Huang et al.,2015). SSR markers have emerged as valuable tools for marker-assisted selection in plant breeding. Their abundance, co-dominant nature, high polymorphism, and transferability make them well-suited for genetic analysis and trait mapping (Amiteye, 2021). The integration of SSR markers in MAS has significantly accelerated the breeding process, allowing breeders to make more informed selections and develop improved crop varieties with desired traits. With ongoing advancements in genomics and molecular breeding technologies, SSR markers are expected to continue playing a pivotal role in enhancing agricultural productivity and addressing global food security challenges (Cobb, et al., 2018).

The integration of genomic tools and technologies, such as high-throughput genotyping and phenotyping, can further enhance breeding for organic performance. These tools enable breeders to identify and select individuals with desirable traits more accurately and efficiently, leading to the development of improved organic varieties (Guo et al., 2021). By employing a combination of participatory breeding, genetic diversity utilization, nutrient-use efficiency enhancement, marker-assisted selection, and genomic tools, breeders can develop varieties that meet the specific needs and challenges of organic farming systems. These breeding strategies contribute to the long-term sustainability and success of organic agriculture (Bohra et al.,2022).

## EXPERIMENTAL ACTIVITIES

### General premises and objectives

Historically, unintentional plant selection and subsequent crop domestication coupled with the need and desire to obtain more food and feed products provided by the continuous development of plant breeding and genetic efforts. Plants are susceptible to a range of environmental challenges during their growth and development in both natural and agricultural environments. Biotic and abiotic stresses pose severe risks to the sustainability of plant production and global food security under current climate and environmental changes. Drought stress is an abiotic stress that is gaining attention because it has a negative impact on plant growth and development and it significantly reduces plant biomass and production, contributing to global food insecurity.

*B. oleracea* crops and related the wild species (n=9) have received special attention among leafy vegetables because of their high phytochemical content, which includes high levels of vitamins, minerals, dietary fiber, GLS, and phenolic compounds. The leaves are characterized by a typical taste owing to the presence of a wide array of sulfur compounds and they are traditional ingredients of the Mediterranean diet. The easy detection of numerous secondary metabolites makes them an optimal model trait for investigating the complex quantitative genetics ones and to stabilize their variation. Cultivation under stressful conditions can promote the production of bioactive molecules associated with the antioxidant system and the plant defense mechanisms. The abundance of bioactive compounds can differ significantly among species and genotypes, as they exhibit distinct responses to stress conditions. The study of the physiological and biochemical adaptations related to drought resistance in plants provide valuable criteria for selecting and developing drought-tolerant cultivars. Plant breeding aims to establish novel varieties by combining specific traits determined by breeders to meet the demands of both the breeders and consumers. The process of genetic enhancement starts with the introduction of genetic diversity, and the subsequent selection of elite genotypes occurs within diverse genetic pools. To facilitate the early and efficient selection of desired traits, molecular markers can be employed to identify and isolate genetic material carrying the target gene. To develop a new crop variety is required a significant time investment, often spanning over a decade, starting from the initial interspecific and/or intraspecific crosses. The genetic improvement activities need to achieve varietal advancements for organic farming in the upcoming decades, because is crucial to start by crosses between suitable parental lines promptly. The primary constraints faced by the existing varieties in organic production becomes essential in this context. By targeting these limitations through dedicated breeding programs for organic farming, Efforts

can be made to develop improved varieties that are well-suited to the specific requirements of organic agriculture. In addition, it is crucial to ensure the widespread adoption and implementation of newly developed organic varieties by effectively communicating research outcomes to breeders. This collaborative effort will contribute significantly to meeting the challenges of organic farming and fostering sustainable agricultural practices in the future.

With a specific focus on organic agriculture, the enhancement of nutraceutical traits through 'omics technologies are the objective of pre-breeding efforts. Sustainable and nutritious vegetable production practices can be promoted by preserving and utilizing this invaluable heritage. The full potential of this invaluable vegetable heritage for supporting sustainable vegetable production practices is aimed to be unlocked through pre-breeding efforts for Brassica oleracea crops. Contribution to the innovation and diversification of vegetable production is aspired to be made by preserving genetic diversity, enhancing nutraceutical traits, and incorporating principles of organic agriculture. In this way, a brighter and healthier future for our region and beyond is aimed to be ensured.

**The framework of the research lines of activity**

The three-year research project was dedicated to thoroughly evaluating the *B. oleracea* complex species (n=9), analyzing various attributes with the ultimate goal of improving crop resistance to stresses and elevating the nutraceutical potential of the resulting products. The aim of our investigation was to assess various biometric, biochemical, and genetic traits that are of particular interest for improving the resilience of *B. oleracea* crops to drought stresses.

All the activities were supported by the H2020 project “Breeding for resilient, efficient and sustainable organic vegetable production” (BRESOV) aimed to screen the biodiversity of broccoli, for individuating elite materials to use for the genetic improvement of the three studied crops.

Our knowledge has been enriched by in-depth bibliographic studies, which have shed light on the nutraceutical value of these products. The collective efforts of research groups worldwide have centered around exploring bioactive compounds such as polyphenols, carotenoids, vitamins, and GLSs, highlighting their antioxidant properties and contributing to our understanding of their potential health benefits. Extensive research has been dedicated to understanding the significance of GLSs compounds, which are known for their prevalence in the Brassicaceae family. These investigations have spurred targeted improvement programs for

major Brassicaceae crops with the primary goal of enhancing the nutraceutical content in their final products.

The examination of relevant literature on GLSs and their biological activities has been carried out to achieve this objective. Furthermore, specific analysis protocols for assessing the antioxidant compounds present in various genetic materials have been identified during the three years of research. These analysis protocols have been integrated and utilized to evaluate different genetic materials, with the aim of identifying those possessing the most promising traits. This valuable information can then be utilized to refine and enhance ongoing work programs, ultimately leading to the development of *B. oleracea* crops with increased levels of beneficial GLSs. Substantial advancements for the genetic improvement of *B. oleracea* crops have been facilitated by the combined efforts to study GLSs content and profile, implementing the comprehensive analysis of gene expression in relation to drought stress. The potential for offering consumers enhanced and healthier products with increased nutraceutical content, contributing to better overall well-being, is held by these endeavors.

The research work undertaken during the PhD course encompassed a wide array of topics, ranging from understanding the biodiversity and history of *B. oleracea* species to explore their potential for organic farming and for their nutritional benefits. Additionally, significant efforts were devoted to improving crop resilience and nutraceutical attributes, thus contributing to the advancement and sustainability of *B. oleracea* crops in the future.

During this PhD, I pointed my attention to the following research lines:

1) **Genetic Diversity and Germplasm Characterization of *Brassica oleracea* L. complex species (n=9) core collection;**
2) **The effect of water stress on the variation of the biochemical profile of *Brassica oleracea*;**
3) **The variation of Glucosinolate metabolism *Brassica oleracea* in relation to drought stress.**

With regards to the first research line aims we explored the genetic diversity within the *Brassica oleracea* primary gene pool, including its wild relatives. It involves the collection, preservation, and characterization of germplasm resources to understand the genetic variability of the species. Firstly, we aimed to assess the morphological diversity within the core collection of *B. oleracea* complex species, observing carefully and documenting meticulously the variations for the studied traits. After, we employ state-of-the-art molecular techniques to evaluate the genetic diversity among the studied accessions, shedding light on the underlying genetic relationships and patterns. These insights will be unvaluable for targeted traits mapping

and breeding programs, with the ultimate aim to develop improved *B. oleracea* cultivars that boast resilience, high yield, and enhanced nutritional content. By achieving these objectives, we hope to advance our understanding of B*rassica oleracea* diversity and to contribute for the sustainable improvement of sustainable agriculture and conservation efforts.

The second line of activities deals with a comprehensive evaluation for gaining crucial insights for the plant's adaptability and resilience under water-limited conditions. For understanding the adaptability of *Brassica oleracea* accessions to water deficiency we have started to individuate elite genetic materials for developing more resilient and drought-tolerant cultivars. By identifying specific biochemical markers associated with water stress tolerance, we can pinpoint genotypes that exhibit exceptional performance under limited water availability. These elite accessions can then serve as valuable genetic resources for breeding programs, promoting the development of water-efficient and stress-resistant cultivars.

As concerns with the third research, we carried out a comprehensive evaluation of *B. oleracea accessions*, focusing specifically on GLS, a group of secondary metabolites with significant health-promoting properties. By studying both roots and leaves, we gained valuable insights for ascertaining profiles the amount and profiles of the GLSs among different plant organs. Our investigation was related to *B. oleracea* plants grown under controlled water stress conditions, simulating the impact of abiotic stress on the GLSs metabolism. Through this approach, we deepen our understanding of the plant's adaptive responses to water deficiency, shedding light on how these compounds function as part of the plant's defense mechanisms against environmental challenges. These efforts align with the overarching goal of organic and sustainable agriculture and bolstering global food security facing climate change in ac

# 1. Research line I. Genetic Diversity and Germplasm Characterization of *Brassica oleracea* L. complex species (n=9) core collection

## 1.1 Introduction

In recent years, our world has been increasingly confronted by the sobering realities of climate change. Altered weather patterns, rising global temperatures, and a heightened frequency of extreme weather events have collectively underscored the fragility of our agricultural systems. In the face of these unprecedented challenges, the need to identify genetic resources with resilience to environmental stressors has become paramount. Among the most adaptable and economically valuable plant species is *Brassica oleracea*, a member of the Brassicaceae family, which encompasses a diverse array of vegetables such as broccoli, cauliflower, cabbage, and kale.

While considerable progress has been made in decoding the genetic diversity concealed within *Brassica oleracea*, there remains a vast reservoir of untapped genetic potential within the *Brassica oleracea* complex species. This genetic diversity, till now unexplored, holds the promise of addressing some of the most pressing issues in agriculture today. Such issues include enhancing crop yield in the face of changing climate conditions, fortifying crops against emerging diseases and pests, and increasing nutritional content to combat malnutrition in vulnerable populations. To fully harness the potential harbored within the genetic diversity of the *Brassica oleracea*, it is imperative to establish a comprehensive understanding of the genetic underpinnings of functional traits. The intricate interplay between genetics and traits such as yield, disease resistance, abiotic stress tolerance, and nutritional content has the potential to revolutionize the development of improved cultivars. This can, in turn, secure food production in the face of environmental challenges and contribute to global food security.

Additionally, the preservation of genetic diversity within the *Brassica oleracea* complex species (n=9) is fundamental for ensuring the species' adaptability and resilience to a rapidly changing environment. The core collection, a curated subset of the *Brassica oleracea* crops, plays a pivotal role in this preservation effort. Representing the culmination of centuries of human selection and cultivation, the core collection encapsulates the zenith of genetic diversity within the species.

To comprehensively comprehend and effectively harness these genetic resources for breeding programs, a multifaceted approach is indispensable. While traditional agro-morphological characterization offers valuable insights, it can be susceptible to environmental

influences and often lacks specificity. In contrast, DNA-based molecular markers, especially Simple Sequence Repeats (SSRs), stand out as invaluable tools. Their multi-allelic nature, high polymorphism, and ease of manipulation in laboratory settings make them indispensable for genetic diversity studies and marker-assisted breeding. In light of these considerations, the overarching objective of this first research line is to illuminate the intricate interplay between genetic diversity, morphological traits, and the potential for crop improvement within the *Brassica oleracea* complex. To achieve this, we aim to provide a holistic overview of the genetic and morphological diversity resident within the core collection, demonstrating its pivotal role in advancing plant breeding and crop improvement.

## 1.2. Material and Methods

### *1.2.1 Plant Material and Experimental design*

In this chapter, our study focused on a core collection comprising *Brassica oleracea* and related complex species (n = 9), with specific details outlined in table (18 Annex). The experimental phase was conducted in Catania, Italy, where seeds from each accession were meticulously sown in cellular trays within a controlled cold greenhouse. This greenhouse provides a natural light environment, with daily light exposure ranging from 4.6 to 9.2 $MJ \cdot m^{-2} \cdot d^{-1}$, and temperatures that exhibited fluctuations between 15.4°C ± and 5.8°C ±. The trays were thoughtfully filled with Brill soil sourced from Geotec, Italy, and standard irrigation techniques were thoughtfully employed. Each cultivar was represented by three replicates in both treatment groups. Upon reaching the four-leaf stage, the plantlets were carefully transplanted into a certified organic greenhouse located in Santa Croce Camerina, Italy (36°51'13.3" N 14°29'32.0" E, Contrada Randello, Ragusa). The plants were systematically arranged in single rows, maintaining a spacing of 1.0 m between rows and 0.5 m between individual plants along the rows. This arrangement resulted in a crop density of 2 plants/m² (Figure 6). The plants achieved commercial maturity in January 2020, the leaves were promptly subjected to freezing at −80°C for 72 hours. Following this preservation step, the freeze-dried plant material was carefully ground into a fine powder using an IKA-A10 mill (from IKA-Werke GmbH & Co. KG, Staufen, Germany), and stored at −20°C until subsequent analysis.

**Figure 6. Organic experimental field for Brassica oleracea cultivation**

### *1.2.2 Biological material*

In the first research line, we have carefully curated a core collection of biological materials to form the basis of our investigations. This selection encompasses a wide range of both commercial varieties and wild species of Brassica plants, all of which play a crucial role in enhancing our understanding of this diverse plant family within their natural contexts (Table3- Detailed in Table 18 in the annex). The collection includes commercial varieties such as Broccoli, Brussels sprouts, Cabbage, Cauliflower, Kale, Kohl Rabi, as well as self-pollinated varieties. Additionally, we have included wild species like B. *drepanensi*s and *B. rupestris,* which were collected from various locations in Sicily, and *B. villosa* and *B. incana* from different regions. These wild specimens are particularly valuable as they provide us with unique insights into the genetic diversity and adaptability of Brassica plants in their native habitats. Furthermore, our research extends to include a diverse array of cultivars sourced from different geographical regions, including Italy, Tunisia, and China. This geographical diversity empowers us to dissect a wide range of genetic traits and study how these cultivars respond to varying environmental conditions.

**Table 3. Illustrations of Studied Varieties of *Brassica oleracea***

| | |
|---|---|
| **Broccoli** |  |

| | |
|---|---|
| **Cauliflower** |  |
| **kale** |  |
| **Kohl Rabi** |  |
| **Cabbage** |  |
| **Wild species** |  |
| **Brussels sprouts** |  |

### *1.2.3 Bio Morphometric Traits*

The plant's bio-morphological characteristics were meticulously assessed using internationally recognized morphological descriptors (Table 4) provided by organizations such as the IBPGR (International Board for Plant Genetic Resources) and UPOV (The International Union for the Protection of New Varieties of Plants). These descriptors serve as standardized

guidelines for evaluating and categorizing various plant traits. The main morphometric traits included:

**Table 4. Agronomic Traits for Evaluating Varieties of *Brassica oleracea***

| Index | Descriptors |
|---|---|
| **IA** | Inflorescence appereance |
| **PB** | Branches in the plant (0-7) |
| **PS** | Plant shape (1-5) |
| **HH** | Plant grown habit (1-9) |
| **NL** | Number leaves per plant main stem (n) |
| **SL** | Vegetative stem length (cm) |
| **Leaves** | |
| **LA** | Leaf area ($cm^2$) |
| **LL** | Leaf length (cm) |
| **LW** | Leaf width (cm) |
| **Petiole** | |
| **RLA** | Petiole length (cm) |
| **RRA** | Petiole width (cm) |
| **Root** | |
| **LA** | Root left angle |
| **LAN** | Root right angle |
| **RD** | Basal root diameter (mm) |
| **MRD** | Main root diameter (cm) |
| **MRL** | Main root length (cm) |
| **LR** | Lateral root diameter (cm) |
| **RA** | Root area ($cm^2$) |
| **RW** | Root weight (g) |
| **DM** | Dry matter (%) |

Additionally, the nutritional status of the plant was assessed using the Single Photon Avalanche Diode (SPAD) method. This innovative technique involves the use of a portable chlorophyll meter, specifically the SPAD-502 developed by Minolta Camera Co. in Osaka, Japan. Three fully developed leaves from each plant in every replicate were subjected to SPAD measurement. The SPAD index, derived from this assessment, serves as a quantitative indicator of the plant's chlorophyll content. Incorporating these comprehensive morphological assessments into the research not only provides a detailed understanding of the plant's physical characteristics but also sheds light on its vital nutritional status.

### *1.2.4 Genotyping by SSR markers*

#### 1.2.4.1 DNA extraction

For each studied accession, DNA extraction was performed on three leaf samples utilizing the CTAB method, renowned for its efficacy in molecular biology (Bernatzky and Tanksley, 1986). This versatile protocol accommodates both fresh and dried tissues, ensuring the

preservation of DNA integrity. To prepare dried leaf samples, young leaves were subjected to a 48-hour desiccation period in an oven set at 65°C, followed by grinding using the Tissue Lyser (Retsch MM300). The initial step in DNA extraction involved cell lysis. This was achieved by introducing 1 ml of extraction buffer, comprising 0.35 M Sorbitol, 0.1 M Tris-pH (8.0), 5 mM EDTA, 2 M NaCl, and 2 g/L CTAB, to each sample. The samples were subsequently incubated in a water bath at 65°C for 20 minutes, facilitating the breakdown of cellular structures. The second phase of the process aimed at separating proteins from nucleic acids. This was achieved by adding 500 µl of chloroform to the samples, followed by gentle homogenization for 20 minutes. The resultant mixtures were then subjected to centrifugation at 12,000g for 10 minutes. The ensuing supernatants were carefully transferred to new 1.5 ml tubes. To precipitate the nucleic acids, 300 µl of isopropanol was added to the supernatants. After thorough mixing, the samples underwent another round of centrifugation for 10 minutes at 12,000g. Following centrifugation, the supernatants were removed, to get the nucleic acid precipitates**.** The precipitates were then subjected to a washing step with 500 µl of 70% ethanol, followed by another round of centrifugation for 10 minutes at 12,000g. Following centrifugation, the supernatants were discarded, and the remaining precipitates were allowed to air dry for 10 minutes. Subsequently, the dried samples were resuspended in 30 µl of ultrapure water. The quantification of DNA was performed using a ND-1000 spectrophotometer (Nanodrop Technologies, USA). In parallel, the quality of the extracted DNA was assessed based on the DO 260/280 ratio, with a range between 1.8 and 2 indicating the purity of the DNA sample.

#### 1.2.4.2 Primers selection

In the process of primer selection, particular attention was devoted to the identification of SSR primers that could effectively target specific Brassica DNA regions associated with GLS metabolism. The selection criteria prioritized primers with a proven track record in amplifying polymorphic regions within the genomic context of GLS-related genes. The choice of these primers was guided by the seminal work of Hasan et al. (2008), as referenced in this study, which provided invaluable insights into the genetic markers associated with GLS pathways in Brassica species. This deliberate primer selection process was fundamental to the success of the genotyping approach, enabling to explore genetic diversity in Brassica varieties with a specific focus on the crucial GLS-related regions. The sequences of SSR primers are outlined in Table 5.

**Table 5. List of Primers Used for Genotyping the Core Collection of *Brassica oleracea* .L**

| GSL-associated SSR | SSR location | SSR repeat motif | Forward primer sequence | Reverse primer sequence |
|---|---|---|---|---|
| Gi1 | At5: 25182045 | (CTC) | AAACGAATAATGTAGAATCGG | GAGCAAAGTAGAAGAGTCGG |
| Gi5 | At5: 25254743 | (CT) | ACACTCCAGATTCCACGAC | TAAACGCCTCACAAAGACA |
| Gi12 | At5: 24895600 | (TGT) | GAAAGGAAGTGAAGAAAGAGTG | CCAAACCATAGCATAAACAAC |
| Gi13 | At5: 24934614 | (TGA) | AACCATCAAGAAGAAGACGA | CAACATCAAGACAATAAGACCA |
| Gi16 | At5: 24863079 | (TC) | AAGTGATTCTTGGAGTTTGGT | ATTGTTCTGATGTTGTCCTTG |
| Gi17 | At5: 1753540 | (AAG) | TCTCGTTTCTCTCTCTTTCTCT | AGGGTTTGCTTCTTTGATG |
| Gi24 | At5: 1727906 | (TCC) | TCTGAACAATCAATCTCCGT | AGTTTACGATACGCTCTCCTC |
| Gi28 | At5: 7808676 | (TGC) | AACAGAGCATTTGGGTCTT | ACCGAGAACAATCCCTATCT |
| Gi30 | At4: 15237362 | (TCA) | TTCTTTCTTTCTTATCGTCTTTG | CCATTCTTTGTTGTCTCTCTG |
| Gi31 | At4: 15276052 | (AG) | GTCCGCATCGTCAATCTC | AGAAACTGTCCTTCATCTGCT |
| Gi34 | At4: 16505683 | (TCA) | TGTCTATCATCTCTCTCACAACA | TAATCACCGTCCAGTTTCTC |
| Gi38 | At5: 7630182 | (AAG) | AGAAGAAGCCAGCAGAGAA | GATGTCGGGATGGACCTG |

### 1.2.4.3 PCR Reaction Setup

Each accession within the core collection underwent genotyping at twelve polymorphic nuclear microsatellite loci, utilizing a specific mix as detailed in Table 6. To ensure the integrity of the results, a negative control was incorporated into each PCR reaction as a precautionary measure. The amplification reactions were carried out in a final volume of 25 µl, following a modified program adapted for our study. a touch-down PCR cycle was employed, an approach refined from the method outlined by Lorenz (2012), with the following steps:

An initial denaturation step was initiated at 95°C for 2 minutes, setting the stage for subsequent amplification. The touch-down PCR cycle was initiated with five cycles, characterized by a 45-second denaturation phase at 95°C, followed by an annealing step. The annealing temperature commenced at 68°C and then systematically decreased by 2°C in each subsequent cycle. Each annealing step was extended to 5 minutes. Following annealing, there was a 1-minute extension at 72°C. Following the initial five cycles, an additional set of five cycles was conducted. Similar to the previous cycle, these cycles began with a 45-second denaturation at 95°C. The annealing temperature, however, initiated at 58°C and decreased by 2°C in each subsequent cycle, again with a 1-minute extension at 72°C. The PCR continued with the standard amplification phase, encompassing 27 cycles. These cycles comprised a 45-second denaturation at 94°C, followed by a 2-minute annealing step at 47°C, and concluding

with a 30-second extension at 72°C. The entire amplification process was finalized with a 10-minute extension step at 72°C, ensuring the completeness of the PCR reaction.

**Table 6. Composition of the PCR Reaction Mix**

| Mix | Concentrations | Volume |
|---|---|---|
| **Buffer** | 10X | 2.5µL |
| **MgCl2** | 3mM | 2.5µL |
| **dNTPs** | 1Mm | 0.5µL |
| **Forword primer (F)** | 0.25µM | 1µL |
| **Reverse primer (R)** | 0.25µM | 1µL |
| **ADNg** | 50ng | 1µL |
| **Taqpolymerase (QBiogen)** | 1,5U | 0.5µL |
| **$H_2O$** | - | 11µL |

The PCR reactions were conducted employing a thermocycler (TProfessional, TRIO Thermocycler). This step facilitated the amplification of target DNA sequences utilizing the selected microsatellite markers. The PCR products, contained in 0.2 ml strips comprising 12 tubes, were directly transferred from the thermocycler to the QIAxcel System's sample tray. The separation procedure employed the OL700 method, characterized by a sample injection voltage of 8 KV for 20 seconds, a subsequent separation voltage of 3 KV, and a separation time of 700 seconds. This process utilized a 12-channel gel cartridge (GCK5000) obtained from Qiagen USA. The resulting alleles' sizes, as resolved during separation, were automatically computed in base pairs (bp) and extracted using the BioCalculator$^{TM}$ software. This software not only provides a visual representation of the gel but also generates an electropherogram for comparative analysis. For reference, PCR products labeled with WellRed primers and FAM primers were separately subjected to capillary electrophoresis: CEQ 8000, with a capillary temperature of 50°C, an injection voltage of 2.0 KV for 30 seconds, denaturation at 90°C for 120 seconds, separation voltage at 4.8 KV for 60 minutes; and ABI 3130xl, with a capillary temperature of 60°C, a 3-minute pre-run at 15.0 KV, sample injection at 1.2 KV for 23 seconds, followed by a 12.5-minute separation at 15.0 KV.

PCR product sizes were determined through software analysis, with the QIAxcel ScreenGel Software employed for QIAxcel results. In both cases, alleles were identified based on their peak fluorescence, with peaks registering more than 50 RFU (Relative Fluorescence Units) in the electropherogram being considered alleles. Additionally, if an extra peak reached

at least one-third the height of the dominant peak, it was also recognized and scored as an allele (Figure 7).

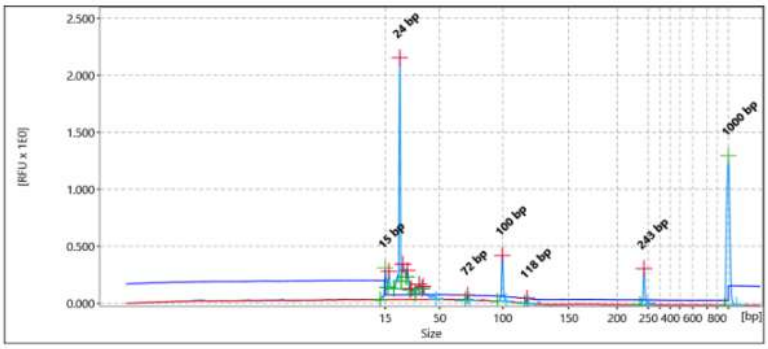



**Figure 7. Allele Size Distribution in a Cauliflower Cultivar Using SSR Primer CEQ TM 8000 genetic analysis system**

The QIAxel system, developed by QIAGEN, employs the capillary electrophoresis technique to meticulously analyze nucleic acid samples. In this method, nucleic acid samples, are loaded into capillaries filled with a gel matrix. An electric current is applied to the capillaries, causing the fragments within each sample to migrate through the gel at varying speeds based on their size and charge. This separation process effectively sorts the fragments by size. A built-in detector monitors the movement of fragments, generating electropherogram data that captures the intensity of detected fragments over time. Subsequently, specialized QIAxel software is used for data analysis, interpreting the electropherogram data to determine fragment sizes and generating peak profiles for individual fragments. The QIAxel system finds widespread use in molecular biology and genetics research, offering high precision and reliability for genotyping, fragment analysis, quality assessment of nucleic acid samples, quantification, and precise sizing of nucleic acid fragments.

### *1.2.3 Data analysis*

The calculation of genetic parameters and indices is performed using functions from the following packages: Adegenet, Poppr, and Vegan.

#### 1.2.3.1 Genetic Variance Analysis

**a. Polymorphism Information Content (PIC):**

This parameter estimates the discriminant power of a given marker, taking into account the number of alleles revealed by a locus and their respective frequencies. From a binary matrix (presence/absence), the Polymorphism Information Content (PIC) of a given marker (primer pair) is calculated using the following formula (Anderson et al., 1993):

$$\mathbf{PIC = 1 - \Sigma\ \mathit{Pij}^2}$$

Where *Pij* is the frequency of marker i revealed by primer pair j.

**b. Percentage of Polymorphic Bands (PBP):**

This parameter is defined as the rate of polymorphism (PBP) revealed for a primer pair. It corresponds to the percentage of polymorphic bands relative to the total number of bands revealed for each tested primer pair, as per the following formula:

**PBP = (number of polymorphic bands / total number of bands) * 100**

**c. Resolving Power of Primers (Rp):**

According to Gilbert et al. (1999), resolving power is an appropriate parameter to evaluate the effectiveness of primer pairs used to differentiate between the studied populations. Resolving power is calculated using the following formula (Gilbert et al., 1999):

$$\mathbf{Rp = \Sigma\ Ib}$$

Where Ib = 1 - (2 × |0.5 - p|), and p is the frequency of populations possessing band I.

**d. Shannon Index:**

The Shannon Index, also known as Shannon-Weaver Index or Entropy Index, is a parameter used to measure species diversity based on the concept of entropy. It is calculated as follows: **H = -Σi pi log pi** (Spellerberg & Fedor, 2003)

Where pi is the proportion of alleles (or genotypes) found in population i, estimated as pi = ni / N, where ni is the number of alleles (or genotypes) in population i, and N is the total number of alleles (or genotypes) across all populations. The Shannon Index values increase as the richness and heterogeneity of the community increase.

**e. Simpson's Index:**

Simpson's Index measures the probability that two individuals (alleles) randomly selected from an infinitely large community belong to the same population (marker):

$D = \Sigma\ P^2$ (Simpson, 1949)

Where Pi represents the proportion of individuals encountered in species i. This index has a value of 0 to indicate maximum diversity and 1 to indicate minimum diversity. To obtain more intuitive values, it's often represented as 1 - D, where 1 indicates maximum diversity, and 0 indicates minimum diversity.

**f. Genotypic Evenness Index:**

This index, also known as Evenness or E.5, assesses the genotypic distribution in a sample. Typically, these indices compare the number of expected genotypes to observed genotypes. An important property of this evenness index is that it should be equal to 0 for a population composed of a single genotype and equal to 1 when all genotypes occur at the same frequency, regardless of their richness. E.5 is calculated using the following formula (Pielou, 1975; Ludwig & Reynolds, 1988; Grünwald et al., 2003):

$$E.5 = 1 - (1 / \lambda) / (H - 1)$$

Where $1/\lambda$ is the Stoddart and Taylor index, H is the Shannon diversity, and E.5 is the genotypic evenness index.

**1.2.3.2 Genetic Differentiation**

**a. Nei's Genetic Differentiation Index (Gst):**

Gst represents the genetic differentiation index, similar to Wright's Fst, considering multiallelic markers (Nei, 1973). It uses allele frequencies and is calculated using the formula:

$$Gst = 1 - Hs / Ht$$

Where Hs is the heterozygosity within populations (intra-population diversity), and Ht is the total heterozygosity (total diversity).

For markers with high mutation rates like microsatellites, mutation quantity can influence differentiation indices like Gst and Fst unfavorably. Therefore, corrected indices like G'st and Dst have been proposed to measure population genotypic differentiation. G'st can be directly related to migration rates between populations, while Dst affects partitioning distances or gene diversity (Verity & Nichols, 2014).

**b. Principal Component Analysis (PCA):**

PCA is used in genetics to obtain a simplified view of genetic diversity among individuals or populations. This analysis is performed using functions implemented in the Ade4 and Adegenet packages in R.

**c. Genetic Distance and Phylogenetic Tree Construction:**

The Neighbor-joining (NJ) method between taxa is used to construct phylogenetic trees based on genetic distances. This method groups genetically closer populations by relating the genetic distances between them. In this study, a similarity matrix based on Euclidean distances between populations is calculated for use in constructing the cladogram using the Neighbor-

joining method. Additionally, the Nei distance matrix (Nei, 1972) is used to generate the phylogenetic tree using the same NJ method. These analyses are carried out using functions from the Poppr and Ape packages.

#### 1.2.3.3 Population Discrimination and Structure

Discriminant Analysis of Principal Components (DAPC) is a multivariate method designed to identify and describe genetically related groups of individuals. DAPC aims to provide an efficient description of genetic groups using a few synthetic variables. These are generated as linear combinations of the original variables (alleles) with the largest inter-population variance and the smallest intra-population variance. The coefficients of the alleles used in the linear combination are called loadings, and the synthetic variables themselves are called discriminant functions. This approach extracts rich information from genetic data to determine the optimal number of genetic clusters and assign individuals to groups. The analysis is performed with the number of clusters (k) ranging from 1 to 8. This visual evaluation of differentiation between populations provides a precise idea of the contribution of individual alleles to population structure. These analyses are conducted using the Adegenet, Poppr, and VcfR packages

## 1.3 Results

### *1.3.1 Morpho-agronomic variability of the core collection*

The bio-morphometric characterization provides a comprehensive description of the core collection, highlighting the range of phenotypic diversity present within the species. It helps in identifying distinct morphotypes, grouping accessions based on similarities in their morphological characteristics, and detecting relationships between different subgroups.

The leaves of *Brassica oleracea* can vary in shape, texture, and arrangement depending on the specific variety as shown in figure 8. Kale leaves are typically large, with a distinctive frilly or curly appearance. However, some varieties of kale, such as Lacinato kale, have flat and elongated leaves that are less curly. The leaves can range in color from deep green to purplish-green and may have a slightly rough texture. Cabbage leaves are generally smooth and have a round or elongated shape, forming a tight head or rosette. The outer leaves are typically thicker and coarser, while the inner leaves are more tender and lighter in color. Cabbage leaves can range in color from light green to deep purple, depending on the variety. Broccoli leaves are typically large and have a somewhat rough texture. They are deeply lobed and often form a loose rosette at the base of the plant. The leaf color is usually medium to dark green. Cauliflower

leaves are similar to those of broccoli, being large, lobed, and forming a rosette. However, cauliflower leaves may have a lighter green color compared to broccoli. Brussels sprouts have leaves that are smaller and more elongated compared to other *Brassica oleracea* vegetables. The leaves are tightly packed around the stem and can have a slightly wrinkled or textured appearance. The color of Brussels sprouts leaves is typically medium green (Figure 8).

**Figure 8. Leaves of different studied accessions of *B. oleracea***

**Figure 9. Variations in Inflorescence Color Among Analyzed Cultivars**

In the graph representing the distribution of Brassica accessions along PC1 and PC2 axes, accessions based on the first three axes PC1, PC2 and PC3 which absorb more variability (Figure 10). The first axis absorbs 37.2% of the total variation while the second absorbs 15.79% of the total variability. It appears that both *Brassica oleracea* var *acephala* and *Brassica oleracea* var *tronchuda* are located at the extreme right end of the PC1 axis. This indicates that these two accessions are positively associated with the variables that contribute to PC1 which are RD, MRD, and MRL. These two varieties share distinct characteristics that set them apart from the other Brassica accessions.

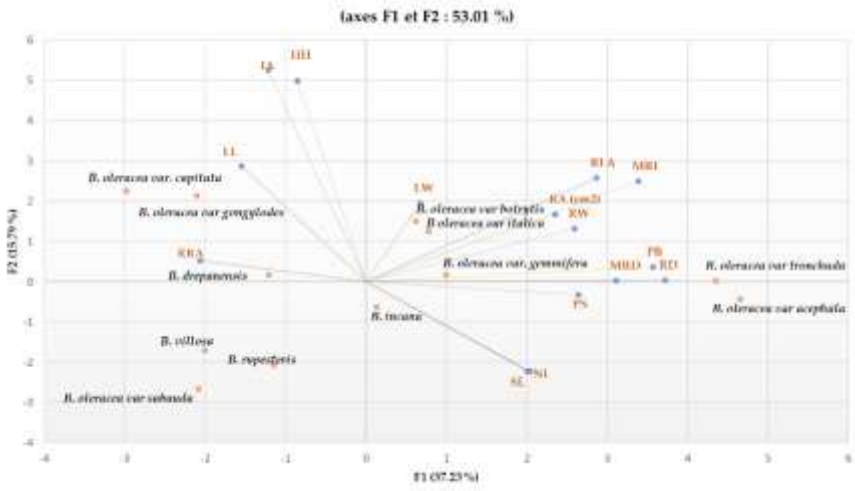



**Figure 10. PCA Analysis of Brassica Based on Morphological Traits**

Table 7 illustrates the correlations between the traits analyzed and the two principal components (PC1 and PC2). These correlations help to understand how each variable relates to the underlying structure represented by the principal components. Notably, PC1 shows a strong positive correlation with RD (0.849), MRD (0.857), and MRL (0.921), suggesting that these variables contribute significantly to the variation explained by PC1 and tend to increase together. In contrast, PC2 exhibits strong positive correlations with IA (0.950) and HH (0.923). Conversely, NL (-0.553) and PB (-0.263) display negative correlations with PC2, suggesting an inverse relationship.

**Table 7. Correlations between Principal Component Axes and Various Morphological Traits**

**CORRELATIONS**

| | PC1 | PC2 |
|---|---|---|
| **IA** | -,018 | ,950** |
| **PB** | ,604* | -,263 |
| **PS** | ,380 | -,004 |
| **HH** | -,024 | ,923** |
| **NL** | ,172 | -,553 |
| **SL** | ,218 | -,278 |
| **LL** | -,076 | ,161 |
| **LW** | -,040 | ,136 |
| **RLA** | ,484 | ,110 |
| **RRA** | -,144 | -,099 |
| **RD** | ,849** | -,282 |
| **MRD** | ,857** | -,158 |
| **MRL** | ,921** | ,092 |
| **RA** | ,689* | -,131 |
| **RW** | ,731** | ,141 |

*, **, and*** indicate respectively that the effect is significant at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively

In the analysis of Brassica crops based on their morphological traits, the dendrogram was constructed using the Euclidean distance measure and Ward's method of aggregation (Figure 11). Each node in the dendrogram represents a cluster, and the branches indicate the degree of similarity between these clusters. The objective was to categorize the core collection of *Brassica oleracea* L. complex species (n=9) into distinct classes, primarily based on specific morphological traits. Four distinct classes were identified, each comprising a varying number of Brassica varieties. Notably, Class 1 included *B. oleracea* var. *acephala* and *B. oleracea* var. *tronchuda*, while Class 2 encompassed *B. oleracea* var. *italica*, *B. oleracea* var. *capitata*, *B. oleracea* var. *gongylodes*, *B. oleracea* var. *botrytis*, and *B. oleracea* var. *gemmifera*. Additionally, Class 3 consisted of wild species *B. drepanensis*, *B. rupesteris*, *B. villosa*, and *B. incana*.It's worth noting that *B. oleracea* var. *sabauda* stands alone in one cluster, which is situated near the cluster containing the wild species. These classifications have shed light on the inherent relationships and variations among the Brassica plant varieties based on their morphological traits.

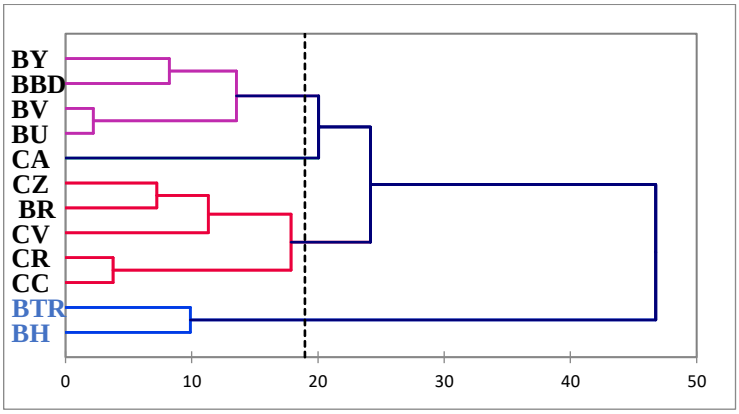



**Figure 11. Dendrogram of set of Brassica crops Varieties Based on Morphological Traits**

### *1.3.2 Analysis of genetic diversity revealed by SSR markers.*

The analysis involved the examination of these SSR markers across the studied accessions to uncover patterns of genetic variation, relatedness, and structure within the *Brassica oleracea* core collection. By assessing the differences and similarities in these genetic markers, a comprehensive understanding of the genetic diversity present in the sample set was gained, serving as a foundational element in the broader research endeavor

#### 1.3.2.1 Genetic Diversity and Allele Distribution

The analysis of amplification profiles unveiled 46 informative alleles, spanning variable lengths from 110 to 450 base pairs (bp). To comprehensively assess genetic diversity, we

subjected the obtained SSR data to a battery of statistical analysis methods, including Shannon's diversity index (H), Simpson's index (1-D), and an evaluation of allelic distribution (as summarized in Table 8). Significantly, Gi12, Gi13, Gi17, and Gi38 were identified as loci with the highest number of observed alleles, each displaying seven unique variants. This observation underscores the substantial genetic diversity present at these specific loci. Gi13, in particular, exhibited an exceptional Shannon-Wiener Diversity Index (H) value of 0.86, underscoring its remarkable diversity of alleles in comparison to the other loci. Moreover, the assessment of observed heterozygosity, which quantifies the proportion of individuals within a population carrying different alleles at a specific locus, revealed values spanning from 0.60 to 1.94, with a mean value of 1.36. This suggests a noteworthy level of moderate to high genetic diversity across the loci. Encouragingly, expected heterozygosity, a theoretical measure based on allele frequencies in the population, closely paralleled observed heterozygosity values, with a range of 0.41 to 0.86 and a mean of 0.71, affirming alignment with anticipated genetic diversity levels. Furthermore, Gi13 exhibited the highest 1-D value of 1.00, signifying the highest probability of two randomly chosen alleles being different within this locus. When examining evenness, which assesses the degree of deviation from genetic equilibrium by comparing observed heterozygosity (H) to expected heterozygosity (H exp), The findings revealed evenness values spanning from 0.81 to 1.00, with an average of 0.92. This implies that, on average, the populations under study maintain a reasonably close proximity to genetic equilibrium. Notably, Gi13 and Gi34 displayed the highest evenness values (1.00 and 0.97, respectively), indicative of a remarkably even distribution of alleles within these loci. Collectively, these results unveil varying levels of genetic diversity and allele distribution across different loci. While Gi13 and Gi17 are noteworthy for their high genetic diversity, Gi5 exhibits relatively lower genetic diversity in comparison. These findings hold substantial implications for the field of population genetics, offering insights that can inform conservation efforts, evolutionary studies, and breeding programs.

**Table 8 .The estimation of allelic diversity of markers generated by SSRs**

| Locus | Allele | H exp | Evenness | 1-D |
|---|---|---|---|---|
| **Gi1** | 4 | 0.7 | 0.88 | 0.7 |
| **Gi5** | 2 | 0.41 | 0.84 | 0.41 |
| **Gi12** | 7 | 0.85 | 0.96 | 0.84 |
| **Gi13** | 7 | 0.86 | 1 | 0.86 |
| **Gi17** | 7 | 0.83 | 0.9 | 0.82 |
| **Gi24** | 3 | 0.62 | 0.9 | 0.62 |
| **Gi28** | 4 | 0.67 | 0.81 | 0.67 |
| **Gi30** | 3 | 0.65 | 0.95 | 0.64 |

| | | | | |
|---|---|---|---|---|
| **Gi31** | 4 | 0.73 | 0.92 | 0.72 |
| **Gi34** | 3 | 0.66 | 0.97 | 0.65 |
| **Gi38** | 6 | 0.82 | 0.93 | 0.81 |
| **Mean** | 4.55 | 0.71 | 0.92 | 0.7 |

**Allele** (Number of observed alleles); **H** (Shannon-Wiener Diversity Index); **H exp** (Expected Heterozygosity);**1-D** (Reciprocal of Simpson's Index); **Evenness** (Allelic Distribution).

Transitioning to the broader context of this study, the results unveiled notable rates of both intra- and inter-population heterozygosity, with Hs encapsulating genetic diversity within subpopulations, showcasing variations within individual populations. Notably, Gi13 exhibited a relatively higher Hs value of 0.1624, signifying greater genetic diversity at this locus. Meanwhile, Ht, the measure of total genetic diversity across all populations, encompassed both within-population diversity (Hs) and among-population diversity (Dst). Gi13, once again, stood out with an Ht value of 0.8579, reflecting substantial overall genetic diversity across populations. Moving to the assessment of genetic differentiation, G'ST, known as Wright's Fst, quantified the genetic differentiation among populations relative to total genetic diversity. Gi1 displayed a G'ST value of 0.9430, indicative of substantial differentiation among populations at this locus. Likewise, G'st, which adjusts for within-population diversity, also highlighted strong differentiation, with Gi1 recording a G'ST value of 0.9902. The D measure underscored considerable genetic differentiation among populations, as exemplified by Gi13's D value of 0.9687. Analyzing the entire set of loci revealed an overall genetic diversity (Ht) surpassing genetic diversity within populations (Hs), consequently elevating the genetic diversity index (GST) based on allele frequencies. Moreover, the corrected Nei's index (G'ST) demonstrated significant genetic variation among studied populations (Table 9). Notably, this differentiation appears to align with the relatively high mutation rates of SSR markers, as indicated by the Jost index (D > 0). These findings collectively shed light on the intricate genetic dynamics and differentiation patterns within the study, providing valuable insights for the thesis on population genetics and evolutionary dynamics.

**Table 9. The estimation of genotypic diversity of markers generated by each SSR.**

| | **Hs** | **Ht** | **Gst** | **G 'st** | **D** |
|---|---|---|---|---|---|
| **Gi1** | 0.039 | 0.699 | 0.943 | 0.990 | 0.801 |
| **Gi5** | 0 | 0.408 | 1 | 1 | 0.476 |
| **Gi12** | 0.099 | 0.843 | 0.882 | 0.996 | 0.963 |
| **Gi13** | 0.162 | 0.858 | 0.811 | 0.995 | 0.969 |
| **Gi17** | 0.038 | 0.821 | 0.953 | 0.998 | 0.950 |
| **Gi24** | 0.032 | 0.616 | 0.949 | 0.988 | 0.725 |
| **Gi28** | 0 | 0.666 | 1 | 1 | 0.8 |
| **Gi30** | 0 | 0.64 | 1 | 1 | 0.8 |

| | | | | | |
|---|---|---|---|---|---|
| **Gi31** | 0 | 0.72 | 1 | 1 | 0.9 |
| **Gi34** | 0 | 0.653 | 1 | 1 | 0.762 |
| **Gi38** | 0.029 | 0.816 | 0.963 | 0.998 | 0.945 |

**Hs** (Heterozygosity within population with population structure), **Ht** (Heterozygosity without population structure), **Gst** (Nei's Diversity Index),**G′st** (Hedrick's Corrected Nei's Diversity Index),**D** (Jost's Index).

### 1.3.2.3 Microsatellite polymorphism and genetic diversity level

The genotypic analysis has identified a total of 58 multi-locus genotypes within the dataset comprising 100 individuals, resulting in an average of 8.33 genotypes per variety. Various parameters were employed to assess the genotypic diversity among the eleven studied populations (Table 10). These parameters include the number of observed multi-locus genotypes (MLG), the Shannon-Wiener diversity index (H), the Stoddart and Taylor's diversity index (G), the Simpson index (λ), genotypic richness (E.5), and Nei's unbiased genetic diversity index (Hexp). Notably, a Shannon index of 1,37, reflecting overall genotypic variability, was reported across all identified genotypes. Specifically, the lowest diversity index (H =0,00) was observed in the *B.o.sabauda, B.incana* and *B.o.tronchuda* conversely, the highest Shannon diversity index (H = 2.71) was noted in *B.o. botrytis* indicative of substantial genotypic diversity within these populations.

**Table 10. Genotypic Variability of *Brassica oleracea* accessions**

| Accessions | N | MLG | eMLG | SE | H | G | λ | E.5 | Hexp |
|---|---|---|---|---|---|---|---|---|---|
| ***B.o.botrytis*** | 15,00 | 15,00 | 10,00 | 0,00 | 2,71 | 15,00 | 0,93 | 1,00 | 0,26 |
| ***B.o.italica*** | 15,00 | 14,00 | 9,57 | 0,00 | 2,62 | 13,24 | 0,92 | 0,97 | 0,18 |
| ***Brassica villosa*** | 2,00 | 8,00 | 2,00 | 0,00 | 2,69 | 2,00 | 0,50 | 1,00 | 0,15 |
| ***Brassica drepensis*** | 2,00 | 6,00 | 2,00 | 0,00 | 2,69 | 2,00 | 0,67 | 1,00 | 0,06 |
| ***Brassica rupesteris*** | 3,00 | 10,00 | 3,00 | 0,00 | 1,10 | 3,00 | 0,83 | 1,00 | 0,10 |
| ***B.o.acephala*** | 15,00 | 7,00 | 6,66 | 0,00 | 1,97 | 5,77 | 0,24 | 0,77 | 0,83 |
| ***B.o.gonglyodes*** | 15,00 | 9,00 | 2,33 | 0,00 | 0,49 | 1,32 | 0,77 | 0,51 | 0,01 |
| ***B.o.capitata*** | 15,00 | 6,00 | 4,62 | 0,00 | 1,53 | 4,41 | 0,50 | 0,94 | 0,35 |
| ***B.o.gemmifera*** | 15,00 | 8,00 | 2,00 | 0,00 | 0,69 | 1,99 | 0,00 | 1,00 | 0,31 |
| ***B.o.sabauda*** | 1,00 | 10,00 | 1,00 | 0,00 | 0,00 | 1,00 | 0,00 | NaN | 0,00 |
| ***B.incana*** | 1,00 | 1,00 | 1,00 | 0,00 | 0,00 | 1,00 | 0,00 | NaN | 0,00 |
| ***B.o.tronchuda*** | 1,00 | 1,00 | 1,00 | 0,00 | 0,00 | 1,00 | 0,00 | NaN | 0,39 |
| **Total** | 8,33 | 7,92 | 3,77 | 0,00 | 1,37 | 4,31 | 0,45 | 0,91 | 0,22 |

N: nombre des individus; MLG: Nombre de génotypes multi-locus observés; eMLG: Nombre de génotypes multi-locus attendus; SE: Erreur Standard; H: Indice de diversité de Shannon-Wiener (Shannon, 1948); G: Indice de diversité de Stoddart et Taylor's (Stoddart & Taylor, 1988); □: Indice de Simpson (Simpson, 1949); E.5: Richesse génotypique (Pielou, 1975; Ludwig & Reynolds, 1988; Grünwald et al., 2003); Hexp: indice de diversité génétique impartiale (non biaisé) de Nei (Nei, 1978).

In the comprehensive analysis of *Brassica oleracea* genetic diversity through Multilocus Genotype (MLG) analysis (Figure 12), the examination of various varieties yielded a multitude

of insights. Notably, *Brassica oleracea botrytis* and *Brassica oleracea italica* each displayed remarkable genetic diversity, featuring 15 distinct Multilocus Genotypes (MLGs). This diversity was reflected in their elevated Shannon and Simpson Indices (H= 2.708 and H= 2.616 respectively and G=15,00 and G=13.24), underscoring the rich genetic variation within these varieties. Moreover, their relatively high evenness (E.5=1 for *Brassica oleracea botrytis* and E.5=0.965 for *B. oleracea italica*) values suggest a balanced distribution of MLGs, implying a more homogeneous genetic landscape. The Simpson Index (G) and Simpson's Lambda (lambda) assess the probability of individuals sharing the same MLG. Higher G values and lower lambda values imply greater genetic diversity. Most populations exhibit higher G values and lower lambda values, emphasizing diverse genetic landscapes. In contrast, *B.oleracea acephala* displayed a lower eMLG=6.66, indicating less genetic diversity within this variety compared to *botrytis* and *italica*. The findings also extended to other varieties within the *Brassica oleracea* group, with varying degrees of genetic diversity and evenness. When considering the entire dataset comprising 100 individuals and 58 MLGs, we observed a moderate level of evenness (E.5) and substantial genetic diversity, as indicated by the high Shannon and Simpson Indices (H and G). This suggests a complex and diverse genetic landscape within the *Brassica oleracea* accessions as a whole. These findings provide valuable insights into the genetic composition and relationships among these varieties, offering opportunities for further exploration into their evolutionary history, adaptation mechanisms, and their potential utility in breeding and conservation efforts.

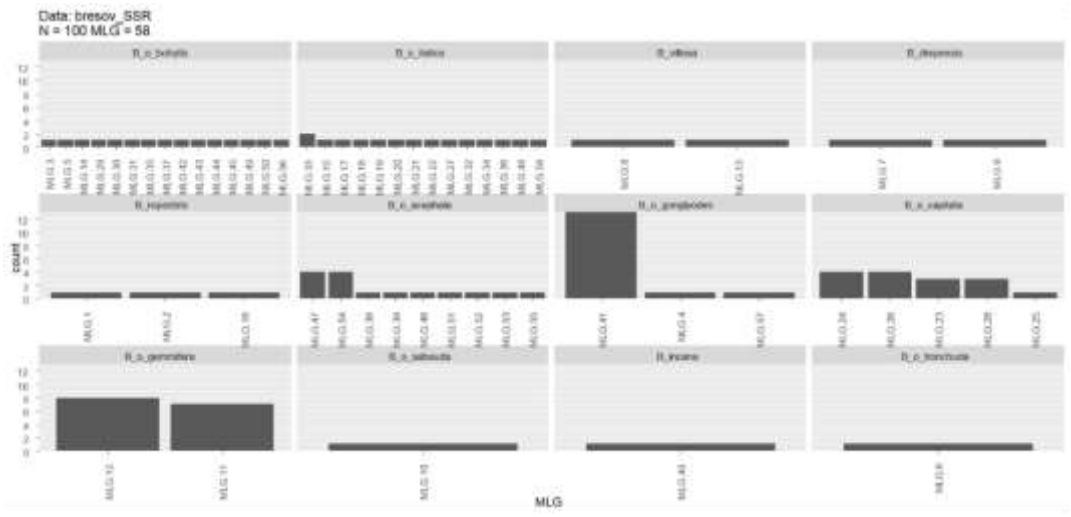



**Figure 12. Composition and Genotypic Distribution of Brassica *oleracea***

As part of the analysis, a Neighbor-Joining dendrogram was constructed. This dendrogram was performed using a dissimilarity matrix that had been calculated through the

Simple Matching method, which is a technique for quantifying the dissimilarity or difference between data points. The resulting dendrogram was provided with a graphical representation of the relationships and clustering patterns among these accessions (Figure 13) and was utilized to offer valuable insights into the genetic similarities and differences within the Brassica population under investigation. When examining the graphical representation of population dispersion in the two-dimensional plane defined by axes 1 and 2 (as shown in Figure 13), four distinct clusters can be observed along these axes. Group 1 stands out, being separated from the other clusters primarily along the first axis. This cluster comprises the varieties *B. oleracea botrytis* and *B. oleracea italica*. In the middle, the wild species are clustered together, representing another distinct group. In contrast, *B. oleracea capitata* is notably separated and forms its own distinct cluster. Additionally, the varieties *B. oleracea gemmifera* and *Brassica B. oleracea*, along with *B. oleracea gongylodes*, are closely grouped together, forming yet another distinct cluster.

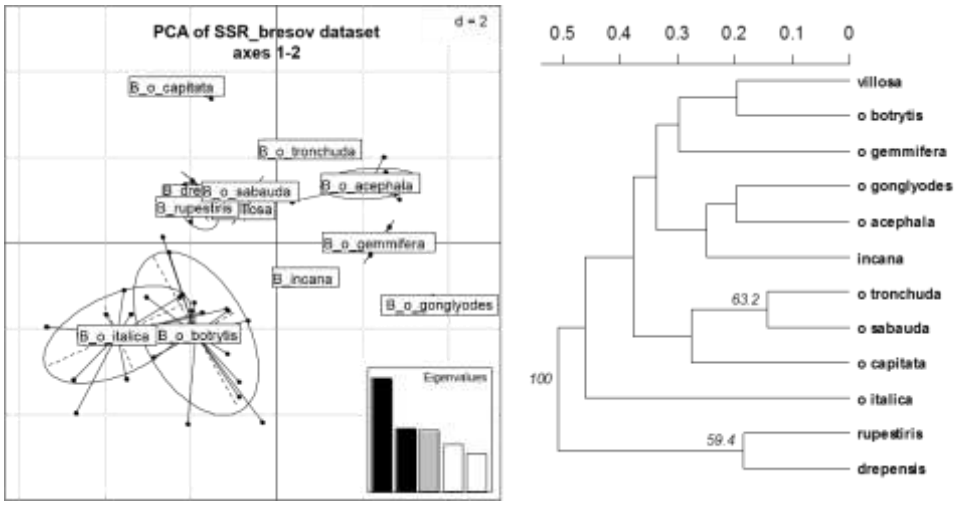



**Figure 13. (A)Principal Component Analysis of 12 varieties of *Brassica oleracea* crops and wild relative's species based on SSR markers. (B) Neighbor-joining dendrogram based on simple matching dissimilarity matrix among the Brassica accessions analyzed.**

### 1.3.2.3 Genetic discrimination and structuring of *Brassica oleracea* populations.

The genetic discrimination of various populations within the *Brassica oleracea* species was encompassed by this study. The goal of the genetic discrimination process was to distinguish and characterize the genetic differences and similarities among these distinct populations. Molecular markers, such as SSRs, were employed to meticulously examine the genetic profiles of these populations, allowing for the discernment of unique genetic patterns, relationships, and structural differences. This investigation yielded valuable insights into the genetic diversity and distinctiveness of *Brassica oleracea* populations, contributing to a

comprehensive understanding of the species' genetic landscape and facilitating future breeding and conservation efforts.

The Discriminant Analysis of Principal Components (DAPC) is a robust statistical method employed to delineate genetic groups using synthesized variables. Within this method, genetic data is initially reduced to principal components, which subsequently serve as the basis for discriminating and classifying individuals into distinct clusters. In this study, the DAPC was executed, guided by the Bayesian Information Criterion (BIC) (Figure14A), a statistical tool for model selection. The BIC played a crucial role in determining the optimal number of genetic clusters for the analysis, ultimately revealing that four clusters (K=4) best characterized the genetic data. The outcomes of the DAPC are visually represented in figure 14-B, where each individual is denoted as a point, and its position on the graph is determined by the values of the principal components. This graphical representation provides a clear visualization of the genetic groupings discerned through the DAPC.

Upon examination of Figure 14, which represents a single discriminant function (axis), it becomes evident that four groups exhibit some degree of overlap. Density plots for individuals within each group are plotted along this axis, with distinct colors denoting separate groups. This overlapping suggests a degree of genetic similarity between these groups, potentially indicating past hybridization events or a complex evolutionary history. The identified genetic groups are as follows:

Group 1: This cluster comprises the wild species *B. villosa* and *B. drepensis*, implying a close genetic affinity between them. Notably, within this cluster, *B. rupestiris* stands out with 3 individuals, signifying its distinct genetic profile. Additionally, *B. o. capitata*, *B. o. sabauda,* and *B. o. tronchuda* share the assignment to Group 1, reflecting varying degrees of genetic similarity within this cohesive cluster.

Group 2: A striking genetic connection is observed between *B. o. acephala* and *B. o. gonglyodes,* as both populations are exclusively assigned to Group 2. This robust grouping underscores shared genetic characteristics or a common genetic background between these populations. The presence of *B. incana* further bolsters this group, indicating a genetic association with the populations within this cluster.

Group 3: Group 3 is distinguished by the exclusive assignment of *B. o. gemmifera,* encompassing 15 individuals. This designation highlights the unique genetic traits that set this population apart from others.

Group 4: The unmistakable genetic link between *B. o. botrytis* and *B. o. italica* is revealed, with both populations exclusively assigned to Group 4, each consisting of 15 individuals. This robust genetic affiliation strongly suggests a high degree of genetic similarity or shared genetic features between these populations.

To elucidate the alleles contributing most significantly to this genetic discrimination, the loading plot is employed. Alleles that exert a substantial influence in distinguishing genetic groups are positioned at opposite ends of the loading plot, thereby emphasizing their significance in characterizing the groups. The loading plot diagram highlights the most influential alleles in this genetic discrimination (Figure 14 C). Four SSR markers have significantly contributed to this genetic discrimination, namely allele 425 of marker Gi28, alleles 390 of marker Gi30, allele 365 of marker Gi31, allele 176 of marker Gi34.

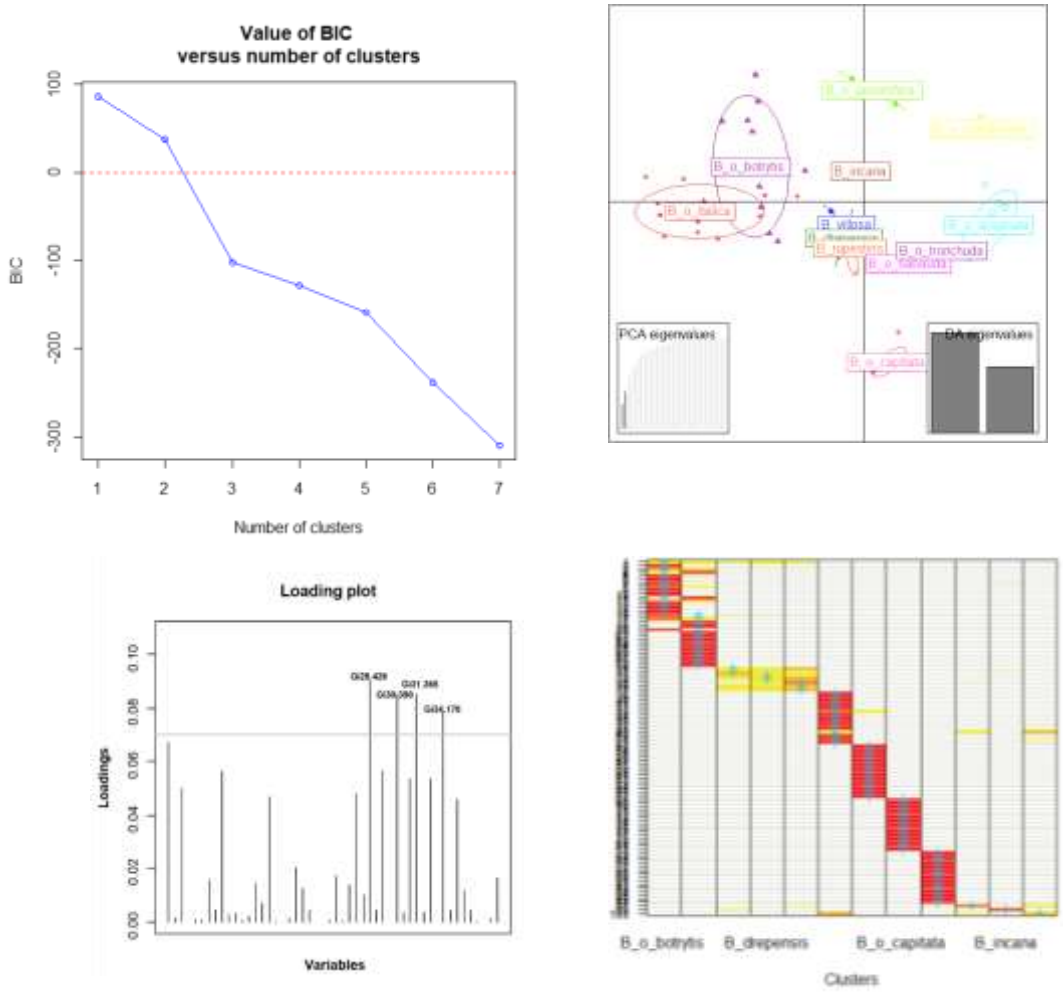



**Figure 14. The genetic discrimination of *Brassica oleracea* populations**

A: BIC (Bayesian Information Criterion) helps determine the value of K. B: DAPC (Discriminant Analysis of Principal Components) represents individuals as points and groups as inertia ellipses. The eigenvalues of the analysis are displayed. At the top of the figure, there is a representation of individual density on a given discriminant function with different colors for

each of the groups. C: The loading plot represents the alleles that contribute the most to discrimination (contributions above the threshold). D: Graphical representation of the degree of link of each individual in each of the four groups (clusters).

In Figure 15, depicting the genetic structure of *Brassica oleracea* populations at K=4 (with K representing the number of inferred genetic clusters), a captivating representation of the genetic relationships among individuals within this species is observed. Each data point on the graph corresponds to an individual plant within the studied populations. The division of the graph into different colors represents the assignment of these individuals to distinct genetic clusters. In this specific analysis, the most suitable number of clusters was determined to be four (K=4), as indicated by the Bayesian Information Criterion (BIC). These clusters offer insights into the genetic subgroups present within the *Brassica oleracea* populations. In the analysis of the genetic structure of *Brassica oleracea* populations, a particularly intriguing observation is the presence of overlapping clusters. This convergence suggests a substantial degree of genetic similarity or shared ancestry among individuals from these clusters. This genetic resemblance among clusters can be attributed to various historical and biological factors. Firstly, historical interbreeding among different genetic clusters is one likely contributor. Over time, individual plants from distinct clusters may have cross-pollinated, leading to the exchange of genetic material and the creation of a shared genetic heritage. Lastly, the presence of common alleles across multiple clusters can also contribute to this observed genetic overlap. When certain genetic markers or alleles are widespread and shared among individuals from different clusters, it fosters a genetic connection among them.

The complex interplay of genetic relatedness and the presence of overlapping clusters highlights the intricate nature of genetic diversity within *Brassica oleracea* populations. This complexity prompts to delve deeper into the historical and ecological forces that have molded the genetic makeup of this species. Understanding these dynamics is crucial, as it informs conservation strategies and enables access to the valuable genetic reservoirs of *Brassica oleracea* to enhance crop breeding initiatives.

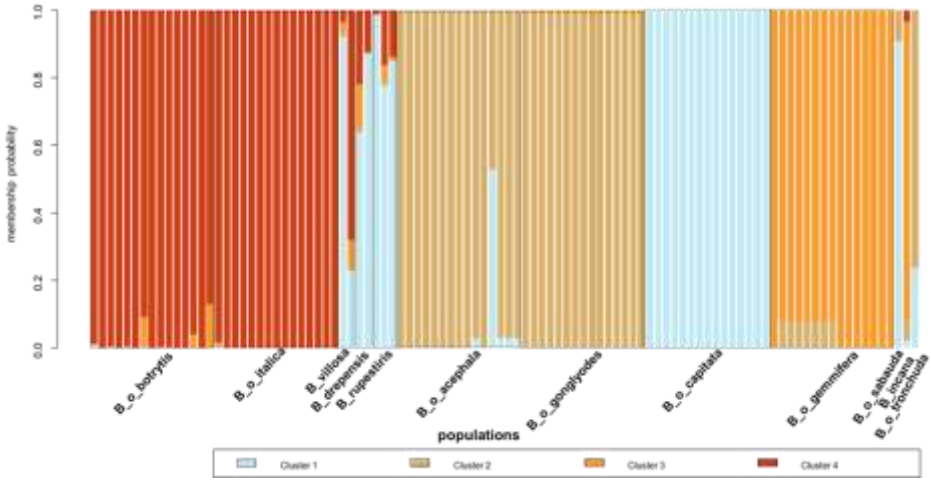



**Figure 15. The genetic structure of *Brassica oleracea* crops**

### 1.4 Discussion

Conducting a comprehensive genetic characterization of specific germplasm is crucial for implementing appropriate management, preservation, and breeding strategies. Among the Brassicaceae family, *Brassica oleracea* commonly known as leafy kale, has garnered significant global interest as a "superfood" and ornamental plant (Šamec, & Salopek-Sondi, 2019). Consequently, numerous studies have been carried out in different countries to explore the genetic diversity of local *Brassica oleracea* accessions, focusing on agro-morphological and nutritional traits. Some notable examples include studies conducted in Portugal (Dias et al., 1994), Spain (Cartea et al., 2010; Padilla et al., 2019), Turkey (Balkaya et al., 2005), Croatia (Šamec et al., 2019), and Italy (Mazzeo et al., 2019). Additionally, several studies have employed molecular markers such as RAPD (Farnham, 1996; Okumus and Balkaya, 2007), AFLP (Christensen et al., 2011), and SSR markers (Treccarichi et al., 2023) for genetic analysis.

SSR markers have consistently been preferred for assessing genetic diversity in plant species, and their continued prominence is well-justified. These microsatellite markers possess a codominant inheritance pattern and display multi-allelic characteristics, granting them robust discriminatory power. Of particular note is the fact that many SSR motifs employed in genetic studies are derived from genic regions, including transcriptome sequences (ESTs). This strategic selection is not arbitrary; rather, it hints at a deeper potential. SSR markers residing within or in proximity to genes have the intriguing capacity to serve as functional genetic markers, potentially linked to functional genetic variations. By virtue of their association with functional genes, these markers could play pivotal roles in regulating crucial plant traits. Consequently, SSR markers derived from such regions offer an exciting opportunity not only

to unravel genetic diversity but also to probe the functional dimensions of genetic variation within plant populations. This dual functionality underscores the enduring significance of SSR markers in the realm of plant genetics.

The study of genetic diversity within *Brassica oleracea* populations, as unveiled through SSR markers, yields valuable insights into the intricate genetic tapestry of this species. The significance of understanding this diversity is underscored by its profound implications for the preservation and management of the genetic resources inherent to this valuable species. The analysis of SSR markers in this investigation unveiled a total of 46 informative alleles across various loci, with allele lengths spanning from 110 to 450 base pairs (bp). This wide spectrum of allele sizes signifies the remarkable genetic richness and variability harbored within *Brassica oleracea*. However, the analysis of SSR markers extends beyond the mere enumeration of alleles; it delves into the quantification and characterization of genetic diversity using a suite of statistical methods.

The analysis of SSR markers in this study has contributed significantly to the understanding of genetic diversity within *Brassica oleracea* populations. Across multiple loci, a total of 46 informative alleles were identified, reflecting a rich genetic diversity. Notably, certain loci, such as Gi12, Gi13, Gi17, and Gi38, exhibited the highest allelic diversity, each hosting seven observed alleles. The presence of such diverse alleles within these loci underlines substantial genetic richness. Among these, Gi13 stood out with a Shannon-Wiener Diversity Index (H) value of 0.86, signaling an exceptional diversity of alleles at this specific locus. Observed heterozygosity values ranged from 0.60 to 1.94, with a mean of 1.36, indicating moderate to high genetic diversity across loci. Expected heterozygosity values closely paralleled observed values, affirming that the observed genetic diversity aligns with expectations. Gi13 also stood out with the highest Reciprocal of Simpson's Index (1-D) at 1.00, signifying the highest likelihood of distinct alleles being randomly selected. Evenness values ranged from 0.81 to 1.00, with a mean of 0.92, indicating a close approximation to genetic equilibrium. Within this context, Gi13 and Gi34 displayed the highest evenness values, pointing to an even distribution of genetic variants within their respective loci. These findings collectively underscore the presence of varying genetic diversity and allele distribution across loci, thereby holding implications for studies in population genetics, conservation biology, evolutionary biology, and plant breeding programs.

The significance of genetic diversity extends beyond the genetic loci; it manifests itself when assessing different populations within *Brassica oleracea*. For instance, the population labeled *B.o.botrytis* exhibited an exceptional level of genetic diversity, featuring 15 unique multilocus genotypes (MLGs). This population's high Shannon-Wiener Diversity Index (H) of 2.708 and Stoddart and Taylor's Index (G) of 15.00 underscore extensive genetic diversity and a substantial probability of distinct multilocus genotypes. Furthermore, the Evenness Index (E.5) at 1.000 indicates an even distribution of genetic variants within this population, further accentuating its genetic richness. Similarly, the variety *B.o.italica* also displayed significant genetic diversity with 14 unique MLGs. The Shannon-Wiener Diversity Index (H) of 2.616 and Stoddart and Taylor's Index (G) of 13.24 reinforce the presence of distinct multilocus genotypes. The relatively high Pielou's Evenness Index (E.5) of 0.965 suggests a relatively even distribution of genetic variants within this population. Even smaller sample populations, such as *B. villosa* and *B. drepensis,* maintained high genetic diversity, each featuring two unique MLGs. Remarkably, the Evenness Index (E.5) at 1.000 underscores the equitable distribution of genetic variants within these groups.

In contrast, varieties such as *B.o.gonglyodes* and *B.o.gemmifera* exhibited reduced MLG richness, indicating comparatively lower genetic diversity. The relatively low Shannon-Wiener Diversity Index (H) values reflect this reduction in genetic diversity. Stoddart and Taylor's Index (G) values of 1.32 and 1.99, respectively, indicate lower probabilities of distinct multilocus genotypes. Varying Pielou's Evenness Index (E.5) values across populations further indicate variations in genetic variant distribution. On the other hand, populations like *B.o.sabauda* and *B.incana* consisted of a single unique MLG each, revealing minimal genetic diversity within these specific groups. Both the Shannon-Wiener Diversity Index (H) and Stoddart and Taylor's Index (G) were 1.00, signifying the absence of genetic variation. In these cases, the Evenness Index (E.5) was not applicable due to the single MLG.

The application of the Discriminant Analysis of Principal Components (DAPC) in this study has further enriched the understanding of the genetic structure and relationships within the studied *Brassica oleracea* populations. Employing Bayesian Information Criterion (BIC)-guided model selection, this method led to the identification of four distinct genetic groups (K=4). The visual representation of these groups in Figure 10, where individual positions are determined by principal component values, offers a snapshot of the genetic landscape. Notably, the overlap observed between three of these genetic groups is an intriguing aspect of this findings. This overlap hints at a degree of genetic similarity that raises questions about potential

historical hybridization events or intricate evolutionary dynamics. Further investigation is warranted to elucidate the genetic mechanisms responsible for this phenomenon. The loading plot has also revealed alleles with significant contributions to genetic discrimination, shedding light on the genetic markers driving group assignments.

These genetic groupings align with and expand upon previous research, such as the work conducted by Izzah et al. (2013), which similarly identified distinct genetic clusters within *Brassica oleracea.* This consistency underscores the robustness of these genetic groupings and suggests broader applicability beyond the specific study region. Various factors, including geographic origin, environmental conditions, historical events, and genetic drift, can influence the genetic structure of *Brassica oleracea* verities. Understanding these factors provides valuable insights into the evolutionary history and dynamics of these populations. Thus, the urgency becomes apparent in the need to preserve a diverse spectrum of *Brassica oleracea* populations to safeguard the overall genetic diversity of the species. When conservation strategies are considered, it becomes evident that special attention must be given to populations with unique genetic profiles, as exceptional traits and genes may be harbored by them, which could prove indispensable for future breeding programs or the ecological restoration of Brassica oleracea populations in their natural habitats.

In summary, the comprehensive analysis of SSR markers has not only highlighted the extensive genetic diversity within *Brassica oleracea* populations but has also unveiled intriguing patterns of genetic structure and relationships. These findings hold profound implications for conservation, evolution, and breeding efforts within this valuable plant species, emphasizing the enduring relevance of SSR markers in plant genetic research.

### 1.5 Conclusion

In this chapter, the bio-morphometric characterization and genetic diversity analysis within Brassica oleracea populations under the influence of drought stress were delved into. The findings presented here establish a foundation for exploring the species' phenotypic diversity and its genetic underpinnings, illuminating the remarkable capacity of these plants to adapt to challenging environmental conditions. The bio-morphometric characterization revealed a rich tapestry of leaf variations within the *Brassica oleracea* species, with kale, cabbage, broccoli, cauliflower, and Brussels sprouts each displaying distinctive leaf shapes, textures, and colors. This comprehensive description of the core collection showcases the invaluable role of morphological traits in identifying and classifying these vegetables, while

also hinting at potential underlying mechanisms driving their diversity. Furthermore, the investigation into the effects of drought stress on morphological traits, such as root weight and root length, unveiled the dynamic response of these plants to water scarcity. The observed variations among accessions underscore the complex nature of drought stress tolerance and the importance of root architecture in mitigating its impact. This knowledge has the potential to guide future breeding and cultivation strategies aimed at enhancing the resilience of *Brassica oleracea* varieties in the face of changing climatic conditions.

In the realm of genetic diversity analysis, SSR markers were utilized to investigate the genetic makeup of the species. The SSR data unveiled a spectrum of alleles, contributing to the characterization of genetic richness and uniformity within the populations. Calculations of heterozygosity rates, fixation indices, and genetic diversity indices provided a nuanced perspective on genetic differentiation and diversity. The Discriminant Analysis of Principal Components (DAPC) emerged as a potent tool for categorizing genetic groups within Brassica oleracea populations. This analysis yielded insights into the genetic structure of the species, emphasizing distinct clusters and intriguing overlaps among groups. These findings lay the foundation for further exploration of genetic relationships and the identification of valuable alleles for breeding programs.

In the first research line, Chapter One focuses on studying morphological traits and genotyping using SSR markers for *Brassica oleracea*. This chapter explores the remarkable phenotypic diversity within the species, cataloging the unique leaf shapes, textures, and colors that distinguish kale, cabbage, broccoli, cauliflower, and Brussels sprouts. Research line I lays a strong foundation, emphasizing the significance of morphological traits and genetic analysis in unraveling the complexities of *Brassica oleracea*.

The second research line is dedicated to assessing the variation of antioxidant compounds in response to water stress in *Brassica oleracea.* This research delves into alterations and fluctuations in biochemical markers, with the aim of acquiring a deeper understanding of the tactics employed by these crops to thrive in challenging environmental conditions. This section sheds light on the complex domain of biochemical reactions, offering insights into the plant's ability to endure and overcome challenges.

## 2.Research line II. The effect of water stress on the variation of the biochemical profile of *Brassica oleracea*

### 2.1 Introduction

The impact of drought on agriculture is exacerbated by dwindling water resources and increasing global food demand. Unfortunately, many crops experience suboptimal conditions due to water scarcity, which is becoming more frequent due to global warming (Orimoloye. 2022) Consequently, it is crucial to research how plants adapt to water stress, particularly in arid and semi-arid regions, to improve agricultural breeding techniques and anticipate the impact of climatic changes on natural vegetation (Rajanna et al.,2023). Metabolic profiling has proven to be a valuable tool for identifying the specific molecular features associated with drought resistance in plants, making it particularly useful for plant breeding purposes. Several studies (Renaud et al.,2014; Biondi et al.,2021) have consistently found that crops grown under conditions of intermediate temperatures, high light intensity, longer days, and limited rainfall exhibit higher concentrations of phytochemicals

Both cultivated and natural vegetation are affected by permanent or temporary water shortages, which have a notable negative impact on various physiological processes. Plants exhibit various strategies to resist water stress, including osmotic adjustment, osmoprotection, antioxidation, and scavenging defense mechanisms (Seleiman et al.,2021). One of the most common responses to water stress is the decrease in photosynthetic activity, which is indicated by a reduction in chlorophyll content and carotenoids (Razi et al., 2021). The decrease in chlorophyll is linked to oxidative stress and can be attributed to photooxidation (Muñoz et al.,2018). Water stress triggers the production of Reactive Oxygen Species (ROS), such as superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen, which can damage cellular components like proteins and lipids, potentially leading to plant death (Cruz de Carvalho. 2008). However, at lower levels, ROS also act as signal transducers, triggering plant defense mechanisms against stress (Huang et al.,2019).

Metabolite profiling is commonly used to study plant responses to abiotic stress, and the increase in biosynthesis of key antioxidant molecules like ascorbic acid (AsA) is expected under drought stress (Arbona et al.,2013). AsA, along with dehydroascorbic acid (DHA), plays a vital role in controlling the redox state of plant cells and regulating abiotic stress responses (Potters

et al.,2004). The AsA to DHA ratio and total AsA levels are considered markers of a plant's response to abiotic stress (Xiao et al.,2021).

The glutathione, a tripeptide with a thiol (-SH) group, is closely related to the regeneration of AsA and participates in antioxidant processes through the Halliwell-Asada cycle (Hasanuzzaman et al.,2017). Additionally, polyphenolic compounds, known for their antioxidant properties and beneficial effects on human health, also contribute to plant defense against oxidative damage (Pandey et al., 2009). Understanding the biosynthesis and distribution of these active compounds in edible plants like *Brassica oleracea* could lead to the development of improved plant varieties with increased bioactive compounds (Le et al.,2020)

Understanding the biochemical responses involved in water stress tolerance in *Brassica oleracea* can provide valuable insights for breeding programs aiming to develop drought-tolerant cultivars. By examining the physiological and biochemical adaptations of the plants to variations in drought resistance, this chapter aimed to establish selection criteria for the development of drought-tolerant cultivars. The identification of genotypes that exhibit favorable responses to water stress can guide breeding efforts in developing new varieties with enhanced drought tolerance.

### 2.2 Material and methods

The primary objective of this experiment was to compare the effects of two distinct irrigation regimes, namely 100% and 35% evapotranspiration (ETc), a methodology established in prior research (Capra et al., 2008). Over the course of the trial period, a total of 20.45 $m^3$ of water was allocated to the 35% ETc treatment, while 51.65 $m^3$ of water was administered to the 100% ETc treatment. Throughout the experiment, daily temperature measurements were meticulously recorded using a high-precision hygro-thermometer (model 445702, Extech Instruments, Nashua, NH, USA). Effective pest and disease management strategies were methodically implemented, encompassing treatments against snails (utilizing Ferramol), aphids (employing Pyganic at 2.5 mL/L), and Pieris brassicae (utilizing Bacillus at 1.5 g/L). In addition to pest control, granular fertilization was judiciously administered as part of the cultivation process to ensure optimal plant nutrition.

Experiments with crossbreeds obtained from Monsampolo del Tronto have been conducted, and the results of these crossbreeding efforts, along with information about their origin, are available in Table 18 (Annex). This comprehensive approach allows for the shedding of light

on the outcomes of crossbreeding within the Brassica family, further enriching the understanding of this important plant group.

### *2.2.1 Extraction and quantification of Pigments*

To determine the concentrations of chlorophyll a, chlorophyll b, and total carotenoids, a method based on Lichtenthaler et al. (2001) was employed. Freeze-dried powder (0.1 g) from each leaf sample was employed as the starting material. These samples were thoroughly homogenized in 2 mL of a 1:1 mixture of ethanol (EtOH) and acetone. To prevent oxidation during extraction, 0.02% BHT (butylated hydroxytoluene) was added to the homogenization mixture. The homogenized samples were subjected to centrifugation at 1200 rpm for 5 minutes. Following centrifugation, the supernatant was collected, and its absorbance was measured after appropriate dilution with ethanol (EtOH) using a spectrophotometer with a 1 cm optical path length. The concentrations of chlorophyll a, chlorophyll b, and carotenoids were determined by measuring the absorbance at specific wavelengths; Chlorophyll a and chlorophyll b were assessed at 664 nm and 649 nm, respectively. Carotenoids were measured at 470 nm. The concentrations of the pigments were calculated using the following equations:

$$\textbf{Chlorophyll a (µg/mL)} = [13.36(A664) - 5.19(A649)]$$

$$\textbf{Chlorophyll b (µg/mL)} = [27.43(A649) - 8.12(A664)]$$

$$\textbf{Total carotenoids (mg/100 g)} = [1000(A470) - 2.13Ca - 97.64Cb]/209$$

Where: Ca and Cb represent the concentrations of Chlorophylls a and b.

These calculations yielded the concentrations of chlorophyll a, chlorophyll b, and total carotenoids in the samples, expressed in the final measure units of milligrams per 100 grams of freeze-dried material (mg/100 g). This meticulous approach provides precise data regarding the pigment composition of the analyzed plant material, offering valuable insights into its photosynthetic and nutritional attributes.

### *2.2.2 Extraction and Quantification of Polyphenols*

The quantification of total phenolic content was carried out employing the Folin–Ciocalteu method, a widely recognized approach for assessing polyphenolic compounds. The Folin–Ciocalteu index (FCI) was calculated based on methanolic extracts, following the method outlined by Di Bella et al. (2020) with slight modifications. Sixty milligrams of lyophilized material were utilized as the starting material. These samples were meticulously homogenized in 1.5 ml of 80% (v/v) methanol to ensure effective extraction of phenolic compounds.

Subsequently, the homogenized mixture was subjected to centrifugation at 15,000 rpm for 10 minutes at 4°C to facilitate the separation of components. An aliquot of 0.2 ml of the resulting supernatant was withdrawn and combined with 0.5 mL of Folin–Ciocalteu reagent, followed by thorough mixing. After a 3-minute incubation period at room temperature, 1 mL of 7.5% sodium carbonate was introduced into each tube. Vigorous vertexing for 20 seconds ensured complete mixing. The tubes were then allowed to stand for 60 minutes in a dark environment at room temperature to facilitate color development.

Following the incubation period, the absorption of the samples was measured at 730 nm against a blank that contained all reagents except for the sample or standard solutions. The quantification of total phenolic content was achieved by establishing a calibration curve using gallic acid solutions at known concentrations (Figure 16). The results were expressed as gallic acid equivalents (GAE) in milligrams per gram of the sample (mg GAE/g sample). The calculation was performed using the following formula:

$$\textit{Total Phenolic Content (TPC)} = ((C \times DF \times mg)/g) \times 100$$

Where **C** represents the concentration of the sample; **DF** signifies the dilution factor of 25; **mg** denotes milligrams of the initial sample; g represents the grams of the sample used.

This approach allowed for the accurate determination of total phenolic content, a crucial parameter in assessing the nutritional and antioxidant potential of the analyzed samples.

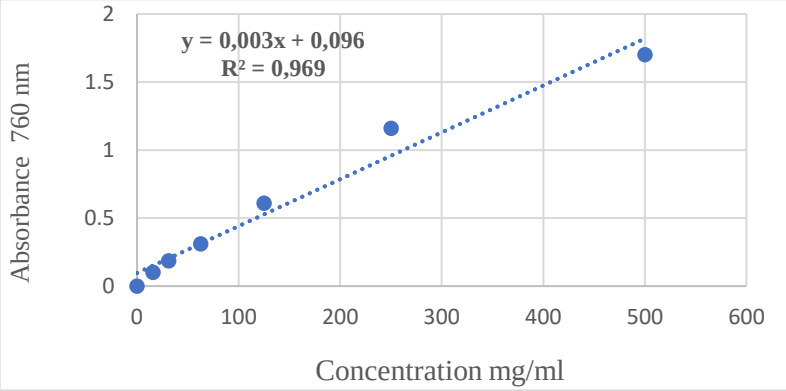



**Figure 16. Gallic acid standard curve for the calculation of total polyphenols content.**

### *2.2.3 Extraction and Quantification of Ascorbic Acid*

The analysis of ascorbic acid, commonly known as vitamin C, was conducted following the methodology outlined by Picchi et al. (2012). Here, we describe the precise steps involved in the extraction and quantification of ascorbic acid from freeze-dried leaves. Freeze-dried leaves (50 mg) were employed as the starting material for the analysis. These leaves were subjected to treatment with 1 mL of cold 3% metaphosphoric acid. Following this treatment,

the suspension underwent thorough agitation for 1 minute. Subsequently, the suspension was centrifuged at 12,000 rpm for 5 minutes to facilitate the separation of components.

From each extract, 100 µl of the resulting supernatant was carefully withdrawn and diluted by adding 900 µl of 0.02 M ortho-phosphoric acid. This step ensured the preparation of suitable samples for subsequent analysis. High-performance liquid chromatography (HPLC) analysis was conducted using an HPLC Agilent 1200 series system equipped with a diode array detector (DAD). A critical aspect of the analysis was the chromatographic separation, which was executed on an LICHROSPHERE-RP C18 column (4 × 250 mm) maintained at a constant temperature of 30 °C.

**Chromatographic Conditions**

Under the chromatographic conditions employed, isocratic elution was achieved using 0.02 M orthophosphoric acid as the mobile phase, with a flow rate set at 0.5 mL/min to ensure efficient separation. Detection was accomplished with a UV detector configured to monitor at 254 nm, precisely capturing the ascorbic acid (AsA) signal. Each sample was introduced into the HPLC system in volumes of 10 µl. These conditions yielded a well-established retention time of 5.96 minutes for ascorbic acid (AsA), providing a robust foundation for accurate quantification.

**Conversion of Dehydroascorbic Acid (DHA)**

To determine the total ascorbic acid content, a reduction reagent, tris-2-carboxy-ethyl phosphine (TCEP), was introduced into the extract. TCEP was dissolved in 0.1 M HCl at a concentration of 0.1 M. The addition of TCEP facilitated the conversion of dehydroascorbic acid (DHA) into its reduced form, ascorbic acid (AsA). This conversion step was allowed to proceed for 10 minutes at room temperature, ensuring the accurate measurement of total ascorbic acid content (Wechtersbach et al., 2007).

**Calibration Curves for Quantitative Analysis**

For precise quantitative analysis, calibration curves were meticulously constructed. This involved the dilution of stock solutions of ascorbic acid (AsA) in 0.02 M metaphosphoric acid (3%) at known concentrations. Subsequently, the peak areas obtained from HPLC analysis (Figure 17) were plotted against the corresponding concentrations (expressed in mg/100 mL). This calibration process yielded a linear equation ($y = 90923x$) with a remarkable coefficient of determination ($R^2 = 0.999$).

### Expression of Ascorbic Acid Concentrations

The concentrations of ascorbic acid were expressed in µmol. $g^{-1}$ dry weight (D.W.), providing a standardized measure that accounts for the dry weight of the plant material.

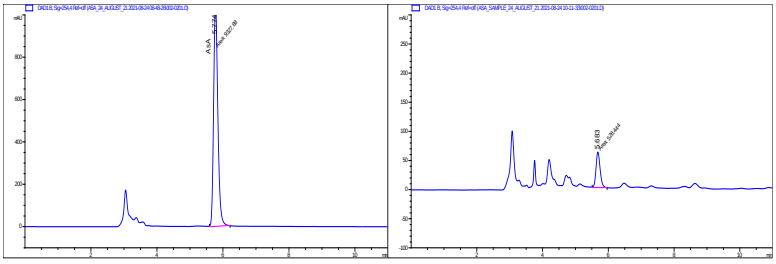


**Figure 17.** High performance liquid chromatography (HPLC) chromatograms of a) Standard of ascorbic acid b) ascorbic acid pick in sample

### *2.2.4 Extraction and quantification of Glutathione*

The precise determination of reduced glutathione (GSH) and oxidized glutathione (GSSG) was conducted employing high-performance liquid chromatography (HPLC) coupled with a coulometric electrochemical detector (ESA mod. 6210, Chelmsford, MA, USA). This method, described by Yap et al. (2010) and previously reported by Picchi et al. (2021), offers a reliable approach for the quantitative analysis of these essential compounds. An isocratic elution method was employed using a mobile phase composed of 25 mM monobasic sodium phosphate, 0.5 mM heptane sulfonic acid as an ion-pairing agent, and 2.5% acetonitrile to enhance separation. A Zorbax C18 column with dimensions of 250 mm × 4 mm was chosen for efficient separation and accurate quantification of two compounds, GSH (Glutathione) and GSSG (Glutathione disulfide). The flow rate was maintained at 0.6 mL/min to ensure consistent sample passage through the chromatographic system. To detect and quantify GSH and GSSG, an electrochemical detector with a four-array electrode system was utilized. Electrodes 1 and 2 served as screening electrodes to oxidize interfering compounds that might be present in the samples. GSH was detected at electrodes 3 and 4, with a retention time of 7.5 minutes, while GSSG was monitored at electrode 4 with a retention time of 9.5 minutes. To ensure accurate quantification of GSH and GSSG in the samples, a calibration curve was constructed. This curve was created using known concentrations of GSH and GSSG, which ranged from 0.001 to 0.004 mg. $ml^{-1}$. The calibration curve served as a reference for relating the detected signal to the precise concentration of these compounds in the analyzed samples. This analytical method allows for reliable and precise quantification of GSH and GSSG in various samples, making it suitable for a range of applications in research and analysis.

### *2.2.5 Statistical Analysis and Data Interpretation*

The results of each analysis were succinctly presented as the mean ± standard deviation (SD) of the replicates, ensuring clarity and transparency in reporting. To extract meaningful insights from the data, a series of statistical analyses and computations were performed.

- **Analysis of Variance (ANOVA)**

Two-way ANOVA was employed, with genotypes and water stress as the variation factors. This statistical approach, conducted using GraphPad Prism version 8.0 (GraphPad Software, Inc., San Diego, CA, USA), allowed for the assessment of how genotypes and water stress influence the observed variations in the data. Subsequently, Tukey's multiple comparisons test was applied to discern specific differences between groups. Statistical significance was established for p-values $< 0.05$.

- **Correlation Analysis**

Exploring the relationships among morphological variables and biochemical compounds, Pearson correlation coefficients were computed. This analysis provided valuable insights into how different traits are interrelated, shedding light on potential dependencies or patterns within the dataset.

- **Multivariate Analysis - PCA and Cluster Analysis**

Multivariate analysis, specifically Principal Component Analysis (PCA), was conducted to succinctly summarize and highlight variations among samples. This analytical approach, carried out using the XLSTAT2018 software (Addinsoft, Paris, France), enabled the identification of key patterns and clusters within the data, offering a holistic view of the dataset's structure.

- **Calculation of Relative Change (RC)**

The relative change, quantified as a reduction percentage, was determined for each trait to assess the impact of stress. The formula used for this calculation was:

$$\textit{Reduction percentage} = (\textit{control} - \textit{stress}) / \textit{control} \times 100$$

This computation elucidated the extent of change induced by the stress conditions relative to the control, providing a quantitative measure of stress response for each trait.

- **Stress Tolerance Index (STI) and Genotype Selection**

To identify better-performing genotypes under water stress conditions, iPASTIC, an online toolset, was employed to calculate the stress tolerance index (STI). Genotypes with the lowest average sum of rankings (ASR) were considered the most tolerant to water stress. This method facilitated the systematic selection of genotypes that exhibited superior stress tolerance, a critical aspect of the study's findings.

## 2.3 Results

### *2.3.1 Morphometric Response to Water Stress*

The assessment of morphometric traits provided valuable insights into the reactions of the studied accessions to water stress treatment. The collected data underscored a pronounced impact of drought stress, unveiling substantial diversity among the genotypes.

Plants undergo morphological and biochemical modifications in response to drought stress as a means of mitigating the adverse water conditions they face. Drought stress tolerance is a complex trait, and various studies have shown that plants can alter their phenotypes to adapt to unfavorable growing conditions caused by abiotic stress. A robust root architecture, characterized by large and elongated roots, plays a crucial role in mitigating the plant's response to drought stress, as indicated by parameters such as root weight (RW) and root length (MRL) in this study. In water stress conditions, resilient genotypes can rapidly reach deeper soil layers to accumulate plant reserves, thereby increasing their root length (RL) and root weight (RW). The effects of water stress on morphological traits were observed in the study, allowing genotypes better tolerate drought conditions to be identified (Figure 18). The assessment of morphometric traits proved to be instrumental in unraveling the effects of drought stress on the studied accessions.

**Figure 18. The effect of water stress in some *Brassica oleracea* genotypes studied**

The data we collected revealed a substantial and statistically significant impact of drought stress ($p < 0.001$) on various growth parameters (Table 11). Notably, drought stress exerted a significant influence on the weight and height of plants across most tested genotypes, resulting in reductions of 31.3% and 10.80%, respectively. However, what makes these findings particularly intriguing is the starkly contrasting response observed in the accession BR5 (*Brassica oleracea* var. *italica*), which exhibited substantial reductions in weight (-52.3%) and height (-31.8%). Similarly, the wild species BU (*Brassica rupestris*) displayed distinctive behavior, with a weight reduction of -9.7% and a height reduction of -35.7%.

While the majority of stressed plants exhibited a collective decrease in stem diameter (% variation = 14.91%), we observed noteworthy exceptions in specific accessions such as BR4, BU, and CI1, which demonstrated increases of -42.7%, -33.4%, and -26.5%, respectively.

**Table 11. Variation (Mean ± SD) in Key Growth Parameters among Twenty Accessions of Brassica oleracea Subspecies under Two Watering Conditions: Control (100% ETc) and Water Stress (35% ETc). Percent Reduction Relative to Control is Also Reported for Each Parameter.**

| accessions | conditions | Plant weight(g) | | Plant height(cm) | | Stem diameter(mm) | | Number of leaves | |
|---|---|---|---|---|---|---|---|---|---|
| | | MEAN±SD | Δ | MEAN±SD | Δ | MEAN±SD | Δ | MEAN±SD | Δ |
| **BH** | **control** | 1367,4± 642,6 | ns | 114,0±64,0 | ns | 18,8±0,3 | ns | 17,0±4,0 | ns |
| | **stress** | 673,0± 194,9 | 49,2 | 97,5±12,5 | 85,5 | 19,7±1,6 | 104,9 | 14,0±2,0 | 82,4 |
| **BR1** | **control** | 1758± 33,4 | ns | 75,6±13,4 | ns | 26,5±4,7 | ns | 23,0±2,0 | ns |
| | **stress** | 1012± 226,6 | 57,6 | 66,1±19,1 | 87,4 | 20,4±5,4 | 76,7 | 18,0±3,0 | 79,7 |
| **BR2** | **control** | 2586,4± 176,2 | *** | 131,8±0,3 | **** | 32,8±4,1 | ** | 22,0±1,0 | ns |
| | **stress** | 990,0± 6,0 | 38,3 | 78,0±4,0 | 59,2 | 18,1±0,7 | 55,1 | 26,0±2,0 | 116,7 |
| **BR3** | **control** | 2732,5±187,3 | *** | 95,0±10,4 | ns | 34,4±5,1 | * | 27,0±8,5 | * |
| | **stress** | 1330,0±223,9 | 48,7 | 81,3±13,4 | 85,6 | 22,4±2,4 | 64,9 | 19,0±1,0 | 70,7 |
| **BR4** | **control** | 1208,1±470,0 | ns | 83,0±15,1 | ns | 15,3±1,7 | ns | 19,0±4 | ns |
| | **stress** | 1421,8± 109,5 | 117,7 | 59,5±0,5 | 71,7 | 21,8±0,1 | 142,7 | 27±3,2 | 137,9 |
| **BR5** | **control** | 1052,4± 280,4 | ns | 85,0±11,0 | * | 28,8±16,3 | ns | 16,0±2,0 | **** |
| | **stress** | 1603,7±184,1 | 152,4 | 112±15,0 | 131,8 | 22,4±1,6 | 77,7 | 35,0±3,0 | 154,3 |
| **BTR** | **control** | 2134,3± 384,3 | ** | 115,0±10,0 | *** | 27,9±0,1 | ns | 21,0±1,0 | ns |
| | **stress** | 918,5±1,6 | 43 | 64,5±1,5 | 56,1 | 29,7±6,5 | 106,3 | 14,0±2,0 | 68,3 |
| **BU** | **control** | 738,6± 4,45 | ns | 70,0±25,0 | ns | 22,7±5,2 | ns | 17,0±1,0 | ns |
| | **stress** | 810,4±400,7 | 109,7 | 95,0±5,0 | 135,7 | 30,3±5,9 | 133,4 | 17±2,0 | 98 |
| **CC** | **control** | 2970,7±221,3 | * | 90,3±1,3 | ns | 31,8±7,9 | | 26,0±3,0 | ns |
| | **stress** | 2155± 192,5 | 72,5 | 81,8±0,5 | 90,6 | 25,5±0,5 | 80,4 | 25,0±3,0 | 93,7 |
| **CI1** | **control** | 5226± 707,4 | *** | 105,7±12,7 | ns | 22,3±2,9 | ns | 21,0±7,0 | ** |
| | **stress** | 3790,2± 240,2 | 72,5 | 108,5±3,5 | 102,6 | 28,2±0,1 | 126,5 | 30,0±1,0 | 142,9 |
| **CI2** | **control** | 2930,2± 990,0 | ns | 92,0±27,9 | ns | 30,6±5,4 | ns | 22,0±1,0 | ns |
| | **stress** | 2593,1±874,9 | 88,5 | 93,8±16,8 | 102 | 24,5±8,1 | 80,2 | 24,0±2,0 | 110,6 |
| **CI3** | **control** | 1928± 196,4 | ns | 118,5±5,5 | ns | 30,6±1,0 | ns | 31,0±3,0 | ns |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **stress** | 1660,2±391,0 | 86,1 | 94,0±5,3 | 79,3 | 21,8±3,3 | 71,4 | 28,0±4,0 | 90,3 |
| **CI4** | **control** | 4544,3± 1271,9 | *** | 124±5,3 | ns | 30,1±5,4 | ns | 29,0±3,0 | ns |
| | **stress** | 1817,9±423,6 | 40 | 101,7±12,6 | 82 | 23,9±3,2 | 79,4 | 26,0±2,0 | 89,8 |
| **CI5** | **control** | 3858,2± 52,5 | *** | 118,5±8,5 | ns | 27,5±1,9 | ns | 23,0±4,0 | * |
| | **stress** | 2197,8±66,2 | 57 | 99,5±10,5 | 84 | 24,9±2,5 | 90,8 | 31,0±1,0 | 136,2 |
| **CI6** | **control** | 4463,2± 413,1 | ns | 118,5±4,5 | ns | 31,5±2,8 | ns | 26,0±3,0 | ns |
| | **stress** | 3686,3±53,7 | 82,6 | 130,9±2,7 | 110,5 | 36,2±7,9 | 114,8 | 27,0±3,0 | 101,9 |
| **CI7** | **control** | 3539± 1439 | ns | 127,6±26,7 | ns | 40,8±1,0 | ns | 28,0±2,0 | ns |
| | **stress** | 3498,4± 2,0 | 98,9 | 133,5±1,5 | 104,7 | 32,3±3,9 | 79 | 22,0±2,0 | 78,8 |
| **CR** | **control** | 651,6±100,5 | ns | 36,5±5,5 | ns | 10,43±0,7 | ns | 7,0±2,0 | ns |
| | **stress** | 541,0± 191,0 | 83 | 28±1,0 | 76,7 | 8,3±0,3 | 79,4 | 6,0±2,0 | 78,3 |
| **CV1** | **control** | 5010,2 ± 866 | *** | 107,3±7,5 | ns | 29,23±0,3 | ns | 29,0±1,0 | ** |
| | **stress** | 2360,5± 266,1 | 47,1 | 94,25±5,75 | 87,8 | 18,1±4,6 | 61,8 | 19,0±5,0 | 65,5 |
| **CV2** | **control** | 5281,1±592,2 | ** | 93,0±7,0 | ns | 32,0±0,9 | ns | 29,0±2,0 | ns |
| | **stress** | 3957,1±440,8 | 74,9 | 92,75±2,75 | 99,7 | 28,2±0,06 | 88,1 | 23,0±3,0 | 79,3 |
| **CV3** | **control** | 775,5± 276,3 | ns | 76,5±23,5 | ns | 26,1±2,6 | ** | 16,0±3 | ns |
| | **stress** | 606,2± 157,2 | 78,2 | 51,5±0,5 | 67,3 | 11,3±0,1 | 43,3 | 16,0±1,0 | 97,9 |
| **ANOVA** | | | | | | | | | |
| **Genotype** | | *** | | *** | | *** | | *** | |
| **Condition** | | *** | | *** | | *** | | **ns** | |
| **G X C** | | *** | | *** | | *** | | *** | |

The variation in morphometric traits resulting from drought stress allowed to identify specific accessions that experienced a decrease, averaging around 30%. Examples of such accessions include BH1, BH2, and BH3 among the kale accessions, BR5 among the broccoli accessions, CCP4 among the cross-composite populations, and CV3 and CV4 among the cauliflower accessions. Overall, this suggests that tolerance to water stress involves different morphological and biochemical characteristics, reflecting diverse underlying stress tolerance mechanisms. Furthermore, this investigation revealed that water stress had a statistically significant impact ($p < 0.05$) on the number of leaves per plant. Some accessions, including BR5 and CI1, experienced remarkable increases of -54.3% and -42.9% in leaf count, hinting at a possible adaptive response to stress. It's worth noting that no significant interaction was observed between factors influencing SPAD readings ($p = 0.05$), indicating consistent chlorophyll levels across conditions.

The examination of soluble solids content (SSC) demonstrated a noteworthy reduction in response to drought stress (% variation = -5.9%). However, substantial variation existed among the twenty accessions under study. For instance, BU experienced a substantial decrease of

38.2% in SSC, while CI2, CV2, and CI5 exhibited increases of -46.2%, -35.3%, and -33.3%, respectively (as summarized in Table 12).

One intriguing exception was noted in the wild species BU, which displayed a -21.9% reduction in shoot fresh weight (FW) when subjected to reduced water supply (35%ETc), in contrast to other Brassica plants. The overall decline in total dry matter under drought conditions can be attributed to compromised plant growth and physiological functions, consistent with the decrease in various growth parameters we observed.

**Table 12. Variation (Mean ±SD) of leaves parameters in the studied accession.**

| Accessions | Conditions | Leaves fresh weigh(g) | | %Dry matter | | SPAD | | SSC (°Bx) | |
|---|---|---|---|---|---|---|---|---|---|
| | | MEAN±SD | Δ | MEAN±SD | Δ | MEAN±SD | Δ | MEAN±SD | Δ |
| **BH** | control | 85,9±24,1 | ns | 26.5±0.9 | ** | 60.4±18.9 | ns | 5.7±0.8 | ns |
| | stress | 30,1±2,5 | 34,9 | 17.0±3.4 | 64,2 | 50.2±7.4 | 83,2 | 5.8±0.4 | 102,6 |
| **BR1** | control | 60,6±90,5 | *** | 17.7±2.3 | ns | 67.9±0.9 | ns | 6.8±0.3 | ns |
| | stress | 50,6±4,8 | 8,3 | 15.1±0.9 | 85,4 | 65.2±7.9 | 96,1 | 6.2±1.0 | 91,4 |
| **BR2** | control | 909,5±2,5 | *** | 10.32±4.2 | ns | 53.2±9.9 | ns | 5.8±0.3 | ns |
| | stress | 319,6± | 35,1 | 10.9±0.7 | 105,3 | 65.0±3.9 | 122,3 | 6.5±1.0 | 111,4 |
| **BR3** | control | 1505,1±5,0 | *** | 11.9±1.7 | ns | 70.4±10.5 | ns | 7.3±0.8 | ns |
| | stress | 157,6±57,9 | 10,5 | 16.6±4.7 | 139,1 | 76.0±13.1 | 108,1 | 8.2±1.0 | 111.4 |
| **BR4** | control | 483,1±4,4 | ns | 11.6±0.7 | ns | 75.8±7.5 | ns | 7.3±1.3 | ns |
| | stress | 305,2±1,4 | 63,2 | 13.1±0.0 | 113 | 65.7±7.9 | 86,7 | 6.2±0.8 | 84,1 |
| **BR5** | control | 197,1±0,0 | ns | 26.1±0.7 | *** | 67.4±9.5 | ns | 6.2±0.8 | ns |
| | stress | 57,7±0,2 | 29,3 | 15.3±2.4 | 58,7 | 77.6±4.2 | 115,2 | 7.9±1.1 | 128,1 |
| **BRT** | control | 255±0,0 | ns | 18.2±1.9 | ** | 47.8±4.6 | ns | 3.5±0.5 | ns |
| | stress | 59,6±0,1 | 23,4 | 8.7±0.7 | 48,1 | 58.5±2.4 | 122,4 | 3.0±0.0 | 85,7 |
| **BU** | control | 123,6±0,0 | ns | 19.9±3.7 | ns | 52.5±6.7 | ns | 5.7±0.8 | * |
| | stress | 150,7±30,7 | 121,9 | 14.5±2.6 | 72,6 | 67±13.0 | 127,5 | 3.5±0.5 | 61,8 |
| **CC** | control | 118,3±0,0 | ns | 32.2±5.8 | *** | 49.2±1.3 | ns | 4.7±0.3 | ns |
| | stress | 64,6±0,2 | 54,4 | 15.4±1.2 | 48 | 66.2±3.8 | 134,6 | 4.2±0.3 | 90 |
| **CI1** | control | 302,3±0,0 | * | 20.9±1.3 | ns | 50.7±7.6 | ns | 5.3±0.6 | ns |
| | stress | 85,45±5,15 | 28,7 | 19.8±1.4 | 94,9 | 51.8±4.5 | 102,2 | 6.5±0.5 | 121,9 |
| **CI2** | control | 215±28,0 | ns | 14.8±4.0 | ns | 58.6±1.9 | ns | 4.8±0.3 | ** |
| | stress | 77,2±7,9 | 35,8 | 20.34±1.9 | 137,1 | 48.2±13.9 | 82,2 | 7.1±1.6 | 146.2 |
| **CI3** | control | 316,3±18,3 | ** | 14.4±1.8 | *** | 66.2±0.7 | ns | 5.3±0.6 | ns |
| | stress | 40,9±7,9 | 12,9 | 3.4±0.8 | 23,5 | 61.9±7.5 | 93,6 | 5.3±0.5 | 100 |
| **CI4** | control | 427,8±0,3 | *** | 17.7±3.1 | *** | 57.5±7.4 | ns | 6.8±1.6 | ns |
| | stress | 74,4±24,4 | 17,4 | 3.8±1.8 | 21,4 | 55.2±5.1 | 96,1 | 6.6±0.4 | 96,1 |
| **CI5** | control | 393,6±193,4 | *** | 9.4±1.8 | *** | 80.6±8.3 | ns | 5.8±0.75 | * |
| | stress | 96±0,0 | 17,5 | 21.8±1.9 | 43,2 | 71.5±3.2 | 88,7 | 7.7±0.3 | 133,3 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | control | 257,3±24,9 | *** | 17.1±3.8 | ** | 71.6±15.5 | ns | 6.1±0.5 | ns |
| **CI6** | stress | 135,6±15,6 | 52,7 | 8.3±3.0 | 48,4 | 58.6±3.6 | 81,8 | 5.5±0.5 | 90,2 |
| | control | 324,9±24,9 | *** | 11.9±2.8 | ns | 67.5±3.9 | ns | 4.3±0.8 | ns |
| **CI7** | stress | 169,8±51,8 | 52,3 | 11.4±0.0 | 95,1 | 58.7±16.4 | 87 | 5.7±0.3 | 130,8 |
| | control | 78,15±7,6 | ns | 29.5±4.5 | ns | 54.8±6.1 | ns | 4.0±0.5 | ns |
| **CR** | stress | 74,9±15,1 | 95,8 | 25.8±4.2 | 87,4 | 49.6±8.2 | 90,5 | 4.5±0.0 | 112,5 |
| | control | 923,6±16,5 | *** | 16.4±1.8 | ns | 58.6±3.8 | ns | 7.2±1.3 | ns |
| **CV1** | stress | 183,45±1,7 | 19,9 | 12.0±3.8 | 73,6 | 59.0±8.9 | 100,6 | 7.8±0.3 | 108,1 |
| | control | 538,1±11,9 | *** | 8.6±2.7 | ns | 54.4±6.6 | ns | 5.7 ±0.8 | * |
| **CV2** | stress | 231,7±11,9 | 43 | 10.7±0.0 | 124,1 | 54±5.9 | 99,2 | 7.7±0.8 | 135,3 |
| | control | 259,1±69,1 | ns | 18.8±4.3 | ns | 61.3±16.8 | ns | 7.0±0.5 | ns |
| **CV3** | stress | 51,0±1,3 | 19,7 | 19.3±3.0 | 102,6 | 55.7±6.3 | 90,8 | 6.3±0.8 | 90,5 |
| **ANOVA** | | | | | | | | | |
| **Genotype (G)** | | **<0.0001*** ** | | **<0.0001*** ** | | **<0.0001*** ** | | **<0.0001*** ** | |
| **Irrigation regime (IR)** | | **<0.0001*** ** | | **ns** | | **ns** | | **0.0160*** | |
| **G X IR** | | **<0.0001*** ** | | **<0.0001*** ** | | **ns** | | **<0.0001*** ** | |

In terms of drought susceptibility, the accessions BR2, BTR, and CI4 exhibited heightened reduction percentages in weight, height, leaves, and stem length of stressed plants. This suggests these accessions are more susceptible to the adverse effects of water stress due to their pronounced reductions in multiple morphometric traits. These findings collectively highlight the varying responses of different Brassica accessions to water stress.

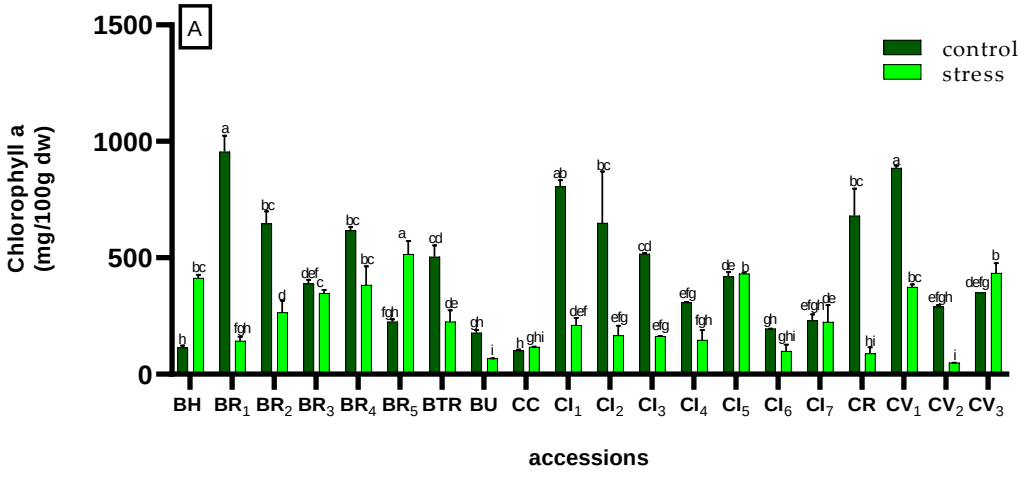
### *2.3.2 Effect of water stress on Photosynthetic Pigment*

The impact of water stress on photosynthetic pigments, including chlorophyll a (Chl a), chlorophyll b (Chl b), and total carotenoids (CAR), was investigated in the leaves of *Brassica oleracea* accessions (Figure19). The study revealed that water stress led to a decrease in the levels of these pigments in most of the analyzed accessions (13.out of 20), although the extent of reduction varied significantly among the accessions ($p<0.0001^{***}$).

Under normal, well-irrigated conditions, the concentrations of Chl a, Chl b, and carotenoids ranged from 101.5 to 955.5, 15.6 to 310, and 12.8 to 148.00 mg/100g D.W, respectively. However, under water stress, the *Brassica oleracea* genotypes responded differently, exhibiting significant shifts in their pigment levels. The reduction in leaf pigments under drought stress was evident, except for three accessions: BH (*Brassica oleracea* var. *acephala*), BR5 (*Brassica oleracea* var. *italica*), and CV3 (*Brassica oleracea* var. *botrytis*). Interestingly, these three accessions showed an increase in the amount of chlorophyll a in response to drought stress. Among the accessions, CC (*B. oleracea* var. *capitata*) exhibited the

lowest pigment contents, with 102, 28, and 129 mg/100g D.W for Chl a, Chl b, and carotenoids, respectively. The accessions BR1, CR, CI1, CI2, and CV2 showed the highest reductions in pigment content, with reductions exceeding 70% compared to the control conditions. However, in five accessions (CC, CI5, CI7, BR3, and CV3), there were no significant changes in photosynthetic pigment content between water-stressed plants and controls. Notably, accessions BH and BR5 displayed a significant increase in pigment content, with BH showing a three-fold increase and BR5 showing a two-fold increase.

These findings highlight the diverse responses of *Brassica oleracea* accessions to water stress in terms of photosynthetic pigment content. While most accessions experienced reductions in pigment levels, a few exhibited resilience or even increases in certain pigments. This variation suggests the presence of genetic variations and adaptive mechanisms within the species. Understanding these responses and identifying accessions with higher pigment stability or increased content under water stress conditions can aid in breeding programs aimed at developing drought-tolerant cultivars of *Brassica oleracea.*



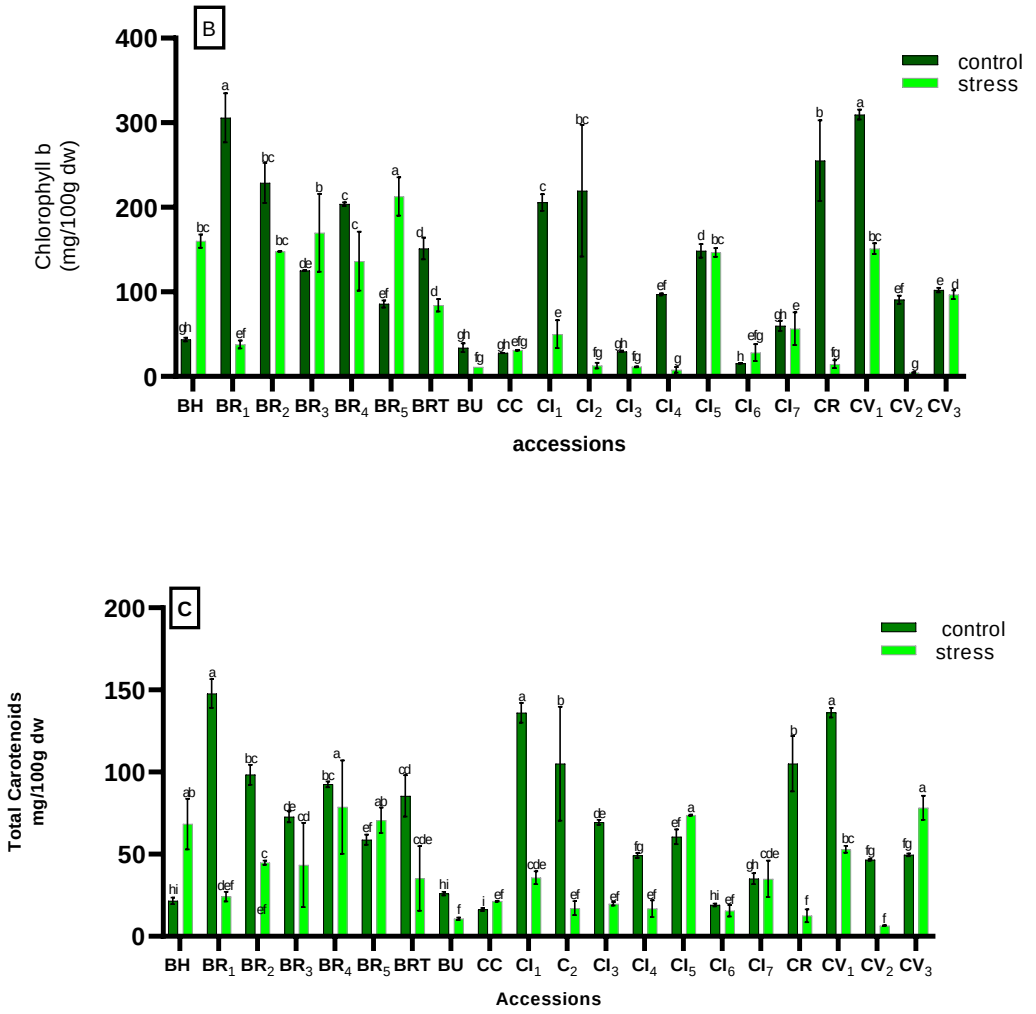



**Figure 19. Variations in Photosynthetic Pigment Content among Twenty Brassica oleracea Accessions under Water Stress Treatment (A) chlorophyll a (Chl a); (B) chlorophyll b (Chl b); (C) total carotenoids (Caro); and Error bars indicate SE (n = 3).**

For each accession, different letters indicate significant differences between accessions subjected to the same treatment according to Tukey's test ($\alpha = 0.05$).

According to previous studies, a reduction in chlorophyll levels during drought stress is considered an indicator of oxidative stress. This reduction can be attributed to the photooxidation of pigments and degradation of chlorophyll. The damage caused by reactive oxygen species (ROS) to chloroplasts is responsible for the decline in chlorophyll content under drought stress conditions (Wang et al., 2018). Furthermore, the decrease in photosynthesis during drought stress leads to an imbalance between light energy absorption and its utilization in carbon fixation, resulting in an excess of energy that can stimulate ROS production (Takahashi et al., 2008). However, a contrasting observation was made by Issarakraisila et al. (2007), where water-stressed plants exhibited an increase in leaf dry matter nitrogen concentration by more than 60% and a doubling of chlorophyll concentration. These plants also displayed a darker green leaf color compared to well-watered plants, indicating an increase in

chlorophyll content. This finding suggests that external factors can influence the physical and optical properties of leaves, leading to deviations from the expected linear relationship between chlorophyll meter readings and actual chlorophyll content. Hence, the correlation between SPAD meter readings, which are commonly used to estimate chlorophyll content, and actual chlorophyll content was not found to be strongly positive (r=0.49).

A decline in chlorophyll levels is commonly associated with drought-induced oxidative stress, the response of chlorophyll content to water stress can vary depending on the specific experimental conditions and the plant species studied (Flores-Saavedra et al.,2023). Factors such as nitrogen concentration and alterations in leaf properties under stress can affect the relationship between chlorophyll content and measurements obtained by chlorophyll meters. These observations highlight the need for careful interpretation and consideration of multiple factors when assessing chlorophyll levels as an indicator of drought stress in plants.

Similar trends in the changes of carotenoid and chlorophyll levels were observed in this study, with a general decrease noted after exposure to drought stress in most of the analyzed genotypes. This observation aligns with the commonly reported response of photosynthetic pigments to drought stress. However, the amount of carotenoids in leafy vegetables can be influenced by various factors, including species, variety, cultivar, maturity, and environmental growth conditions such as light, temperature, and soil quality. Consequently, certain genotypes may exhibit contrasting behavior, with higher pigment concentrations observed in stressed plants. These variations highlight the complexity of carotenoid regulation and suggest that different genotypes may possess unique adaptive mechanisms to cope with drought stress. A general decrease in carotenoid and chlorophyll levels is typically observed in response to drought stress, the specific responses can be influenced by multiple factors, including genetic variation and environmental conditions. The variability in pigment concentrations among genotypes underscores the need for considering the specific characteristics and traits of individual plant varieties when studying the effects of drought stress on carotenoid and chlorophyll content.

### *2.3.3 Effect of water stress on Ascorbic Acid and Glutathione*

The concentration of Ascorbic Acid (AsA), Dehydroascorbic Acid (DHA), Reduced Glutathione (GSH), and Oxidized Glutathione (GSSG) in Brassica leaves was investigated under water stress conditions, in the control plants, the levels of AsA varied between 0.22 and 1.70 μmol $g^{-1}$ DW, with the minimum and maximum values observed in CI3 and CV2,

respectively. However, when subjected to water stress, approximately half of the studied accessions (10 out of 20) exhibited a decrease in AsA content (Figure 20). Notably, CI3, BR3, BR4, and CV3 displayed a substantial increase in AsA content, ranging from three to more than thirty-fold compared to the control plants (Figure 21).During the drought-stress trial, the total Ascorbic acid (Asa+ DHA)content in the leaves ranged from 0.02 µmol $g^{-1}$ DW to 1.74 µmol $g^{-1}$ DW, with the largest variation observed in accession BR3, which experienced an increase from 0.08 to 1.74 µmol $g^{-1}$ D.W.

Three distinct sources of variation were examined: genotype (G), irrigation regime (IR), and their interaction (GX IR). The results indicate that each of these factors significantly contributes to explaining the observed variations. The genotype demonstrates a highly significant p-value of <0.0001 (****) in contributing to the total variation.

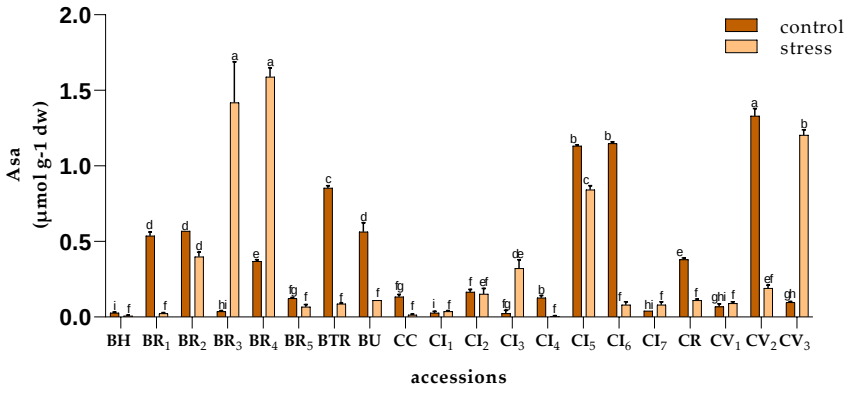



**Figure 20. The variation of ascorbic acid AsA in relation to water stress**

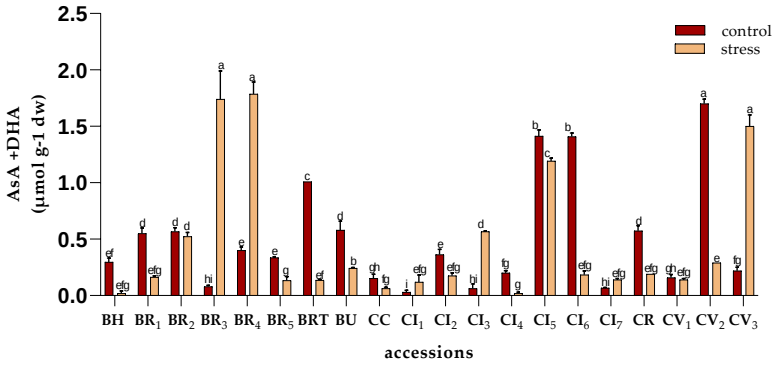



**Figure 21. Fluctuations in Ascorbic Acid (AsA) + DHA Levels Under the Influence of Water Stress**

The GSH content in Brassica leaves appeared to differ among the genotypes, as evident from the results shown in Figure 22. In the control trial, GSH content ranged from 0.02 to 0.75 µmol $g^{-1}$ DW for CI5 and CI7, respectively. Interestingly, almost half of the studied accessions

(9 out of 20) displayed an increase in GSH after experiencing drought stress, with levels ranging from 1.5 to approximately 7-fold higher than the control plants. Among the genotypes, the wild species BU exhibited the highest GSH content under stress conditions, with an increase from 0.65 to 1.35 µmol $g^{-1}$ DW. Conversely, accessions CI1, CI4, and CI7 demonstrated a significant decrease in GSH levels during the water stress trial, with percentage variations of 64.29%, 76.67%, and 41.34%, respectively.

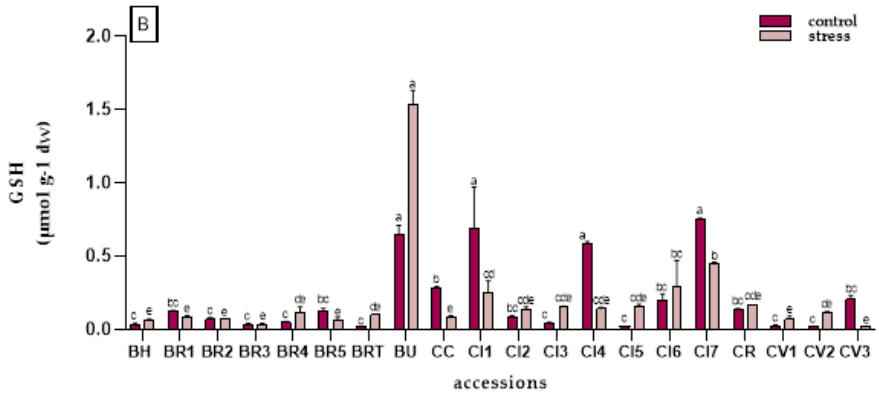



**Figure 22.** The variation of Gluthation GSH in relation to water stress

In the control conditions, the GSSG levels ranged from 0.32 µmol g−1 DW for the accession BR3 to 1.6 µmol g−1 DW for the accession CI7. However, in the drought stress trial, the lowest amount was recorded in the accession CR (0.3 µmol g−1 DW), while the highest was observed for CI6 (1.33 µmol g−1 DW) (Figure 22).

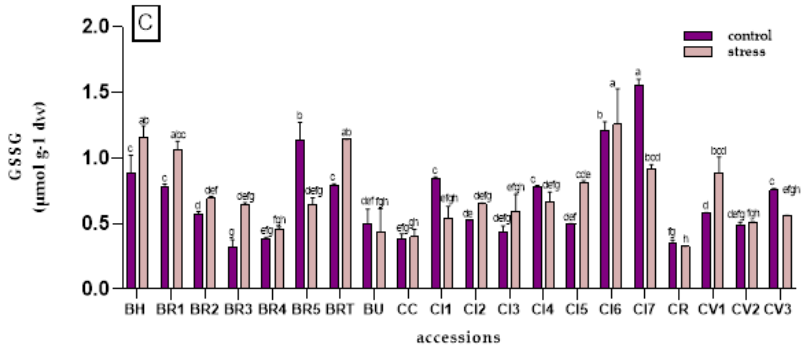



**Figure 23.** GSSG Variation in Response to Water Stress

The data presented in the study are expressed as mean ± standard error (S.E.). Different letters are used to indicate significant differences among accessions that underwent the same treatment, as determined by the Tukey test ($p < 0.05$). This statistical analysis allows for the identification of significant variations between accessions and provides valuable insights into the variability and significance of the observed results.

Regarding the glutathione content, the study observed lower levels of GSH (glutathione) under drought stress conditions. However, an interesting finding was that the genotype BU exhibited a distinctive response among the genotypes. It exhibited a significant increase in GSH

content following drought stress. Additionally, this genotype demonstrated greater morphological plasticity, showcasing its ability to adapt and respond to water stress. This particular observation highlights the importance of considering wild Brassica genotypes, such as BU, due to their high phytochemical content and potential tolerance to abiotic stresses like drought. These wild genotypes possess valuable traits that can contribute to the development of crop varieties with enhanced nutritional profiles and increased resilience to adverse environmental conditions. Overall, the findings of this study emphasize the significance of selecting and utilizing wild Brassica genotypes for their phytochemical composition and their ability to withstand and adapt to abiotic stress factors. Incorporating such genotypes into breeding programs can lead to the development of improved cultivars with enhanced nutritional value and increased tolerance to environmental challenges.

### *2.3.4. Effect of water stress on Total Phenolic Compound (TPC)*

The total phenolic content (TPC) of the studied *Brassica oleracea* accessions was analyzed, and the results are presented in Figure 24. Interestingly, TPC levels were significantly higher under drought conditions compared to control conditions for all the Brassica accessions analyzed ($p < 0.001$). There were notable variations in TPC levels among the different genotypes. Under control conditions, TPC concentrations ranged from 177 to 710 mg gallic acid equivalent (GAE) per gram of dry weight (D.W.). However, under water stress conditions, the TPC range expanded to 259-1594 mg GAE $g^{-1}$ D.W., representing a decrease of approximately 62.78% compared to control. It is worth mentioning that CV1 (*B. oleracea* var. *botrytis*) exhibited the highest TPC value under water stress, measuring 1594.1±108.5 mg GAE $g^{-1}$ D.W., which was more than double of the control. On the other hand, the wild genotype BU (*B. rupestris*) displayed the lowest TPC value, with 259.4±13.1 mg GAE $g^{-1}$ D.W.

Notably, the accessions CR (*B. oleracea* var. *gongylodes*) and CI5 (*B. oleracea incrocio*) showed the most significant increase in TPC values under water stress, with percentage variations of -376% and -361.6% respectively. This suggests that these genotypes exhibited a substantial enhancement in phenolic content in response to water stress. Additionally, BR1 (*B. oleracea* var. *italica*) displayed a moderate tolerance to water stress, as indicated by a percentage variation of -188.7% in TPC levels.

The total phenolic content in the studied Brassica accessions was significantly higher under drought stress conditions, with considerable variations observed among genotypes. Some genotypes exhibited a remarkable increase in TPC levels, surpassing the control values, while

others showed lower TPC values under water stress. These findings highlight the influence of water stress on the phenolic composition of Brassica crops, suggesting a potential role for phenolic compounds in the adaptive responses to drought conditions.

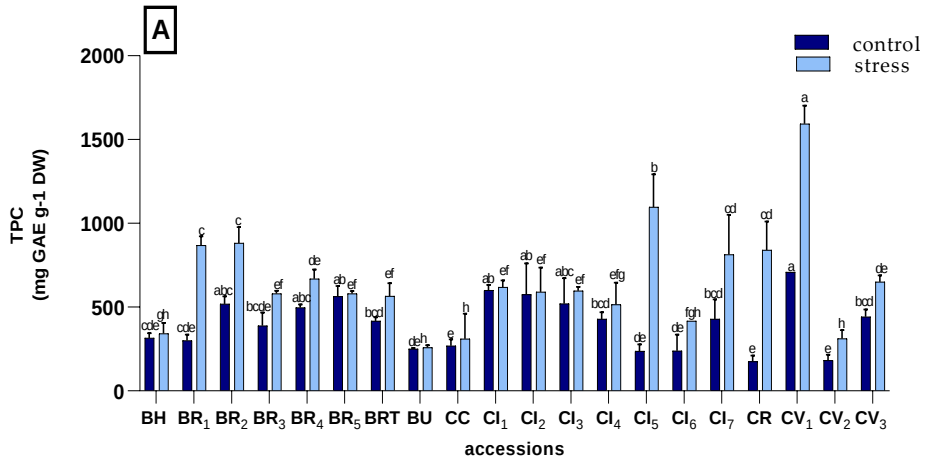



**Figure 24.** The varition of total phenolic compounds in relation to water stress

### Principal Component Analysis (PCA)

PCA (Principal Component Analysis) was performed on the biochemical composition of *Brassica oleracea* (as shown in Figure 25) in order to better understand how water stress affects different subspecies. The results of the analysis revealed that the first two principal components, PC1 and PC2, together explained 54% of the overall variability in the total. PC1, which accounted for 31.43% of the variability, played a significant role in distinguishing certain phytochemical parameters. Specifically, it separated GSH (Glutathione), GSSG (Oxidized Glutathione), and the carotenoid-to-chlorophyll ratio from the other phytochemical parameters. These substances had negative values along PC1, while the rest of the phytochemical parameters had positive values. This suggests that PC1 highlights a distinction between these specific compounds in response to water stress.PC2, contributing 22.57% of the variability, further enhanced the discrimination among the different biochemical parameters. It likely captured additional variations related to the effects of water stress on *Brassica oleracea* subspecies.

Under drought stress conditions, distinct and genotype-specific associations within the biochemical composition of B*rassica oleracea* were observed. Certain genotypes exhibited noticeable connections with specific biochemical components. In particular, genotypes BR3, BR4, and CV3 were marked by a pronounced correlation with Ascorbic Acid (AsA), highlighting their unique responsiveness to this antioxidant. CV1 showcased a distinct

inclination toward polyphenols, indicating a specialized metabolic response. Furthermore, BR2 and CV5 genotypes displayed robust links with carotenoids, signifying their specific involvement in the biosynthesis or accumulation of these pigments under stress conditions. Importantly, a significant number of other genotypes exhibited strong associations with both forms of glutathione, suggesting a more general and shared response mechanism. This underscores the significant impact of drought stress on Brassica metabolism. Interestingly, CC and BH performed consistently across normal and stressed conditions, implying water stress tolerance. The biplot (Figure 25) visually demonstrates varied responses of cultivars to drought stress, indicating diversity in their adaptive strategies.

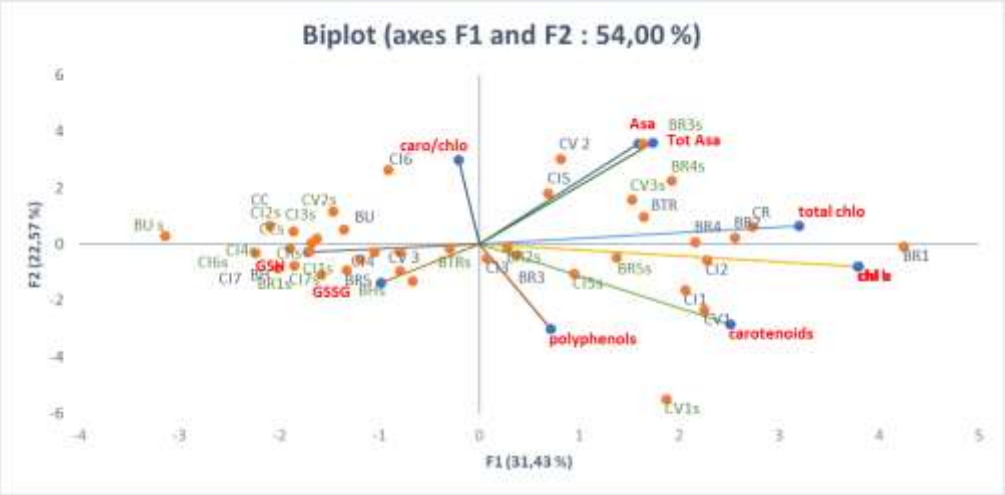



**Figure 25. Two-Dimensional Principal Component Analysis (2D-PCA) Demonstrating Water Stress Biomarker Biochemical Compounds.**

### Pearson's Correlation Coefficients

To assess the relationships between morphological and biochemical traits, Pearson's correlation coefficients (r) were calculated based on mean values separately for control and stress conditions. This allowed for a comprehensive understanding of drought stress effects on the studied traits. The correlation heatmap (Figure 26) illustrates these associations.

In control conditions, a strong positive correlation was evident between carotenoids and chlorophylls ($r = 0.9$, $p < 0.05$). However, this correlation weakened under drought conditions ($r = -0.058$). Notably, both control and drought conditions displayed positive correlations between polyphenols and chlorophyll a (Chl a) as well as chlorophyll b (Chl b).

In drought conditions, a significant positive correlation emerged between polyphenols and carotenoids ($r = 0.792$, $p < 0.05$). Additionally, total ascorbic acid exhibited positive correlations with Chl a ($r = 0.483$, $p = 0.05$) and Chl b ($r = 0.501$, $p = 0.05$), with the significance

varying across control and drought conditions. Intriguingly, a negative correlation was observed between Total Phenolic Content (TPC) and Ascorbic Acid (AsA) under control conditions ($r = -0.587$, $p < 0.05$).

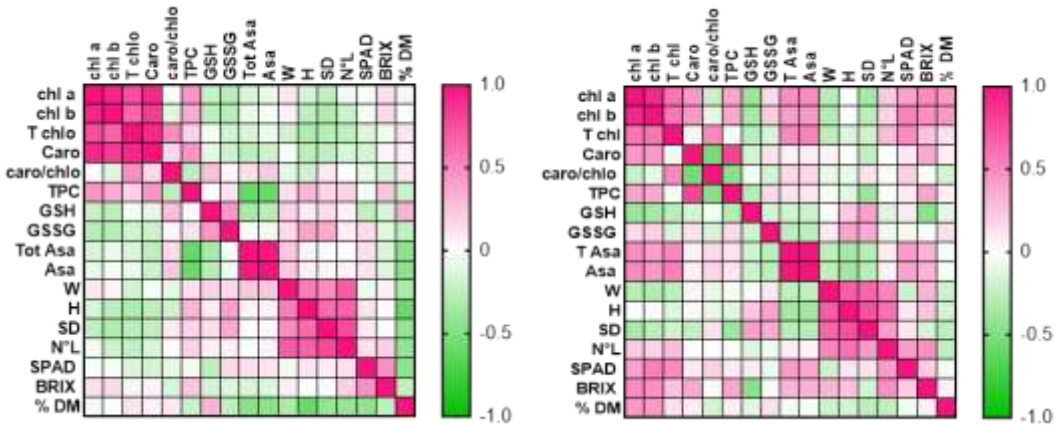



**Figure 26. Heat maps of Pearson's correlation between morphological and biochemical traits in a) control conditions; b) drought conditions.**

Chl a: chlorophyll a; Chl b: chlorophyll b; TChl: total chlorophyll (a+b); Caro: carotenoids; TPC: Total phenolic compounds; GSH: Reduced glu-tathione; GSSH: oxidized glutathione; Asa: ascorbic acid; Tot Asa: DHA+Asa; W: plant weight ; H: plant hight; SD: stem diameter; N°L: number of leaves ; % DM: dry matter ; SSC: Soluble Solids Content (° BRIX).

### *2.3.5 Screening of Brassica Genotypes using Stress Tolerance Index (STI)*

The drought tolerance of various Brassica genotypes was systematically assessed using the Stress Tolerance Index (STI), a method that quantifies drought stress based on dry matter. This evaluation utilized the iPastic online toolbox, a tool developed by Pour et al. The results, presented in Figure 27 as a bar graph, provided valuable insights into the drought tolerance levels of different accessions within the Brassica genus.

Eight genotypes, CR (STI value = 3.14), CC (2.05), BH (1.86), CI1 (1.71), BR5 (1.65), CV3 (1.50), CI2 (1.25), and BU (1.19), emerged as notably drought-tolerant. These genotypes exhibited the highest STI values, indicating their resilience to drought stress conditions. Four other genotypes, BR1 (1.10), CI5 (0.85), BR3 (0.82), and CV1 (0.81), displayed moderate tolerance to drought stress. Their STI values, while not as high as the drought-tolerant group, still suggested a reasonable level of resilience. The remaining genotypes, were classified as susceptible to drought stress. This diversity in response underscores the need for tailored strategies to enhance drought resilience in Brassica crops, taking into account the varying degrees of tolerance observed among these genotypes.

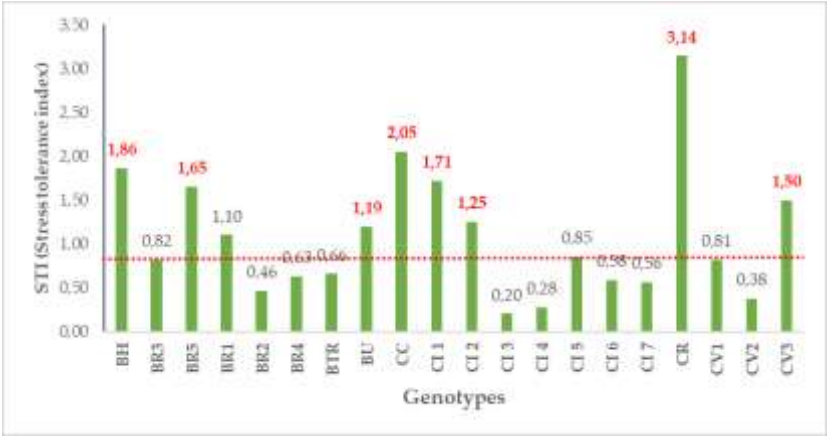



**Figure 27.** Stress tolerance index (STI) of 20 *Brassica oleracea* genotypes.

The horizontal red line indicated the median value (0.83). The red font values above the vertical bar are the highest STI which indicated the most drought tolerant genotypes**.**

To further assess the overall drought tolerance of the genotypes, a multifaceted analysis of various genotypes was conducted (Table 14), assessing their performance across critical parameters related to stress tolerance and biochemical composition. This finding revealed a diverse range of adaptations among the genotypes. Notably, genotypes CI 5 and CV1 emerged as exceptional accessions, displaying outstanding overall stress tolerance, as indicated by the Stress Tolerance Index (STI). These genotypes have proven to be well-suited for thriving in a variety of stress-prone environments. Furthermore, the emphasis on specific traits, such as chlorophyll content, antioxidants like GSH (Reduced Glutathione) and AsA (Ascorbic Acid), and sugar content, facilitated the identification of genotypes with specialized adaptations. For instance, BR4 exhibited remarkable assimilation rates, while CI 7 displayed high levels of Reduced Glutathione (GSH).

In the pursuit of a comprehensive genotypic assessment, the Average Sum of Ranks (ASR) was utilized to evaluate drought tolerance. Genotypes were categorized as possessing high drought tolerance when lower ASR values were observed, whereas higher ASR values indicated greater sensitivity to drought stress. Remarkably, CR (*B. oleracea* var. *gongylodes*), CC (*B. oleracea* var. *capitata*), and BH (*B. oleracea* var. *acephala*) consistently emerged as the top-performing genotypes in terms of drought tolerance, underscoring their resilience to drought conditions. Conversely, CI5 (*B. oleracea* var. *botrytis x italica*), BU (*Brassica rupesteris*), and CV1 (*B. oleracea* var. *botrytis*) exhibited higher ASR values, signifying their vulnerability to drought-induced stress.

**Table 13. Stress tolerance index**

| Genotypes | PW | PH | SD | N°L | DM | SPAD | SSC | chla | chlb | Tchl | caro | Polyphenols | GSH | GSSG | AsA | Tot AsA | ASR | Rank |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CR | 0.05 | 0,10 | 0,11 | 0,09 | 3,14 | 0,72 | 0,54 | 0,29 | 0,20 | 0,28 | 0,30 | 0,91 | 0,54 | 0,24 | 0,28 | 0,44 | 8,25 | 20,00 |
| CC | 0.85 | 0,76 | 1,07 | 1,28 | 2,05 | 0,87 | 0,59 | 0,06 | 0,05 | 0,05 | 0,08 | 0,51 | 0,57 | 0,33 | 0,01 | 0,04 | 9,16 | 19,00 |
| BH | 0.12 | 1,14 | 0,49 | 0,47 | 1,86 | 0,81 | 0,99 | 0,23 | 0,37 | 0,26 | 0,06 | 0,67 | 0,04 | 2,16 | 0,00 | 0,02 | 9,69 | 18,00 |
| CI 3 | 0.43 | 1,14 | 0,88 | 1,70 | 0,20 | 1,09 | 0,85 | 0,41 | 0,02 | 0,27 | 0,31 | 1,91 | 0,16 | 0,55 | 0,05 | 0,14 | 10,11 | 17,00 |
| BTR | 0,26 | 0,76 | 1,09 | 0,59 | 0,66 | 0,74 | 0,32 | 0,55 | 0,68 | 0,58 | 0,68 | 1,45 | 0,05 | 1,89 | 0,49 | 0,55 | 11,35 | 16,00 |
| CI 2 | 1,01 | 0,88 | 0,99 | 1,05 | 1,25 | 0,75 | 1,03 | 0,53 | 0,15 | 0,45 | 0,41 | 2,09 | 0,28 | 0,72 | 0,17 | 0,26 | 12,01 | 15,00 |
| CI 4 | 1,10 | 1,29 | 0,95 | 1,52 | 0,28 | 0,84 | 1,35 | 0,22 | 0,04 | 0,18 | 0,19 | 1,36 | 1,92 | 1,09 | 0,00 | 0,02 | 12,34 | 14,00 |
| BR1 | 0,24 | 0,51 | 0,71 | 0,83 | 1,10 | 1,18 | 1,25 | 0,67 | 0,62 | 0,66 | 0,81 | 1,61 | 0,25 | 1,75 | 0,08 | 0,36 | 12,62 | 13,00 |
| CV3 | 0,06 | 0,40 | 0,39 | 0,51 | 1,50 | 0,91 | 1,34 | 0,74 | 0,53 | 0,69 | 0,87 | 1,77 | 0,10 | 0,90 | 0,78 | 1,32 | 12,80 | 12,00 |
| BR 3 | 0,48 | 0,79 | 1,02 | 1,04 | 0,82 | 1,42 | 1,81 | 0,66 | 1,13 | 0,77 | 0,71 | 1,39 | 0,02 | 0,44 | 0,35 | 0,56 | 13,40 | 11,00 |
| CV2 | 2,79 | 0,88 | 1,19 | 1,31 | 0,38 | 0,78 | 1,31 | 0,07 | 0,02 | 0,06 | 0,07 | 0,35 | 0,05 | 0,52 | 1,69 | 1,97 | 13,45 | 10,00 |
| BR 5 | 0,23 | 0,97 | 0,85 | 1,08 | 1,65 | 1,39 | 1,47 | 0,57 | 0,97 | 0,65 | 0,94 | 2,01 | 0,19 | 1,55 | 0,05 | 0,18 | 14,75 | 9,00 |
| CI 6 | 2,20 | 1,59 | 1,51 | 1,35 | 0,58 | 1,12 | 1,01 | 0,09 | 0,02 | 0,08 | 0,07 | 0,61 | 1,34 | 3,20 | 0,61 | 1,03 | 16,41 | 8,00 |
| BR2 | 0,34 | 1,05 | 0,78 | 1,11 | 0,46 | 0,92 | 1,14 | 0,84 | 1,81 | 1,04 | 0,99 | 2,81 | 0,11 | 0,83 | 1,52 | 1,19 | 16,95 | 7,00 |
| CI 1 | 2,64 | 1,17 | 0,83 | 1,24 | 1,71 | 0,70 | 1,04 | 0,82 | 0,55 | 0,76 | 1,09 | 2,29 | 4,05 | 0,96 | 0,01 | 0,01 | 19,88 | 6,00 |
| BR4 | 0,23 | 0,50 | 0,44 | 1,01 | 0,63 | 1,32 | 1,36 | 1,15 | 1,48 | 1,23 | 1,64 | 2,05 | 0,13 | 0,38 | 3,93 | 2,86 | 20,34 | 5,00 |
| CI 7 | 1,65 | 1,74 | 1,74 | 1,24 | 0,56 | 1,05 | 0,74 | 0,25 | 0,18 | 0,23 | 0,28 | 2,15 | 7,83 | 3,00 | 0,02 | 0,04 | 22,72 | 4,00 |
| CI 5 | 1,13 | 1,21 | 0,91 | 1,42 | 0,85 | 1,53 | 1,33 | 0,88 | 1,16 | 0,94 | 0,35 | 1,61 | 0,08 | 0,85 | 6,38 | 6,75 | 27,37 | 3,00 |
| BU | 0,08 | 0,68 | 0,91 | 0,56 | 1,19 | 0,94 | 0,60 | 0,06 | 0,02 | 0,05 | 0,06 | 0,40 | 23,32 | 0,46 | 0,41 | 0,56 | 30,30 | 2,00 |
| CV1 | 1,58 | 1,03 | 0,70 | 1,08 | 0,81 | 0,92 | 1,67 | 1,61 | 2,50 | 1,80 | 8,97 | 6,96 | 0,03 | 1,09 | 0,04 | 0,09 | 30,89 | 1,00 |

## 2.4 Discussion

The findings reveal a notable trend of moderate to high reductions in morphometric traits, with variations discernible among the different accessions. This pattern aligns closely with a prior study conducted by Issarakraisila et al. (2007), where significant decreases in leaf area, fresh area, and dry weight were documented in Chinese kale under conditions of water deficiency. These outcomes underscore the sensitivity of morphometric parameters to water scarcity stress. Similarly, the research conducted by Souza et al. (2018) provides further support to the observations. In their study, cauliflowers subjected to water stress at 40% ETc (evapotranspiration crop coefficient) exhibited diminished plant height and reduced leaf number compared to those maintained at higher irrigation levels. This outcome underscores the impact of water deficit on the growth and development of cauliflower plants.

However, within the prevailing context of diminished morphometric traits in response to water stress conditions, this study illuminated a captivating aberration. Specifically, accession CV3 displayed a noteworthy contrast by showcasing higher plant weight under water stress conditions compared to the control group. This outcome hints at the existence of a unique resilience mechanism within the cauliflower CV3, as it managed to maintain or even enhance its biomass despite the imposed water deficit. This distinct behavior invites further investigation into the underlying physiological and molecular factors that contribute to cauliflower's resilience.

Chlorophyll concentration is commonly used as an indicator of drought stress, as drought can cause significant damage to photosynthetic pigments, leading to accelerated chlorophyll degradation. Both chlorophyll a and b are susceptible to drought stress. In this study, the impact of water stress on chlorophyll a and b was found to be highly significant. The current findings align with previous research, where genotype effects on chlorophyll a and b content in broccoli were observed. Under water stress conditions, the concentration of chlorophyll a was higher than that of chlorophyll b, which is consistent with other studies. Certain genotypes, such as BH and BR5, exhibited higher chlorophyll content, possibly indicating greater stress resistance. The reduction in chlorophyll is associated with oxidative stress and can result from photooxidation of pigments and chlorophyll degradation. Damage to chloroplasts caused by reactive oxygen species during drought stress contributes to the decline in chlorophyll levels. This decrease in photosynthesis can lead to the absorption of more light energy than can be utilized for carbon fixation, potentially increasing the production of reactive oxygen species.

(Foyer. 2018). However, there have been cases where water stress actually increased leaf dry matter nitrogen concentration and doubled chlorophyll concentration, resulting in darker green leaf color compared to well-watered plants. These observations suggest that the relationship between chlorophyll meter readings and absolute chlorophyll content can be influenced by external factors affecting the physical and optical properties of leaves (Padilla et al.,2019). Therefore, in this study, we did not find a strong positive correlation between the SPAD value (chlorophyll meter readings) and chlorophyll content.

This chapter highlights the significant impact of water stress on chlorophyll a and b concentrations, with genotypic variations playing a role. The reduction in chlorophyll levels during drought stress is linked to oxidative stress and chlorophyll degradation. However, the relationship between chlorophyll meter readings and absolute chlorophyll content may be influenced by other factors, emphasizing the complex nature of assessing chlorophyll levels under water stress conditions. Extensive research has been conducted on the composition of polyphenols in Brassicaceae plants, and various studies have investigated the concentrations of phytochemicals in organic and conventional fruits and vegetables. In this study, we observed that, in general, polyphenol levels increased following exposure to drought stress. Some genotypes even displayed two- to three-fold increases in polyphenol content compared to control conditions. These results underscore the complexity of the relationship between environmental stressors like drought and the production of valuable phytochemicals in Brassica crops, providing valuable insights for further research and agricultural practices. According to Heimler et al. (2006), an examination of various Brassica oleracea crops revealed that broccoli and kale exhibited the highest concentrations of total phenolics and flavonoids. It's well-established that the biosynthesis and accumulation of phenolic compounds in plants are influenced by environmental factors. Numerous studies have emphasized significant variability in antioxidant phytochemicals within the Brassica species, both across and within species, and even among different cultivars of the same species. Hence, the potential health benefits associated with cruciferous crops are primarily contingent upon their genetic characteristics. The composition of phenolic compounds can vary significantly among cultivars and even within the same plant, a phenomenon observed in turnip greens (Cartea et al., 2010) and tronchuda cabbage (Ferreres et al., 2008). These observations echo the findings of Heimler et al. (2006), where the total phenolic content spanned from 4.30 to 13.80 mg gallic acid per gram of dry weight. In a study conducted by Sousa et al. (2005), a comparison was made between the levels of phenolic compounds in leaves of *B. oleracea* var. *tronchuda* cabbage grown under

organic and conventional conditions. The outcome indicated that leaves from organically cultivated plants displayed higher levels of phenolic compounds. This distinction could potentially be attributed to the influence of mineral fertilizers and pesticides on phenolic compound production. Furthermore, Kaulmann et al. (2014) observed noteworthy differences in total phenolic compounds (TPC) among diverse Brassica cultivars. White Brassica cultivars exhibited the lowest amounts (ranging from 5.4 to 61.5 mg GAE per 100 grams of fresh weight), while red and purple Brassica types demonstrated the highest concentrations (ranging from 5.4 to 61.5 mg GAE per 100 grams of fresh weight). Green Brassica cultivars recorded TPC values spanning from 13 to 139 mg GAE per 100 grams of fresh weight. Remarkably, reports of up to a 200% disparity in total phenolic content among different broccoli cultivars have been documented.

One of the initial metabolic responses to both biotic and abiotic stressors is the generation of reactive oxygen species (ROS). The vitamin C content in Brassica plants exhibits significant variation both within and between species. Furthermore, understanding the variability of each chemical constituent within or among subspecies is crucial as it aids in estimating the maximum achievable concentration of each compound through genetic manipulation. In terms of quantitative analysis, kale has been reported to possess the highest concentration of vitamin C (82.14 mg/100 g) according to Singh et al. (2007). Another investigation found the ascorbic acid content to be 94.18 mg/100 g and 107 mg/100 g in fresh produce. Among Brassica genotypes, Brussels sprouts (76–192 mg/100 g FW) and kale (92–186 mg/100 g FW) exhibited the highest levels of vitamin C, followed by broccoli (34–146 mg/100 g FW), cauliflower (17–81 mg/100 g FW), and white cabbage (19–47 mg/100 g FW). The variation in vitamin C levels was more than four-fold in broccoli and cauliflower, 2.5-fold in white cabbage, and twice in kale. White cabbage generally displayed the lowest vitamin C content among Brassica crops.

In the present study, *Brassica oleracea* L. var. *botrytis* and var. *italica* genotypes demonstrated diverse behaviors. Three genotypes (CI5, CI6, and CV2) exhibited the highest vitamin C content under control conditions, but experienced a significant decrease under drought stress. Conversely, three genotypes (BR3, BR4, and CV3) showed a substantial increase in vitamin C content following drought stress. This differential response aligns with previous findings observed in other plant systems. Hence, multiple factors such as cultivar selection, harvest date, growth conditions, soil quality, and postharvest storage conditions influence the vitamin C content in Brassica crops. Considering the findings of, ascorbic acid (AsA) levels appear to be significantly affected by rapid oxidation to dehydroascorbic acid

(DHA) under unfavorable growth conditions. Broccoli has been identified as a good source of both ascorbic acid and glutathione, two natural plant antioxidants that offer various health benefits when consumed, as reported by Raseetha et al. (2013). The total ascorbic acid and total glutathione content in broccoli florets were found to be 5.18 and 0.70 µmol/g D.W, respectively. In terms of glutathione content, we observed generally low levels of reduced glutathione (GSH) following drought stress.

### 2.5 Conclusion

In conclusion, this study shed light on the complex effects of drought as a multifactorial stressor on *Brassica oleracea* cultivars. This chapter specifically focused on how water stress impacted the biochemical profile of these cultivars. The findings highlighted the diverse responses exhibited by different Brassica genotypes under water stress conditions. Notably, drought stress had a significant impact on various morpho-physiological and biochemical parameters crucial for plant growth and development, such as plant height, leaf number, fresh weight, dry weight, pigments, and antioxidant compounds. The study emphasized the importance of selecting cultivars with an efficient antioxidant system, as this trait was associated with higher chances of survival and improved performance under limited water conditions. By considering morphological and biochemical traits as indicators of abiotic stress response, the genotypes CR, CC, BH, CI, and BTR were identified as the most water stress-tolerant among the studied cultivars.

This finding accentuates the existence of genetic diversity among different genotypes in their ability to withstand drought stress, signifying the substantial role that breeding programs can play in enhancing tolerance to water stress. However, despite these significant findings, it is important to acknowledge that the understanding of the underlying molecular and physiological mechanisms governing plant responses to drought remains incomplete. Further research is needed to delve deeper into these pathways and gain a more comprehensive understanding of how plants navigate drought stress. Such knowledge will be valuable in developing effective strategies for improving drought tolerance in agricultural crops and mitigating the negative impacts of water scarcity on plant productivity and food security. While the investigation into the effects of drought stress on *Brassica oleracea* has yielded valuable insights into its responses to abiotic stressors, the research activities doesn't conclude here. The next phase of the study delves into a different aspect of *B. oleracea* evaluation, focusing on agronomic traits, particularly GLSs

Continuing from the previous research focusing on genetic variability among cultivars in response to drought, the objective is to uncover distinct GLS profiles within Brassica genotypes. These profiles have the potential to impact various aspects of plant performance, including resistance to pests and pathogens, nutritional quality, and flavor. This understanding of GLS composition in *Brassica oleracea* enhances comprehension of this species and offers potential agricultural and nutritional benefits. As the exploration of agronomic traits progresses, the commitment remains to advance knowledge of *Brassica oleracea* from both ecological and agricultural perspectives. The research activities aim to contribute to a broader understanding of this remarkable plant and, ultimately, to the development of improved cultivation practices and crop varieties that are resilient against diverse environmental challenges while delivering nutritional and culinary value.

## Research line III: Evaluation of *Brassica oleracea* Based on Agronomic Trait: Glucosinolates (GLSs)

### 3.1. Introduction

The tolerance of different *B. oleracea* varieties to water stress is intricately linked to the accumulation and composition of specialized metabolites. These compounds serve dual purposes as defensive mechanisms for the plant and essential nutritional components for human consumption. Within this context, GLSs emerge as central factors, which are secondary metabolites present in Brassica species, have diverse roles in plant defense mechanisms and have gained attention for their potential health benefits. Assessing GLS content typically involves a combination of chemical analysis and the use of molecular markers associated with GLS biosynthesis genes (Coves et al., 2023).

The selection process of *B. oleracea* materials for this study involves a meticulous screening of numerous accessions or breeding lines to identify those with desirable GLS profiles. While a high GLS content is often preferred, it's important to note that the specific composition and balance of different GLS types can vary, depending on the intended use of the crop, whether it is for human consumption or as livestock feed. Within the realm of breeding programs, the selection of elite *Brassica oleracea* materials, guided by agronomic traits, particularly GLS content, holds paramount importance. This study has been designed to conduct a comprehensive examination of GLS variations in both the roots and leaves of a diverse accessions of *B. oleracea* landraces (LRs), encompassing 17 distinct genotypes, in addition to a composite cross population (CCP). The primary objective is to identify variations in GLS profiles that can be attributed to genetic factors and environmental conditions, particularly in response to abiotic stress, such as water scarcity. By closely examining both roots and leaves, this study aims to offer insights into GLS profiles and quantities across different plant tissues. Additionally, the goal is to investigate the expression of genes related to GLS accumulation, further enhancing the understanding of the plant's adaptive responses to abiotic stress. These insights have the potential to illuminate the intricate interplay between genetic factors and environmental cues in shaping GLS profiles within *B. oleracea.*

Ultimately, the findings from this study will provide invaluable insights for breeding programs focused on enhancing GLS content and composition within *Brassica oleracea.* This,

in turn, can improve the nutritional value of the crop, fortify its defense mechanisms against pests and diseases, and contribute to sustainable Brassica cultivation practices.

## *3.2.* Material and methods

### *3.2.1 Experimental design: Water Stress Trial*

The accession list consisted of three kale accessions (BH1-BH2-BH3), five broccoli accessions (BR1-BR2-BR3-BR4-BR5), five cauliflower accessions (CV1-CV2-CV3-CV4-CV5), and four composite cross populations (CCP1-CCP2-CCP3-CCP4). All the tested accessions were part of the Brassica collection at the Department of Agriculture, Food, and Environment (Di3A) of the University of Catania (UNICT). The experiment started with sowing the seeds in cellular trays using organic substrate. The trays were kept under cold greenhouse conditions on the experimental farm of the University of Catania, with natural light, starting from the beginning of September. After one month, the plantlets were transplanted into pots and allowed to grow for four weeks (Figure 28). Subsequently, the plants were divided into two groups: irrigated (IRR) and not irrigated (NIR). The IRR plants were watered to reach pot capacity, while the NIR plants did not receive any additional irrigation. After subjecting the NIR plants to two weeks of drought stress, all the plants were collected for recording morphometric and biochemical traits. Leaves and root samples were collected, washed, and dried before being stored at -80°C for one week. They were later freeze-dried for biochemical analysis.

**Figure 28. *Brassica oleracea* Pot Experiment in Greenhouse (Water Stress Trial)**

### *3.2.2 Extraction of Glucosinolate*

The extraction method of GLSs followed the International Standard Method ISO 9167-1, 1992, with some modifications and formal adoption by the European Commission (European Commission, 1990). The detailed procedure is as follows, 200mg of freeze-dried samples were boiled in 5 mL of 70% methanol at 70°C for 10 minutes. This step aimed to deactivate myrosinase, an enzyme responsible for the hydrolysis of GLSs. After boiling, the samples were centrifuged at 12,000 rpm for 20 minutes at 4°C. The resulting supernatant was collected. To obtain desulfoglucosinolates, 2 mL of the collected supernatant was loaded into a 25 × 8 mm inner diameter column containing 0.5 mL of DEAE-Sephadex A-25 resin (50% w/v). The resin was pre-conditioned with a 0.02 M buffer of acetic acid and pyridine. The GLSs present in the sample were hydrolyzed within the column by adding 75 µL (5 U/mL) of sulfatase E.C.3.1.6.1 from *Helix pomatia*. The column was then incubated overnight. Desulfoglucosinolates were eluted from the column using 1.5 mL of ultrapure water. The eluted desulfoglucosinolates were subjected to high-performance liquid chromatography (HPLC) analysis with a diode array detector.

### *3.2.3 Quantification of Glucosinolate and HPLC analysis:*

A stock of standard solution was prepared by dissolving ten intact GLSs standards (SIN, GRA, SIB, GNA, GAL, GER, GBS, GBN, NGBS, and GST) at a concentration of 0.2 M in 2 mL of Milli-Q water. The GLSs standards used in the study were obtained from ChromaDex (Santa Ana, CA, USA) Calibration standard solutions were prepared by diluting the stock solution to concentrations of 0.1, 0.2, 0.4, and 1.0 µmoles. $ml^{-1}$(Figure 40 annexe).

The desulphoglucosinolate extracts were injected into an HPLC-DAD system equipped with a Kinetech C18 column (250 × 4.6 mm, particle size 5 µm). The mobile phase consisted of ultrapure water (solvent A) and acetonitrile: water (20:80, v/v) (solvent B) at a flow rate of 1.1 mL/min. The injection volume was 20 µL. A binary gradient was used, starting with 100% solvent A for 5 minutes, followed by a gradual increase to 70% solvent A and 30% solvent B from 5 to 17 minutes. The composition was then maintained at 30% solvent A and 70% solvent B for 3 minutes. The total run time was 40 minutes. Chromatograms were recorded at 229 nm, and quantification was based on calibration curves of the external standards by comparing retention time (RT) and UV spectra (Figure 29). The results were expressed in micromoles per gram of dry weight. The mean and standard deviation (SD) were calculated based on triplicate experiments.

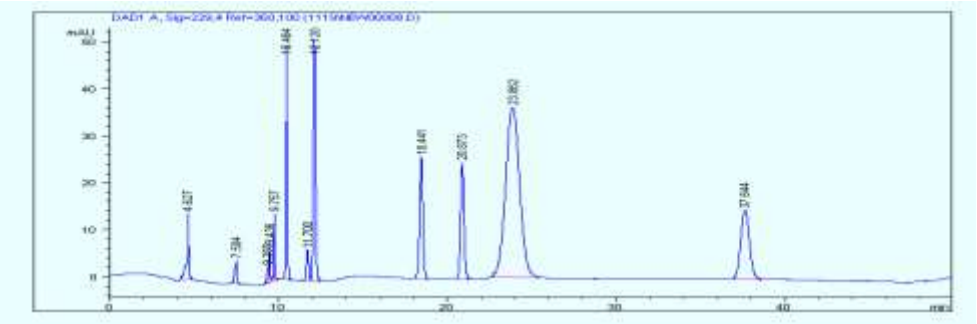


**Figure 29.** Chromatogram of the standards used to analyze the Glucosinolate profile

**1=glucoiberin, 2=progoitrin, 3=sinigrin, 4=glucoraphanin, 5=sinalbin, 6=gluconapin, 7=glucoerucin, 8=glucobrassicanapin, 9=glucobrassicin, 10=gluconasturtin.**

### *3.2.4 Transcriptomic Analysis*

#### 3.2.4.1 RNA extraction

Total RNA was extracted from the samples using the plant RNeasy mini kit (Qiagen), which included a DNase digestion step to remove any contaminating DNA. The concentration and purity of the RNA samples were assessed using a Nanodrop 1000 Spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE, USA). The integrity of the RNA was evaluated using a modified method based on Masek et al. (2005). In this method, 2 µg of total RNA were denatured at 65°C for 5 minutes in a mixture containing 50% (v/v) formamide, 1× TAE buffer, 5% (v/v) glycerol, and 0.025% (w/v) bromophenol blue. After denaturation, the samples were immediately chilled on ice and then loaded onto a 1% (w/v) agarose gel. To synthesize cDNA, 1.5 µg of total RNA from each sample was used in a reverse transcription reaction with the oligo(dT)$_{18}$ primer and the Revert AidTM H Minus First-strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany), following the manufacturer's instructions.

#### 3.2.4.2 Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Quantitative real-time PCR was carried out following the manufacturer's guidelines. A 20 µl reaction mixture was prepared as follows: 10 µl of 2x QuantiSpeed SYBR mix (Qiagen), 1 µl each of forward and reverse gene-specific primers (Table 15), 1 µl of template cDNA (100 ng), and 7 µl of distilled-deionized water. The thermal cycling protocol consisted of an initial denaturation at 95°C for 10 minutes, followed by 40 cycles comprising denaturation at 95°C for 15 seconds, annealing at 59°C for 15 seconds, and extension at 72°C for 10 seconds. The final extension step included 15 seconds at 95°C and 1 minute at 60°C.

**Table 14. Primer sequences and efficiency for GLS biosynthesis related genes used in the relative expression analysis through qPCR**

| Gene Name | Accession Number | cDNA Size (bp) | Forward Primer Sequence | Reverse Primer Sequence | Product Size (bp) | Primer efficiency values (%) |
|---|---|---|---|---|---|---|
| | **Transcription factor-related genes** | | | | | |
| ***MYB28*** | Bol007795 | 558 | CCACACCAGTTCAGAGAGGT | GGGAAATGGATCGAAGTCAGC | 221 | 98 |
| ***MYB29*** | Bol008849 | 513 | CGCCCAAGACTTCTGAGTT | TGATATTGCCCATGGAAGCTG | 234 | 95 |
| ***MYB34*** | Bol017062 | 951 | AAGGTGGATGGCGTACTCTC | TGTGAGTGGTTGGATCGACA | 279 | 98 |
| ***MYB122*** | Bol026204 | 981 | GACCATTCCGAGACATTGCC | GCATCGTGGATCATGTGGAG | 284 | 94 |
| | **Aliphatic biosynthesis-related genes** | | | | | |
| ***ST5b*** | Bol026202 | 1035 | AAGCCTTGACTTTCGCCATC | ACTTCACAACTGAGTCCGGT | 204 | 100 |
| ***FMOGS-OX6*** | Bol031350 | 1380 | ATGGCACCCTCTTGCAGTCC | AGTCGTAGACGCTAGAGTGG | 226 | 99 |
| ***GSL-OH*** | Bol033373 | 243 | GATTGTGCAAAAGGCTTGT | AGAGCATTAGGATTAGGAGGA | 188 | 96 |
| | **Indolic biosynthesis-related genes** | | | | | |
| ***ST5a*** | Bol026200 | 1017 | GTCCGGTTGCAAGATGGTTT | CCTCTCCGGGTTCTCTTTGT | 214 | 100 |
| ***CYP81F1*** | Bol028914 | 1497 | CTTTCCAACTGACGGCCAAA | CGTTAGGTCCGAGAAAAGCG | 257 | 99 |

To ensure robustness and reliability, three independent biological replicates were conducted, each with technical replicates. As part of quality control, we utilized the actin gene as a reference gene. This reference gene allowed us to normalize the expression levels of genes involved in GLS biosynthesis and breakdown. To calculate the relative expression levels for each sample, Livak's comparative $2^{-\Delta\Delta CT}$ method was employed. This method involves determining the average Cq value of the actin gene, which serves as a baseline. The relative expression levels of genes related to GLS biosynthesis and breakdown were accurately quantified by comparing their Cq values to that of the actin gene.

### *3.2.5 Data Analysis*

#### 3.2.5.1 Analysis of the Glucosinolate amount

The GLSs were analyzed and the data were presented as means ± standard deviation (SD). The statistical significance was determined by performing triplicate measurements and conducting a two-way analysis of variance (ANOVA) using CoStat software version 6.4. Tukey's multiple comparisons test was then applied, and p-values below 0.05 were considered statistically significant. To facilitate analysis, the data were transformed using the percentage rank of the analyzed matrix. To assess the correlation among individual GLSs, Pearson's correlation coefficient was calculated using SPSS software version 27. This coefficient helped determine the strength and direction of the relationships between different GLSs. The variation index (VI) was employed to describe the percentage of variation in morphometric traits between

the plants subjected to near-infrared (NIR) treatment and those under irrigated (IRR) conditions. The VI was calculated using the formula:

$$\mathbf{VI = -(100 - (Stress/Control \times 100))}$$

where Stress and Control represent the respective measurements under NIR and IRR conditions.

Principal component analysis (PCA) was conducted to highlight the contribution of each detected GLS, presented as a percentage relative to the total amount detected. This analysis aimed to discriminate different varietal groups of *B. oleracea* and identify the main GLSs associated with each group. The percentage values were normalized using the angular coefficient (DEGRES(ASIN(RACINE(x/100)))).

Additionally, the percentage of variation was determined for leaves collected from the NIR plot compared to the IRR plot by calculating ((NIR/IRR) × 100). This measurement helped evaluate the relative differences in leaf composition between the two plots.

### 3.2.5.2 Analysis of qRT-PCR parameters

- Reaction Efficiency

It is necessary to determine the efficiency of the reactions with the different primers used for accurate gene expression quantification. For this purpose, a real-time PCR reaction is performed with increasing dilutions of cDNA sample for each primer pair. The reaction efficiency is calculated based on the slope of the standard curve. This curve is established for each primer pair using the points from the calibration range (range of cDNA concentrations) and their corresponding qPCR Ct values, which are represented as Ct = f(LogN0), where N0 is the initial DNA concentration. This curve allows determining the coefficient of correlation $R^2$ (which should be > 0.98) and the reaction efficiency ($E = 10^{-1}$), which should be between 90% and 105% (Devers et al., 2004).

- Quantification of Relative Gene Expression

Real-time PCR is a fluorescence-based quantification method. The quantification kinetics rely on the real-time detection of the fluorescent signal, whose intensity is proportional to the amount of PCR product generated during successive cycles. The fluorescence detector is connected to software that translates the results into curves. The comparison of expression profiles among different samples and their technical replicates was performed using the QuantStudio™ Real-Time PCR software. For this purpose, the $2^{-\Delta Ct}$ method was applied, where ΔCt represents the difference between the Ct value of the target gene and the Ct value of the

reference gene. The Ct value of the reference gene is defined as the harmonic mean of the Ct values of the three commonly used reference genes. The analysis of relative quantification for each gene was performed by applying the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), where ΔΔCt represents the difference between the ΔCt value of the gene under control conditions and the ΔCt value of the gene under experimental conditions:

**ΔCt = Ct target gene - Ct reference gene**

**ΔΔCt = (ΔCt) control condition - (ΔCt) experimental condition**

The gene expression is expressed as $2^{-\Delta\Delta CT}$.

Promoter Analysis for Cis-Acting elements promoter regions harbor cis-acting elements (CAEs) that regulate the expression of genes. The cis-acting elements in the 1500bp region upstream of the studied genes were predicted using the Plant CARE online tool. (http://bioinformatics.psb.ugent.be/webtools/plantcare/h. The results obtained from PlantCARE can provide insights into the putative roles of specific cis-acting elements in gene regulation and their involvement in plant responses to various environmental stimuli.

### 3.3 Results

#### *3.3.1 Effects of drought stress on Morphometric Traits in Diverse Genotypes of Brassica oleracea*

Throughout the growth cycle, the average temperature remained relatively stable at 22.4 ± 5.8 °C, with an average daily solar radiation of 5.9 MJ/m². In this context, the study explores the complex interplay between environmental conditions and genetic diversity, revealing significant differences among the genotypes examined (Figure 30).

**Figure 30. phenotypical differences between the studied accessions in relation to water stress**

Plant Weight (PW): Notably, there was a compelling interaction between these two crucial experimental factors regarding plant weight (PW). The range of PW values was substantial, ranging from 535.0 g for BR3 when cultivated in the irrigated (IRR) main plot to a more modest 112.0 g for CV1 under non-irrigated (NIR) conditions. Genotypes CCP4 and CV3 exhibited a positive variation index (VI), indicating their ability to thrive in diverse conditions, with VI values ranging from 33.1 to 8.9. Conversely, BH3, BH2, CV4, BR5, and BH1 displayed negative VI values, ranging from -8.4 to -29.7, suggesting their adaptability and limited reduction in PW when grown in the NIR plot compared to IRR.

Plant Height (PH): Furthermore, the observations revealed a significant interaction between irrigation (IRR) and genotype (GE) concerning plant height (PH). PH values displayed a wide range, from a towering 69.8 cm for BH2 in IRR to a more diminutive 13.7 cm for CV2 in NIR. The variation index (VI) for PH ranged from -6.1 to -27.1, with BR2 and BR4 showing the most pronounced negative VI values.

Stem Diameter (SD): Similarly, stem diameter (SD) exhibited a remarkable interaction between IRR and GE (IRR × GE). SD values varied from 5.1 mm for BR1 under the conventional irrigation system to a slender 1.3 mm for BR1 and CCP4 under drought stress conditions. The genotypes displayed negative VI values, ranging from -20.7 to -29.4, emphasizing the substantial reduction in stem diameter for BH1 and BR5.

Number of Leaves (NL): The number of leaves (NL) witnessed a significant interaction between IRR and GE. NL values ranged from a prolific 15.0 leaves for CV3 under the IRR system to a modest 4.0 leaves for BR2 and CCP2 when grown in the NIR environment. The VI value for NL exhibited a diverse range, from 0.0 for CV4, indicating no observed variation compared to drought-stressed plants, to a notable -28.6 for BR1 and CCP4.

The SPAD index exhibited a significant interaction between the two experimental factors (IRR × GE). It ranged from 62.8 to 35.1 for CV5 and BH2 when grown using IRR and NIR protocols, respectively. The VI values for this index ranged from 0.0 to -28.5 for CCP2 and BH2, respectively

Regarding root weight (RW), we did not observe significant variation with IRR alone, but there was a notable interaction between the two experimental factors (IRR × GE). The VI ranged from 43.9 to 8.0 g for BR3 grown in the IRR plot and CV5 in the NIR system, respectively. The VI values ranged from -0.7 to -28.2 for BH2 and BR1, respectively (Figure31).

As for the main root length (MRL), a significant interaction between IRR and GE was also observed. MRL values ranged from 21.0 to 2.8 cm for BH2 and BH3 under the IRR and NIR regimes, respectively (Figure31). The highest VI value observed among all the genotypes was -30.0, indicating substantial variation among the genotypes, which was higher than the variations observed in the other traits analyzed.

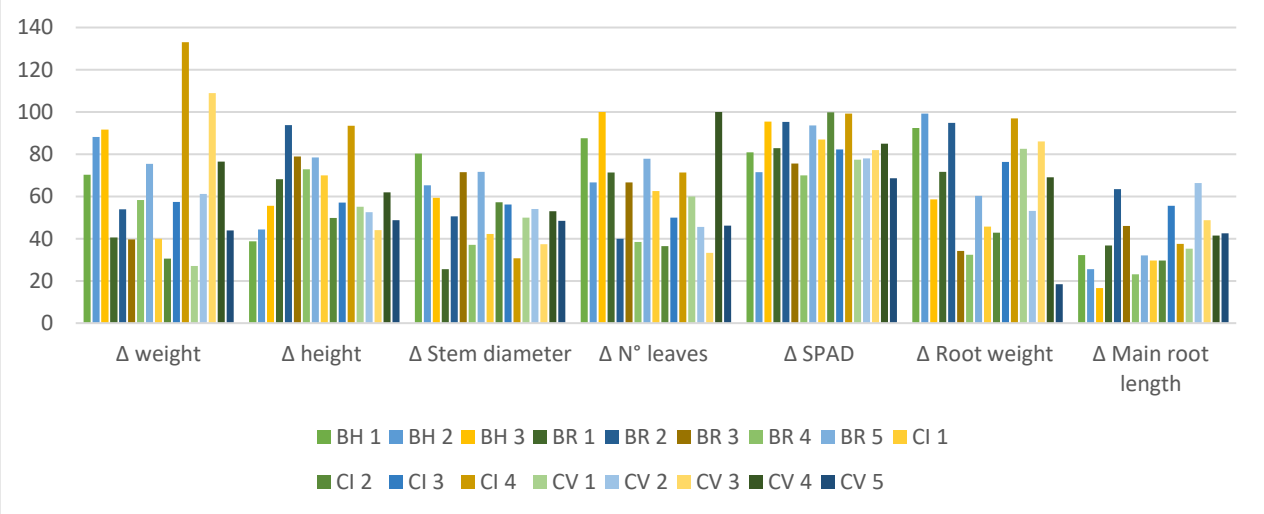



**Figure 31. Morphometric Trait Variations in Response to Water Stress**

### *3.3.2 Comparison between the Total Amount of GLS between Roots and Leaves*

The results indicate that water stress significantly impacted the amount and composition of GLSs in the different genotypes of *Brassica oleracea* studied. The roots of the plants exhibited a significant interaction between the experimental factors, specifically the genotype (G) and irrigation regime (IR). The concentrations of GLSs in the roots varied widely (Table 14), ranging from 38.2 to 2.5 µmol·g$^{-1}$ dry weight (D.W) for BH2 in non-irrigated (NIR) plots and CCP1 in irrigated (IRR) plots. Among the genotypes, BR4, CCP3, CV1, and BH1 had the highest GLSs concentrations in the roots, with concentrations ranging from 38.2 to 11.8 µmol·g$^{-1}$ D.W for BH2 in NIR and IRR plots, respectively (Table14).

Moving on to the leaves, they exhibited a higher total concentration of GLS compared to the roots across all the genotypes studied. The total amount of GLSs in the leaves was significantly influenced by the interaction between irrigation regime (IR) and genotype (GE). The concentrations ranged from 578.9 to 35.8 µmol·g$^{-1}$ D.W. for BR4 in the NIR plot and CV3 in the IRR plot. Specifically, BR4, BR5, BR2, CCP3, and CV1 displayed the highest GLS content in the leaves, ranging from 578.9 to 111.8 µmol·g$^{-1}$ D.W. for BR4 in the NIR plot and CV1 in the IRR plot. On the other hand, BH1, CV2, and BR3 showed the least variation in total

GLS content among the genotypes in both IR plots, with concentrations ranging from 126.2 to 66.9 μmol·g$^{-1}$ D.W. for BH1 in the NIR plot and CV2 in the IRR plot.

**Table 15. Variation in the total amount of GLSs (μmol g$^{-1}$ D.W.) in the roots and leaves in relation to the two experimental factors studied.**

| | Roots | | | Leaves | | |
|---|---|---|---|---|---|---|
| **Genotypes** | **IRR** | **NIR** | **Mean** | **IRR** | **NIR** | **Mean** |
| **BH 1** | 5.1 ± 1.0 | 9.7 ± 0.3 | 9.7 ± 3.6 | 122.7 ± 3.0 | 126.0 ± 3.7 | 124.4 ± 2.3 |
| **BH 2** | 11.8 ± 0.6 | 38.2 ± 1.6 | 38.2 ± 18.7 | 39.1 ± 2.5 | 82.5 ± 2.4 | 60.8 ± 30.7 |
| **BH 3** | 3.5 ± 0.2 | 5.1 ± 0.2 | 5.1 ± 1.1 | 48.4 ± 5.0 | 76.9 ± 1.7 | 62.7 ± 20.2 |
| **BR 1** | 4.7 ± 0.6 | 32.2 ± 1.8 | 32.2 ± 19.4 | 39.8 ± 2.8 | 72.7 ± 3.6 | 56.3 ± 23.3 |
| **BR 2** | 4.2 ± 0.0 | 6.2 ± 0.0 | 6.2 ± 1.4 | 184.3 ± 14.9 | 264.9 ± 4.2 | 224.6 ± 56.9 |
| **BR 3** | 8.8 ± 1.6 | 10.7 ± 0.1 | 10.7 ± 1.3 | 81.6 ± 3.2 | 98.2 ± 0.0 | 89.9 ± 11.7 |
| **BR 4** | 28.9 ± 5.4 | 36.1 ± 6.0 | 36.1 ± 1 | 291.4 ± 91 | 578.9 ± 33.5 | 435.2 ± 203.3 |
| **BR 5** | 9.2 ± 1.9 | 20.1 ± 1.9 | 20.1 ± 7.7 | 222.2 ± 17.9 | 336.7 ± 51.5 | 279.5 ± 80.9 |
| **CCP 1** | 2.5 ± 0.1 | 2.7 ± 0.2 | 2.7 ± 0.1 | 45.5 ± 1.0 | 66.2 ± 2.6 | 55.9 ± 14.7 |
| **CCP 2** | 4.3 ± 0.1 | 6.6 ± 0.6 | 6.6 ± 1.6 | 103.9 ± 67.3 | 138.7 ± 16.8 | 121.3 ± 24.6 |
| **CCP 3** | 19.6 ± 7.5 | 23.6 ± 5.3 | 23.6 ± 2.8 | 115.3 ± 35.9 | 400.1 ± 10.5 | 257.7 ± 201.4 |
| **CCP 4** | 5.2 ± 0.7 | 8.3 ± 2.1 | 8.3 ± 2.2 | 48.9 ± 0.0 | 99.2 ± 4.6 | 74.1 ± 35.6 |
| **CV 1** | 29.0 ± 0.1 | 32.1 ± 3.2 | 32.1 ± 2.2 | 111.8 ± 23.7 | 411.6 ± 5.1 | 261.7 ± 211.9 |
| **CV 2** | 4.9 ± 1.1 | 18.2 ± 1.2 | 18.2 ± 9.4 | 66.9 ± 7.2 | 78.6 ± 2.9 | 72.8 ± 8.3 |
| **CV 3** | 6.1 ± 0.9 | 16.6 ± 2.3 | 16.6 ± 7.4 | 35.8 ± 0.0 | 88.1 ± 0.0 | 62.0 ± 36.9 |
| **CV 4** | 10.9 ± 0.4 | 12.8 ± 1.6 | 12.8 ± 1.3 | 39.02 ± 2.4 | 66.9 ± 7.3 | 53.0 ± 19.7 |
| **CV 5** | 14.8 ± 1.1 | 15.3 ± 4.4 | 15.3 ± 0.4 | 114.6 ± 0.0 | 245.7 ± 0.0 | 180.2 ± 92.7 |
| **Mean** | 10.2 ± 8.4 | 8.4 ± 11.4 | | 100.6 ± 72.7 | 190.1 ± 156.0 | |
| **Significancy of the differences by ANOVA Student–Newman–Keuls** | | | | | | |
| **IR** | | ** | | | ** | |
| **GE** | | ** | | | ** | |
| **IR × GE** | | ** | | | *** | |

** and *** indicate that the correlation is significant at $p < 0.01$ and $p < 0.001$, respectively

The variation in GLS composition between roots and leaves provides the plant with adaptability in its defense responses. This flexibility in GLS distribution can influence how effectively the plant defends itself against different types of threats. Furthermore, the unique GLS profiles in these organs can have far-reaching consequences, impacting not just pest resistance but also the nutritional quality of various plant parts. Understanding the divergence in GLS accumulation between roots and leaves holds significant implications for the allocation

of defense resources within Brassica plants. This insight could guide efforts to breed and improve plants with optimized defense mechanisms and nutritional attributes. These improved varieties would not only possess fortified defense mechanisms but also improved nutritional value and overall performance.

The fold change in GLS levels in response to drought stress is utilized as a critical metric for assessing the extent of increase or decrease in GLS concentrations when compared to a non-stress or control condition. This measurement provides crucial insights into how drought stress impacts the abundance of GLSs within a plant. A positive fold change signifies an elevation in GLS levels under drought stress, as exemplified by the case of GBS in BR1, BH3, CCP3, and CCP1. Conversely, a negative fold change indicates a reduction, as observed in the instance of GBS in BR2, CV1, BR3, and CV2.

Notably, within the same plant variety, instances of both increased and decreased GLS levels are observed. This phenomenon can be attributed to genotype-specific variations in GLS biosynthesis. These findings are invaluable for gaining insights into how plants respond to drought stress and how their defense mechanisms, particularly those related to GLSs, are modulated by environmental stressors such as drought.

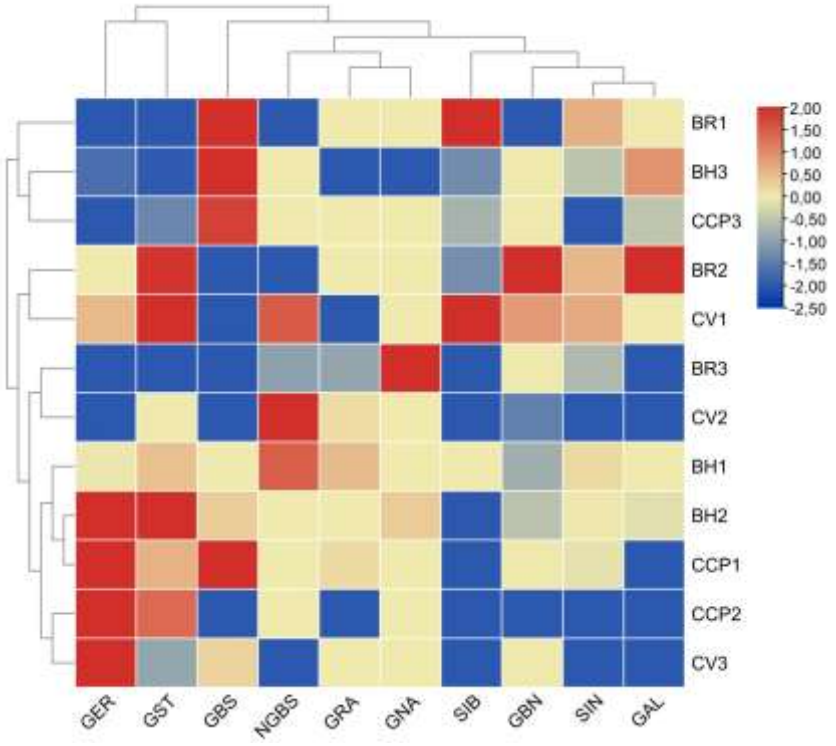



**Figure 32. Fold change of glucosinolates under drought stress**

### *3.3.3 Evaluation of Glucosinolate Variation in Roots*

In the profile of GLSs in the roots, each compound was influenced by the significant interaction between the two experimental factors, namely IR (irrigation regime) and GE (genotype). Here is a summary of the findings for each compound:

- Aliphatic Glucosinolates:

In this study, a substantial variation in aliphatic GLS content was observed, influenced by the intricate interplay of irrigation (IR) and genotype (GE). Specifically, SIN content exhibited significant disparities among genotypes and irrigation conditions (Table17). BR4 displayed the highest SIN content when cultivated in the NIR plot, whereas several genotypes, including CCP4, CV1, and CV5, exhibited minimal SIN content under IRR conditions. Notably, BR3 displayed substantial fluctuations in SIN content between the NIR and IRR plots, indicating its sensitivity to irrigation. Similarly, the data revealed intriguing fluctuations in GRA content across different genotypes and irrigation conditions. CCP4 exhibited a substantial increase in GRA content under IRR conditions, while GRA content remained undetectable in BR4 under both NIR and IRR conditions, highlighting the pivotal role of genotype in GRA synthesis. Additionally, BR3, CPP1, and CCP3 displayed noteworthy increases in GRA content when grown in both NIR and IRR plots.

The variability in GNA content was also apparent, with BR2 and BR4 displaying no detectable GNA content under both IR conditions, while CCP2 exhibited the highest GNA content under IRR conditions, emphasizing the genotype-dependent and irrigation-influenced nature of GNA synthesis. In terms of GER content, it exhibited substantial variation, with genotypes like BH3 and CV1 showing no detectable GER content under either IR condition, while BR5 displayed the highest GER content in the NIR plot, underlining genotype-specific differences in GER synthesis. Additionally, GBN and GAL content exhibited significant variations across genotypes and irrigation conditions, with BR4 recording the highest GBN and GAL content in the NIR plot. Conversely, CCP1 and CCP4 exhibited negligible GAL content in the NIR plot. These findings underscore the dual importance of genotype and irrigation conditions in determining GBN and GAL content.

Statistical analysis (ANOVA) confirmed the significance of these differences, with both genotype and irrigation conditions (IR x GE) playing pivotal roles. These results highlight the intricate nature of GLSs synthesis, where genotype-environment interactions exert a substantial influence on their content.

**Table 16.** Variation of the aliphatic glucosinolates content (µmol $g^{-1}$ D.W.) in roots in relation to the two experimental factors studied.

| Genotype | SIN | | | GRA | | | GNA | | | GER | | | GBN | | | GAL | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | IRR | NIR | Mean | IRR | NIR | Mean | IRR | NIR | Mean | IRR | NIR | Mean | IRR | NIR | Mean | IRR | NIR | Mean |
| **BH 1** | 1.1±0.8 | 1.3±1.6 | 1.2±0.1 | 0.3± 0.1 | 1.4±0.4 | 0.9±0.3 | 1.3±0.7 | 0.0±0.0 | 0.6±0.4 | 0.2±0.0 | 0.0±0.0 | 0.08±0.0 | 0.8±0.0 | 2.2±1.4 | 1.5±0.7 | 0.8±0.4 | 0.4±0.0 | 0.6±0.2 |
| **BH 2** | 1.3±0.5 | 0.8±0.4 | 1.1±0.4 | 0.4± 0.2 | 0.2± 0.0 | 0.3±0.1 | 0.2±0.0 | 0.2±0.0 | 0.2±0.0 | 0.7±0.0 | 6.9± 0.0 | 3.8±0.0 | 1.5±0.1 | 2.6±1.6 | 2.1±0.9 | 1.3±0.9 | 1.6± 0.0 | 1.5±0.5 |
| **BH 3** | 0.7±0.3 | 1.3±0.6 | 1±0.4 | 0.5± 0.0 | 0.3±0.1 | 0.4±0.1 | 0.7±0.1 | 0.4±0.0 | 0.6±0.1 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.8±0.2 | 0.3 ±0.0 | 0.6±0.1 | 0.5±0.1 | 0.5±0.0 | 0.5±0.1 |
| **BR 1** | 0.5±0.1 | 1.1±0.0 | 0.8±0.4 | 0.8±0.1 | 1.0±0.0 | 0.9±0.1 | 0.0±0.0 | 1.0±0.0 | 0.5±0.0 | 0.0±0.0 | 0.1±0.0 | 0.1±0.0 | 1.5±0.0 | 0.3±0.0 | 0.9±0.0 | 0.4±0.0 | 0.5±0.0 | 0.5±0.0 |
| **BR 2** | 0.0±0.0 | 1.3±0.0 | 0.7±0.9 | 0.4±0.0 | 0.8±0.0 | 0.6±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 2.2±0.0 | 1.1±0.0 | 0.8±0.2 | 1.3±0.0 | 1.1±0.1 | 7.6±0.9 | 0.6±0.2 | 4.1±0.5 |
| **BR 3** | 1.0±0.5 | 5.5±0.7 | 3.3±3.2 | 0.3±0.1 | 1.2±0.4 | 0.8±0.3 | 0.6±0.0 | 0.0±0.0 | 0.3± 0.0 | 0.0±0.0 | 0.3±0.0 | 0.2±0.0 | 1.3±0.2 | 3.3±1.5 | 2.3±0.9 | 0.3±0.0 | 15.7±8.2 | 8.0±4.1 |
| **BR 4** | 9.2±2.8 | 10.8±4.8 | 10±1.1 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0±0 | 0.0±0.0 | 0.0±0.0 | 0.1±0.0 | 1.6±0.1 | 0.9±0.1 | 3.0±2.3 | 12.5±0.1 | 12.5±1.2 | 5.8±2.1 | 8.6±0.7 | 7.2±1.4 |
| **BR 5** | 3.6±2.7 | 0.4±0.0 | 2.1±2.3 | 1.0±0.7 | 0.5±0.1 | 0.8±0.4 | 0.4±0.1 | 0.0±0.0 | 0.2± 0.1 | 1.3±0.6 | 7.1±0.1 | 4.2±0.4 | 1.7±1.0 | 1.6±0.2 | 1.7±0.6 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **CCP 1** | 0.2±0.0 | 0.4±0.1 | 0.3±0.1 | 0.2±0.0 | 0.4±0.1 | 0.3±0.01 | 0.1±0.0 | 0.1±0.0 | 0.1± 0.0 | 0.1±0.0 | 0.0±0.0 | 0.1±0.0 | 0.6±0.2 | 0.3±0.1 | 0.5±0.2 | 0.5±0.0 | 0.7±0.1 | 0.6±0.1 |
| **CCP 2** | 2.0±0.4 | 0.9±0.6 | 1.5±0.8 | 0.0±0.0 | 0.5 ±0.1 | 0.3±0.02 | 0.7±0.3 | 1.6±0.4 | 1.2±0.4 | 0.6±0.1 | 0.9±0.6 | 0.8±0.4 | 0.7±0.3 | 0.0±0.0 | 0.4±0.2 | 0.3±0.0 | 0.8±0.5 | 0.6±0.3 |
| **CCP 3** | 2.1±0.2 | 0.0±0.0 | 1.1±1.5 | 7.1±6.8 | 1.3±0.9 | 4.2±3.9 | 0.4±0.1 | 0.9±0.2 | 0.7±0.2 | 0.0±0.0 | 0.9±0.1 | 0.5±0.1 | 0.8±0.2 | 3.0±2.1 | 1.9±1.2 | 0.2±0.0 | 0.0±0.0 | 0.1±0.0 |
| **CCP 4** | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.3±0.2 | 1±0.0 | 0.7±0.1 | 0.2±0.1 | 0.0±0.0 | 0.1±0.1 | 0.2±0.1 | 0.0±0.0 | 0.1±0.1 | 0.8±0.1 | 2.3±1.6 | 1.6±0.9 | 0.6±0.1 | 2.3±1.1 | 1.5±0.6 |
| **CV 1** | 0.0±0.0 | 0.3±0.0 | 0.2±0.2 | 0.2±0.1 | 0.4±0.1 | 0.3±0.1 | 0.2±0.1 | 0.6±0.2 | 0.4±0.1 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.7±0.2 | 0.5± 0.1 | 0.6±0.2 | 0.0±0.0 | 1.6±0.6 | 0.8±0.3 |
| **CV 2** | 0.4±0.0 | 1.3±0.7 | 0.9±0.6 | 0.6±0.4 | 0.7±0.5 | 0.7±0.6 | 0.2±0.1 | 0.3±0.2 | 0.3±0.2 | 0.0±0.0 | 0.2±0.1 | 0.1±0.1 | 0.8±0.1 | 0.2±0.1 | 0.5±0.1 | 0.8±0.3 | 3.7±3.0 | 2.3±1.7 |
| **CV 3** | 0.2±0.1 | 0.5±0.5 | 0.4±0.2 | 0.3±0.1 | 0.3±0.2 | 0.3±0.2 | 0.3±0.2 | 0.2±0.2 | 0.2±0.2 | 0.0±0.0 | 0.1±0.1 | 0.1±0.1 | 0.0±0.0 | 0.1±0.1 | 0.1±0.1 | 0.9±0.1 | 0.3±0.1 | 0.6±0.1 |
| **CV 4** | 1.8±0.9 | 2.6±1.8 | 2.2±0.6 | 0.7±0.0 | 0.3±0.1 | 0.5±0.1 | 0.3±0.2 | 1.0±0.9 | 0.6±0.6 | 0.0±0.0 | 0.5±0.1 | 0.3±0.1 | 0.0±0.0 | 0.7±0.5 | 0.4±0.3 | 1.3±0.7 | 1.1±0.1 | 1.2±0.4 |
| **CV 5** | 0.0±0.0 | 1.0± 0.3 | 0.5±0.7 | 0.2±0.1 | 0.5±0.2 | 0.4±0.2 | 0.5±0.0 | 0.4±0.2 | 0.5±0.1 | 0.0±0.0 | 0.9±0.0 | 0.5±0.0 | 0.0±0.0 | 1.6±0.3 | 0.8±0.2 | 2.2±1.1 | 1.1±0.1 | 1.7±0.6 |
| **Mean** | 1.4±0.5 | 1.7±0.7 | | 0.8±0.5 | 0.6±0.2 | | 0.4±0.1 | 0.4±0.1 | | 0.2±0.1 | 1.3±0.1 | | 0.8±0.3 | 1.9±0.6 | | 1.4±0.4 | 2.3±0.9 | |
| **Significancy of the differences by ANOVA Student-Newman-Keuls** | | | | | | | | | | | | | | | | | | |
| **IR** | | *** | | | n.s. | | | n.s. | | | ** | | | n.s. | | | ** | |
| **GE** | | *** | | | *** | | | *** | | | *** | | | *** | | | *** | |
| **IR x GE** | | *** | | | *** | | | *** | | | *** | | | *** | | | *** | |

**Table 17.** Variation of the indolic and aromatic glucosinolates content (µmol $g^{-1}$ D.W) in roots in relation to the two experimental factors studied.

| Genotype | GBS | | | NGBS | | | SIB | | | GST | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | IRR | NIR | Mean | IRR | NIR | Mean | IRR | NIR | Mean | IRR | NIR | Mean |
| **BH 1** | 4.8±1.8 | 0.2± 0 | 2.5±0.9 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.4±0.0 | 0.4±0.0 | 0.4±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **BH 2** | 5.5±1.0 | 7.0±0.1 | 6.3±0.6 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.7±0.1 | 3.2±0.0 | 2.0±0.1 | 0.0±0.0 | 14.2±0.0 | 7.1±0.0 |
| **BH 3** | 0.3±0.0 | 1.1±0.4 | 0.7±0.2 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.3±0.1 | 0.3±0.0 | 0.3±0.1 | 0.0±0.0 | 0.3± 0.0 | 0.2±0 |
| **BR 1** | 0.0±0.0 | 0.9±0.0 | 0.5±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.8±0.2 | 1.1±0.1 | 0.6±0.2 |
| **BR 2** | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 4.5 ±0.1 | 2.3±0.1 |
| **BR 3** | 0.2±0.0 | 4.1±0.0 | 2.1±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.3±0.1 | 0.0±0.0 | 0.2±0.1 | 0.5±0.0 | 1.9±0.7 | 1.2±0.4 |
| **BR 4** | 6.0±0.3 | 0.6±0.1 | 3.3±0.2 | 0.0±0.0 | 0.3±0.0 | 0.2±0 | 2.8±1.3 | 1.2±0.3 | 2.0±0.8 | 2.4±0.6 | 0.7±0.2 | 1.5±0.4 |
| **BR 5** | 5.1±2.7 | 7.4±0.4 | 6.2±1.6 | 1.3±0.3 | 0.0±0.0 | 0.7±0.2 | 0.8±0.0 | 13.5±0.7 | 7.2±0.4 | 4.8±0.8 | 6.1 ±0.3 | 5.5±0.6 |
| **CCP 1** | 0.2±0.0 | 0.5±0.2 | 0.4±0.3 | 0.2±0.0 | 0.3±0.1 | 0.3±0.1 | 0.5±0.0 | 0.0±0.0 | 0.3±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **CCP 2** | 0.0±0.0 | 1.5±0.0 | 0.8±0.0 | 0.0±0.0 | 0.4±0.2 | 0.2±0.1 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **CCP 3** | 8.7±2.1 | 0.4±0.1 | 4.6±1.1 | 0.0±0.0 | 0.6±0.5 | 0.3±0.3 | 0.4±0.1 | 0.3±0.0 | 0.4±0.1 | 0.0±0.0 | 17.0±8.5 | 8.5±4.3 |
| **CCP 4** | 0.4±0.0 | 0.5±0.1 | 0.4±0.1 | 0.4±0.0 | 2.2±0.1 | 1.3±0.1 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 2.3±0.2 | 0.0±0.0 | 1.2±0.1 |
| **CV 1** | 0.6±0.3 | 2.1±1.3 | 1.4±0.8 | 0.4±0.4 | 2.0±1.2 | 1.2±0.8 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 27.0±1.4 | 25.0±0.9 | 26.0±1.2 |
| **CV 2** | 0.8±0.1 | 8.0±6.5 | 4.4±3.3 | 1.3±1.0 | 3.5± 0.5 | 2.4±0.8 | 0.1±0.1 | 0.4±0.1 | 0.3±0.1 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **CV 3** | 3.3±1.1 | 11.7±5.2 | 7.5±3.2 | 0.6±0.2 | 3.4±0.3 | 2.0±0.3 | 0.4±0.4 | 0.0±0.0 | 0.2±0.2 | 0.4±0.4 | 0.0±0.0 | 0.2±0.1 |
| **CV 4** | 6.5±1.7 | 0.0±0.0 | 6.5±1.7 | 0.0±0.0 | 5.9±1.4 | 3.0±0.7 | 0.3±0.3 | 0.4±0.1 | 0.3±0.2 | 0.0±0.0 | 0.5±0.1 | 0.3±0.1 |
| **CV 5** | 3.2±0.1 | 0.8±0.0 | 2.0±0.5 | 8.3±1.5 | 8.6±4.2 | 8.5±2.9 | 0.4±0.3 | 0.3±0.1 | 0.3±0.2 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **Mean** | 2.7± 0.7 | 3.1±1.0 | | 0.7±0.2 | 1.6±0.5 | | 0.4±0.2 | 1.2±0.1 | | 2.2±0.2 | 4.2±0.6 | |
| **Significancy of the differences by ANOVA Student-Newman-Keuls** | | | | | | | | | | | | |
| **IR** | | n.s. | | | * | | | * | | * | | |
| **GE** | | *** | | | *** | | | *** | | *** | | |
| **IR x GE** | | *** | | | *** | | | *** | | *** | | |

- Indolic Glucosinolates:

This investigation revealed substantial variations in the content of aliphatic and aromatic GLSs, underscoring the complex interplay between irrigation (IR) and genotype (GE). Indolic glucosinolates, including Glucobrassicin (GBS) and Neoglucobrassicin (NGBS), exhibited significant fluctuations influenced by these factors. GBS content ranged from 11.7 to 0.0 $\mu mol \cdot g^{-1}$ D.W., with notable variations among genotypes and irrigation conditions. NGBS, detected in lower concentrations, displayed content fluctuating from 8.6 to 0.0 $\mu mol \cdot g^{-1}$ D.W. Across all tested accessions, it was evident that genotype-specific differences and irrigation conditions played pivotal roles in determining indolic GLSs content (Table18).

- Aromatic Glucosinolates:

Shifting the focus to aromatic GLSs, Sinalbin (SIB) and Gluconasturtiin (GST) content also exhibited significant variations. SIB content ranged from 13.5 to 0.0 μmol·g−1 D.W., with notable differences observed among genotypes, particularly BR5, BR3, CCP1, and CV3, when grown in the NIR plot. Conversely, SIB was not detected in the roots of plants from several genotypes, irrespective of the IR conditions. Genotype BR 5 maintained certain levels of SIB even under low irrigation conditions (NIR), suggesting its capacity to endure SIB production in conditions of water scarcity. Similarly, GST content in the roots varied widely, with concentrations ranging from 27.0 to 0.0 $\mu mol \cdot g^{-1}$ D.W. The genotype CV1, when grown in the IRR plot, displayed the highest GST content, while several genotypes, including BH2, BH3, BR2, CCP3, and CV4 under IRR conditions, had no detectable GST. Remarkably, GST was not found in CCP4 and CV3 in the NIR plot, highlighting both genotype and irrigation regime's influence on GST synthesis (Table18).

The content of aromatic GLSs, specifically SIB and GST, exhibited notable diversity among genotypes and was significantly influenced by the irrigation regime. Genotype BR 5 demonstrated resilience in maintaining SIB levels under low irrigation conditions, while genotype CCP 1 displayed sensitivity to water stress. Additionally, GST content varied widely among genotypes and was affected by both genotype and irrigation conditions, underscoring the complex interplay of genetic and environmental factors in determining these GLSs levels.

These findings demonstrate that the content of various GLSs in the roots was influenced by both the irrigation regime and the genotype, resulting in significant variations in their concentrations. The investigation revealed substantial variations in the content of aliphatic and aromatic GLSs, underscoring the complex interplay between irrigation (IR) and genotype (GE).

Among the correlations observed, several pairs of variables stand out for their deep and significant relationships. Firstly, the correlation between SIN and GBN is notably strong, with a coefficient of 0.882. This suggests that these two variables increase or decrease in tandem. Similarly, the correlation between SIN and SIB is even stronger, at 0.896, indicating an even more profound positive association. Furthermore, GBN and GBS exhibit a robust positive correlation of 0.811, signifying that changes in one of these variables are strongly mirrored by the other. GER and GBN demonstrate a moderate positive correlation of 0.394, implying that these two variables share a noteworthy association. In contrast, GER and GAL exhibit a moderate negative correlation of -0.188, signifying an inverse association, where an increase in GER is linked to a decrease in GAL, and vice versa (Figure 33-A).

In water stress conditions, the strongest correlation observed is between GBN and SIN with a remarkably high correlation coefficient of r= 0.845. This strong positive correlation suggests that changes in SIN are closely tied to changes in GBN. When SIN increases, GBN tends to increase in tandem. Another notable correlation is between GER and GBS, which shows a substantial positive correlation coefficient (r= 0.809). This finding implies a significant link between GBS and GER When GER increases, GBS tends to increase as well (Figure 33-B). Regarding GAL and SIN also have a strong positive correlation, indicating that they tend to move together. An increase in SIN is associated with a substantial increase in GAL, and vice versa, revealing a notable positive association. GNA and GBN have a strong negative correlation (r=-0.39). An increase in GNA is associated with a substantial decrease in GBN, and vice versa. This indicates a notable inverse association between the two variables.

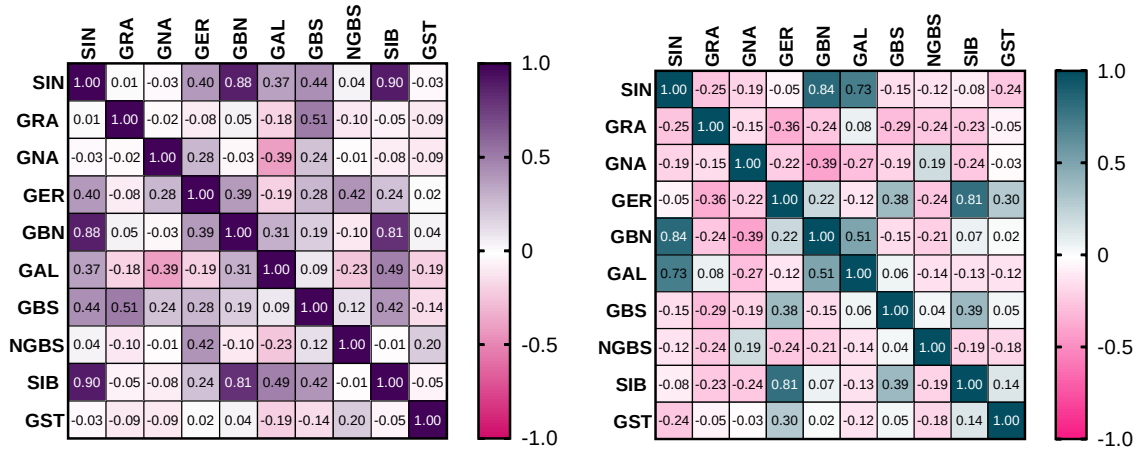



**Figure 33. Pearson correlation between the different Glucosinolate in roots (A) control conditions (B) in drought stress condition**

When comparing the correlations of different GLSs observed in the roots of *Brassica oleracea* under normal and water stress conditions, distinct patterns emerge. Under normal conditions, SIN and GBN display a strong positive correlation, as do SIN and SIB, highlighting their closely linked biosynthesis. Similarly, GBN and GBS exhibit a robust positive association, indicating synchronicity in their changes. GER and GBN share a moderate positive correlation, while GER and GAL display a moderate negative association. In contrast, under water stress conditions, GBN and SIN exhibit the strongest positive correlation, emphasizing their close interdependence, whereas GER and GBS reveal a substantial positive link. GAL and SIN also maintain a strong positive correlation. Notably, GNA and GBN establish a strong negative correlation, signifying an inverse connection. This comparison illustrates how water stress can alter the correlations between GLSs in *Brassica oleracea* roots, potentially reflecting the plant's adaptive responses to environmental challenges.

### *3.3.4 Evaluation of Glucosinolate Variation in Leaves*

The GLSs profile detected in the leaves showed a highly significant interaction between the two experimental factors studied (IRR × GE) for all compounds registered.

- Aliphatic Glucosinolate

The quantification of sinigrin (SIN) displayed a decreasing trend, ranging from 185.7 to 0.0 µmol·$g^{-1}$ D.W. The highest SIN levels were observed in BR4 when grown in the NIR plot, followed by CCP1 and CV3 under the same conditions.

Regarding glucoraphanin (GRA), concentrations varied from 36.4 to 0.0 µmol·$g^{-1}$ D.W. with the lowest levels detected in CV5 from the NIR plot and in BR2, BR4, CCP2, and CV2 from the IRR plot. Remarkably, GRA was not detected in the leaves of BH2, CCP1, and CV4 from the NIR plot, as well as BH3 and CV3 from both irrigation regimes.

Gluconapin (GNA) consistently displayed low levels across all genotypes, ranging from 83.1 to 0.0 µmol·$g^{-1}$ D.W. The highest concentrations were observed in BR3 from the NIR plot, followed by BR4, CCP2, CCP4, CV1, and CV2 from the IRR plot. CCP3 and CV3 did not exhibit any measurable GNA levels in the NIR conditions, while BH1, BR1, BR2, BR5, CCP1, and CV4 had no detectable GNA in both irrigation regimes.

The analysis of glucoerucin (GER) content revealed a range from 331.8 to 0.0 µmol·$g^{-1}$ D.W., with the highest levels observed in BR2, BR3, BR5, CCP1, CCP2, and CV4 in the NIR

conditions. CV2 did not exhibit any GER in the plants grown in the IRR plot, while BR4 did not register GER in either irrigation regime.

Glucobrassicanapin (GBN) content varied from 100.6 to 0.0 μmol·g$^{-1}$ D.W., with the highest levels observed in CCP4 from the IRR plot, followed by BH2, BR3, CCP3, and CV3 in the NIR conditions. Moreover, CV1 did not exhibit any GBN in the plants grown in the IRR plot, and BR1 did not register GBN in both irrigation regimes.

Finally, the quantification of glucoalyssin (GAL) revealed concentrations ranging from 77.8 to 0.0 μmol·g$^{-1}$ D.W. in CCP1, BR3, BR4, CCP3, and CV3 grown in the NIR conditions. GAL was not detected in BH1, BR1, and CV1 in both irrigation regimes.

This comprehensive analysis demonstrates the significant variability in the content of various GLSs (SIN, GRA, GNA, GER, GBN, and GAL) across different genotypes and irrigation regimes. It highlights how genetic factors and environmental conditions can influence the synthesis of these important compounds in plant tissues.

- Indolic Glucosinolates

The glucobrassicin (GBS) detected varied from 125.3 to 0.0 μmol·g$^{-1}$ D.W. for BR4 in the NIR condition and for BR2 and CV2 in the IRR plot. The GBS was not detected for BR3, BR5, CCP1, CV1, and CV4 grown in the NIR plot. For BH1, BR1, and CCP4, the GBS was not registered for the leaves of the plants grown in both IRs studied. The neoglucobrassicin (NGBS) varied from 63.6 to 0.0 μmol·g$^{-1}$ D.W. for CV2 in the NIR plot and for BH1, CCP1, CCP2, and CCP4 grown in the IRR plot, in decrescent order, respectively. The NGBS was not detected for BH2, BR1, BR3, and BR5 in both IRs studied (IRR and NIR). For BH3, BR2, and CCP3, we have not detected the NGBS in the leaves of the plants grown in the NIR plot (Table S5).

- Aromatic Glucosinolates

Different cultivars of Brassica exhibited a wide range of sinalbin (SIB) concentrations, varying from 20.1 to 0.0 μmol·g$^{-1}$ D.W., under different growth conditions. Among the cultivars, BR4 grown in the NIR plot displayed the highest SIB content, followed by BH1, BR1, CV2, and CV5 grown in the IRR condition, in descending order. However, SIB was not detected in BR3, CCP1, CCP3, CV3, and CV4 cultivated in the NIR plot, and BR2 was not found in either growth condition. Regarding the aromatic GLSs compound glucosinasturtiin (GST) displayed notable variability across the examined cultivars, with concentrations ranging

from 65.0 to 0.0 μmol·$g^{-1}$ D.W. CV1, BR1, BR3, and CCP2 cultivated in the NIR condition exhibited the highest levels of GST. However, neither BR4, CV2, nor CV4 showed detectable levels of GST in either of the growth conditions investigated. This chemotaxonomic analysis highlights the diversity of GLSs profiles among the different accessions.

The highly significant interaction effect (IR) for these traits highlights the genotype-specific responses to different irrigation regimes. Some genotypes demonstrate sensitivity to reduced water availability, with no measurable values under low irrigation conditions, while others exhibit adaptability, maintaining certain trait levels even with limited water. These examples illustrate the genotype diversity in how they respond to irrigation.

In a normal irrigation system (Figure 33-A), the correlation between GRA and GNA stands at approximately 0.518. This value suggests a moderately strong positive correlation between these two variables. In other words, when GRA increases, there is a tendency for GNA to also increase. Similarly, the correlation between SIN and GER is approximately 0.466, indicating a moderate positive correlation. This suggests that there is a positive association between SIN and GER, meaning that as SIN values increase, we tend to see an increase in GER values as well. Conversely, there are certain pairs of variables, such as GBN and GAL, which have correlations very close to 0. This indicates that there is little to no linear relationship between these variables. In practical terms, changes in GBN are unlikely to predict changes in GAL, and vice versa. Essentially, these variables are independent of each other, and one does not influence the other. Furthermore, it's worth noting that there exists a negative correlation between GER and GBS, with a coefficient of approximately -0.409. This negative correlation signifies an inverse relationship between GER and GBS. When GER values increase, there is a tendency for GBS values to decrease, and vice versa.

In drought stress conditions (Figure 33-B), the most prominent positive correlation is between GER and GST, with an exceptionally high coefficient of approximately 0.982. This signifies an extremely strong direct relationship between the two variables. A substantial negative correlation exists between SIB and GBS at approximately -0.222, indicating a significant inverse relationship. When SIB increases, GBS tends to decrease, and vice versa. There is also a notable negative correlation between SIB and NGBS at around -0.206, Aside from their strong negative correlation with other variables, SIB and GST exhibit a relatively strong positive correlation of approximately 0.982. This suggests that these two variables tend to move together, possibly indicating some underlying relationship. GER and GRA have a

relatively low positive correlation of about 0.031, implying a weak linear relationship between them. The two indolic GLSs GBS and NGBS have a moderate negative correlation of around -0.205, suggesting that as one variable (GBS) increases, the other (NGBS) tends to decrease.

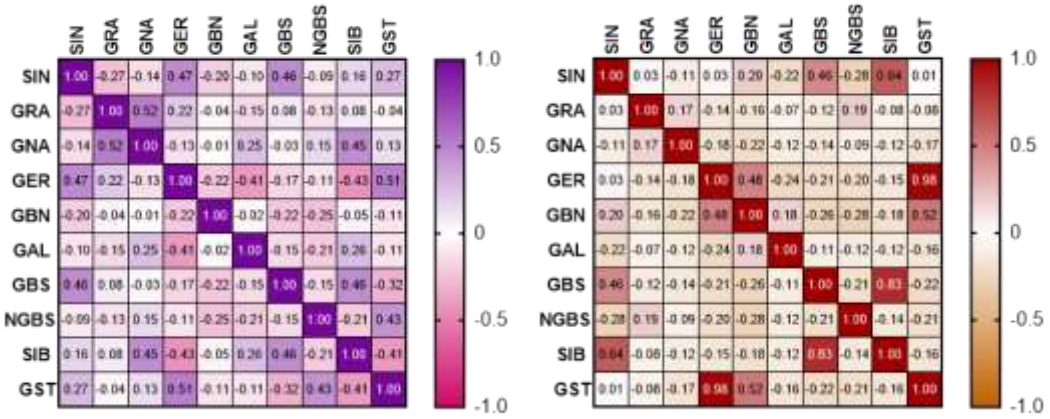



**Figure 34. Pearson correlation between the different Glucosinolate in leaves (A) control conditions (B) in drought stress condition**

The comparison of correlations between the GLSs compounds under normal irrigation conditions and during drought stress reveals several interesting findings: GRA and GNA (Normal vs. Drought Stress): Under normal irrigation, GRA and GNA exhibit a moderately strong positive correlation of approximately 0.518. However, during drought stress, GER and GST show an exceptionally high positive correlation of about 0.982. This suggests that while GRA and GNA are positively related under normal conditions, GER and GST become significantly more strongly linked during drought stress. The shift from a moderate to an extremely strong positive correlation may indicate a unique response of these compounds to water stress.

SIN and GER (Normal vs. Drought Stress): In normal conditions, SIN and GER display a moderate positive correlation of about 0.466. This indicates some positive association between these variables. However, during drought stress, GER and GST show a highly positive correlation of approximately 0.982, which is much stronger than the correlation between SIN and GER under normal conditions. This suggests that the relationship between GER and GST becomes notably stronger during drought stress, potentially signifying their shared response to water stress.

GBN and GAL (Normal vs. Drought Stress): In normal irrigation conditions, GBN and GAL have correlations very close to 0, indicating little to no linear relationship. This independence remains consistent during drought stress. In practical terms, changes in GBN are

unlikely to predict changes in GAL, or vice versa, both under normal conditions and during drought stress.

GER and GBS (Normal vs. Drought Stress): Under normal conditions, GER and GBS have a negative correlation of approximately -0.409, indicating an inverse relationship. However, during drought stress, GER and GST show an extremely strong positive correlation of about 0.982. This transition from a negative to a highly positive correlation is noteworthy and may signify a unique response of these variables to drought stress.

SIB and GBS / SIB and NGBS (Normal vs. Drought Stress): In both normal and drought stress conditions, SIB and GBS exhibit a significant negative correlation of around -0.222 and SIB and NGBS have a notable negative correlation of approximately -0.206. This indicates a consistent inverse relationship between these variables in both scenarios.

SIB and GST (Normal vs. Drought Stress): Interestingly, SIB and GST show a relatively strong positive correlation of approximately 0.982 during both normal and drought stress conditions. This suggests that these two variables tend to move together, indicating a robust relationship that persists regardless of water availability.

GER and GRA / GBS and NGBS (Normal vs. Drought Stress): GER and GRA have a weak positive correlation of about 0.031 under normal conditions, and GBS and NGBS have a moderate negative correlation of around -0.205. These relationships remain relatively stable during drought stress, suggesting that water stress does not significantly alter the associations between these pairs of variables.

### *3.3.5 Chemotaxonomy of the Different Accessions*

Chemotaxonomy, which involves classifying plants based on their chemical characteristics, reveals interesting insights into different *Brassica oleracea* varieties and their response to water stress conditions (Figure 34).

In the roots of broccoli (*B.oleracea* var. *italica*) and kale (*B.oleracea* var. *acephala*), aliphatic GLSs were the dominant group, comprising 61.8% and 55.3% of the total GLSs, respectively (Figure 34). However, when subjected to water stress, the percentage of aliphatic GLSs increased to 65.3% in broccoli, while indolic GLSs decreased from 16.3% to 10.9%. Aromatic GLSs also showed a slight increase from 21.9% to 23.8%. In kale, the percentage of aliphatic GLSs decreased to 48.0%, while aromatic GLSs increased significantly from 6.2% to 35.3%. Indolic GLSs also decreased from 38.5% to 17.8%.

In cauliflower (*Brassica oleracea* var. *botrytis*), indolic glucosinolates were the predominant group, constituting a substantial 66.0% of the total GLS content. Specifically, glucobrassicin, a type of indolic GLSs, made up 37.5% of the total GLSs. However, under water stress, glucobrassicin decreased to 32.4%, while neoglucobrassicin increased from 28.7% to 34.0%. Aliphatic GLSs accounted for 31.0% of the total GLSs in cauliflower, while aromatic GLSs were present in negligible amounts.

In CCP (*Brassica oleracea* var. *cross*) roots, there was a notably higher percentage of aromatic GLSs, comprising 50.0% under well-watered conditions, which rose to 56.0% under water stress. Simultaneously, the proportions of both aliphatic and indolic GLSs decreased from 31.0% to 28.6% and from 18.2% to 14.7%, respectively.

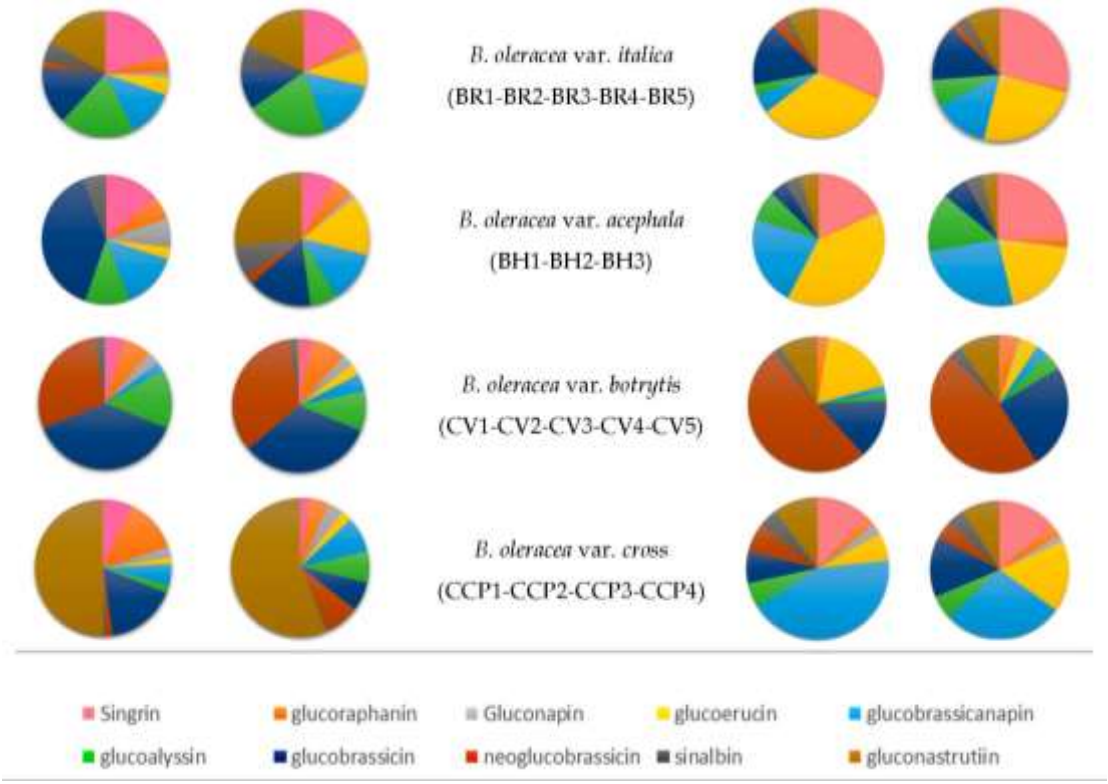



**Figure 35.** Varietal Classification of GLSs in Roots and Leaves under Water Stress: A Chemotaxonomic Analysis

These findings highlight the distinct chemotaxonomic profiles of different Brassica crops. Broccoli and kale are characterized by predominantly aliphatic GLSs, while cauliflower features indolic GLSs as the primary component. For CCP, aromatic GLSs are more prevalent. Additionally, it's worth noting that water stress conditions have a significant impact on the relative proportions of GLS groups, resulting in changes in their percentages for each variety.

### *3.3.6 Principal Component Analysis of Glucosinolate Profile in Leaves and Roots*

In this study, the aim was to assess the potential utility of GLS profiles as reference markers for the chemotaxonomic classification and distribution of four *B.oleracea* varieties: kale, broccoli, cauliflower, and a composite cross population. To visually represent the discerned variations in GLS profiles among the samples. The visual representation of the PCA (Figure 36) distinguishes different tissue types by color (leaves in green and roots in brown), and specific cultivar labels are thoughtfully provided for each group (kale, broccoli, cauliflower, and CCP). In the PCA results, PC1, accounting for 25.6% of the total variance, effectively segregated the crops based on their tissue types (roots and leaves). PC2, explaining 22.0% of the total variance, primarily captured the variability in GLS profiles between leaves and roots. These tissue-specific and cultivar-related differences were linked to specific GLSs, as illustrated in the PCA-loading plots, which depict the distribution of individual GLSs across the various cultivars.

In the left cluster of the biplot, the control and stressed roots clustered together and were associated with the majority of GLSs, including aliphatic, indolic, and aromatic types. Conversely, all the leaves formed another cluster in a different part of the biplot and correlated with three predominant aliphatic GLSs: singrin, glucoerucin, and glucobrassicanapin. However, it's noteworthy that the leaves of cauliflower under control conditions exhibited a positive correlation with the indolic glucosinolate neoglucobrassicin.

The loading plots derived from the PCA provided additional insights into the specific GLS compounds contributing to the observed patterns. In the case of the leaves, a cluster of GLS compounds, including singrin, glucoerucin, and glucobrassicanapin, emerged as dominant. These compounds displayed strong correlations with each other and with the leaves of the analyzed Brassica crops. In contrast, the roots displayed a distinct cluster of GLS compounds encompassing aliphatic, indolic, and aromatic types. These compounds demonstrated correlations with the roots of the Brassica plants, underscoring their prevalence in this tissue type.

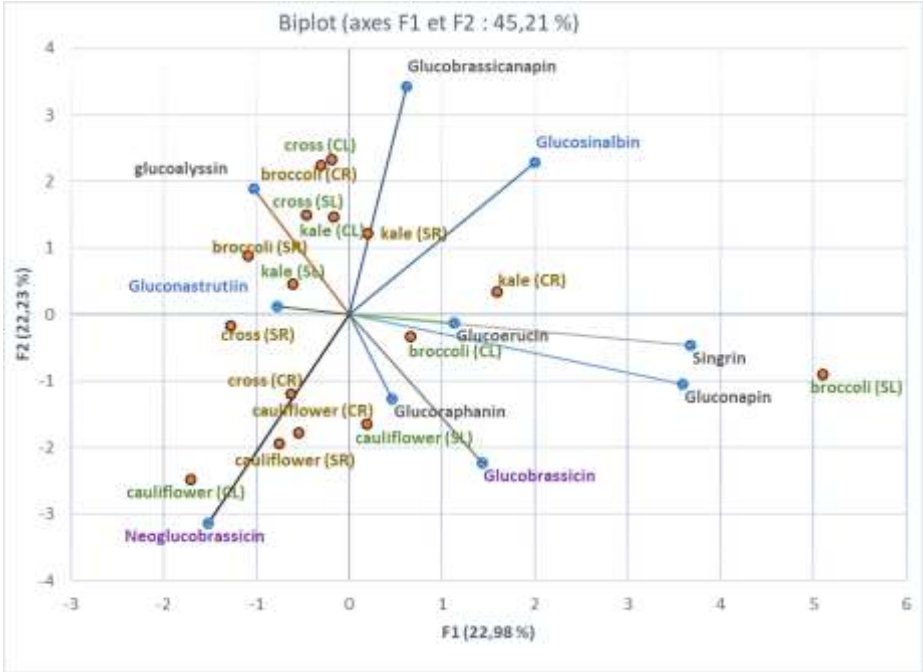



**Figure 36. Two-dimensional principal component analysis (2D-PCA) of GLS profile in leaves and roots.**

The PCA analysis served as a powerful tool for unraveling the utility of GLS profiles as markers for chemotaxonomic classification in *B. oleracea* varieties. These findings elucidate how these profiles can effectively differentiate between tissue types (leaves and roots) and offer profound insights into the intricate relationships among various *B. oleracea* cultivars.

### *3.3.7 Expression of genes related to Glucosinolate*

The heatmap presented here illustrates the co-expression patterns among genes and transcription factors (TFs) associated with GLSs biosynthesis. This analysis helps identify clusters or groups of genes that are co-regulated, shedding light on potential regulatory mechanisms. The colors or intensity values in the heatmap correspond to the expression levels of each gene and TF in different samples. Brighter colors represent higher expression, while darker colors indicate lower expression. Notably, the expression of these genes showed significant changes in response to drought stress, with a more pronounced impact on gene expression observed in stressed plants compared to the control group (Figure 37).

In addition, positive transcriptional regulators belonging to the MYB family were identified within the transcriptome. Specifically, MYB28 and MYB29 serve as transcription factors responsible for regulating the biosynthesis of aliphatic GLSs, whereas MYB34 and MYB122 play roles in overseeing the synthesis of indolic GLSs

The heatmap offers a clear representation of the varying expression levels of genes and TFs associated with Glucosinolate biosynthesis (Figure 37). The analysis provides valuable insights into the expression patterns of genes and transcription factors (TFs) related to GLS biosynthesis. Among these, MYB 122 and MYB 28 exhibit robust expression patterns in CCP2, CV1, and BR1. This suggests their significant roles in these specific tissues, potentially serving as key regulators of GLS biosynthesis. MYB 34, which play roles in overseeing the synthesis of indolic GLSs, stands out prominently in CCP2, CV1, and BR1, indicating its high expression levels and its crucial role in this biosynthetic process. Additionally, ST5a exhibits notable expression in CV2, CCP2, and CV1, highlighting its significance in these tissues for GLS biosynthesis. CYB81 F4 plays a pivotal role in regulating GLS biosynthesis in BH1, BR1, and BR2. Conversely, FMOG-ox5 is particularly noteworthy due to its pronounced upregulation in BR1 and BR2, emphasizing its significant role in regulating this biosynthetic pathway in those specific tissues. Moreover, CYP 97 F1 shows increased expression in CCP2, CV1, and BR, indicating its active participation in GLS biosynthesis within these particular tissues.

The heatmap analysis also reveals distinct clusters of elements with similar expression profiles: Cluster one includes CCP2, CV1, BR1, and BR2, which are closely grouped together, indicating they share similar patterns of gene expression. This cluster suggests that CCP2, CV1, BR1, and BR2 may have common regulatory mechanisms, possibly related to specific aspects of Glucosinolate biosynthesis. In the other hand, cluster two comprises CV2, BH1, CCP3, CV3, CCP1, BH3, BH2, and BR3. These accessions form another distinct cluster, suggesting that they share different gene expression patterns compared to those in cluster one. This cluster indicate a separate set of genes or conditions related to a different aspect of GLS biosynthesis.

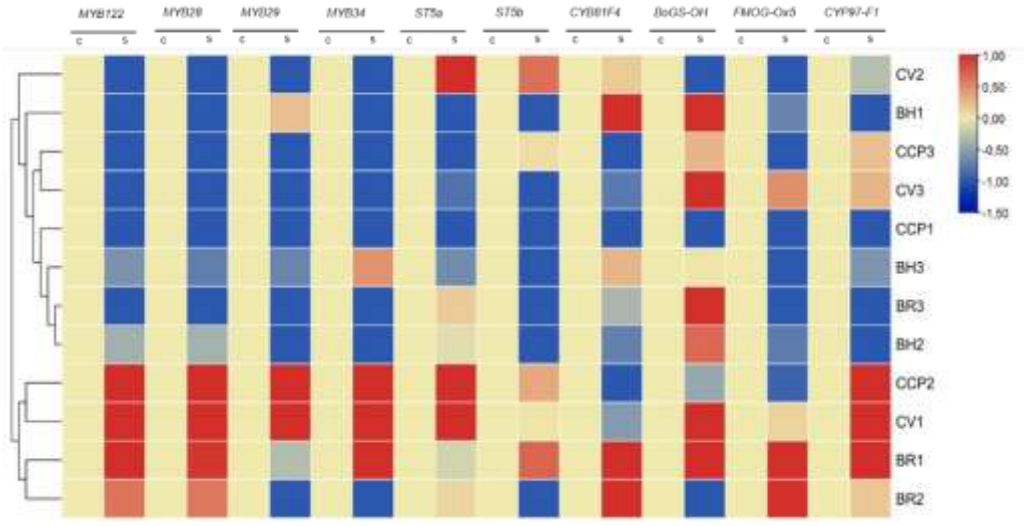



**Figure 37.** Heatmap of expression of genes and TFs involved in the biosynthesis of Glucosinolate

The heatmap of gene expression provides valuable insights into the regulatory dynamics of biosynthesis pathways, including Glucosinolate production. By analyzing the expression patterns of key genes involved in Glucosinolate biosynthesis, we can uncover critical transcription factors (TFs) that orchestrate this intricate process. Transitioning from gene expression to a broader perspective, the protein-protein interaction network complements this understanding by revealing the intricate molecular dialogues that underpin pathway regulation. TFs, as central players in gene expression, often occupy pivotal positions in these interaction networks, further elucidating their roles in shaping the Glucosinolate biosynthesis pathway. Connecting the dots between the heatmap of gene expression and the protein-protein interaction network, we gain a comprehensive view of how TFs drive the synthesis of GLSs, shedding light on the molecular intricacies that govern this essential metabolic pathway.

These findings underscore the intricate orchestration and harmonious regulation of genes central to GLSs biosynthesis. The presence of distinct protein clusters suggests a collaborative, integrated response to various cues, ultimately fine-tuning the production of GLSs. This precision is paramount for the plant's defense mechanisms against herbivores and pathogens, and it profoundly influences the flavor profile and nutritional value of cruciferous vegetables. In essence, the network analysis provides a meticulously structured and scientifically enlightening overview of the multifaceted functions and interplay within the GLSs biosynthesis pathway, offering profound insights into the underlying molecular mechanisms governing this indispensable metabolic process in plants. In the protein-protein interaction network analysis, distinct clusters of proteins closely associated with the regulation of genes relevant to GLSs biosynthesis have been identified (Figure 38).

Cluster 1: Aliphatic Glucosinolate Biosynthesis Regulation, this cluster is characterized by the presence of CYP79F1, a multifunctional enzyme responsible for catalyzing the conversion of short-chain elongated methionine into aldoxime, specifically 5-methylthiopentanaldoxime, 6-methylthiohexanaldoxime, and 7-methylheptanaldoxime. This cluster also encompasses MYB28 and MYB29, transcription factors known to be major regulators of aliphatic GLSs biosynthesis. The close proximity of CYP79F1 with MYB28 and MYB29 suggests a potential role in the GLS biosynthesis pathway.

In cluster 2, a functional module comprising SOT18, FMOGS-OX5, CYP81F4, and AT2G25450 comes into focus. SOT18, an aliphatic desulfoglucosinolate sulfotransferase, plays a pivotal role in the sulfate conjugation of desulfo-glucosinolates, particularly long-chain ones.

OX5, a flavin-monooxygenase glucosinolate S-oxygenase, is responsible for the conversion of methylthioalkyl glucosinolates into methylsulfinylalkyl glucosinolates, thereby enhancing glucosinolate diversity. CYP81F4 is involved in indole GLSs biosynthesis, catalyzing hydroxylation reactions. AT2G25450 contributes to the hydroxylation of but-3-enyl GLS, yielding the toxic 2-hydroxybut-3-enyl GLSs.

Cluster 3 is Transcriptional Regulators, this cluster unites MYB122, MYB34, and MYB29, all of which function as transcription factors impacting GLSs biosynthesis. MYB122, belonging to the R2R3 factor gene family, likely exerts transcriptional control over the network. MYB34 plays a pivotal role in modulating the expression of ASA1, a key control point in the tryptophan pathway. MYB29, while having a minor role in aliphatic GLSs biosynthesis, is important for promoting GLSs production and thwarting insect herbivores.

These findings underscore the intricate coordination and regulation of genes involved in GLS biosynthesis. The protein clusters suggest a cooperative response to various cues, fine-tuning the production of GLSs. This is of paramount importance for plant defense mechanisms against herbivores and pathogens, as well as for the flavor profile and nutritional value of cruciferous vegetables. In essence, this network analysis provides a structured and scientifically informative overview of the key functions and interactions within the GLS biosynthesis pathway, elucidating the underlying molecular mechanisms governing this essential metabolic process in plants.

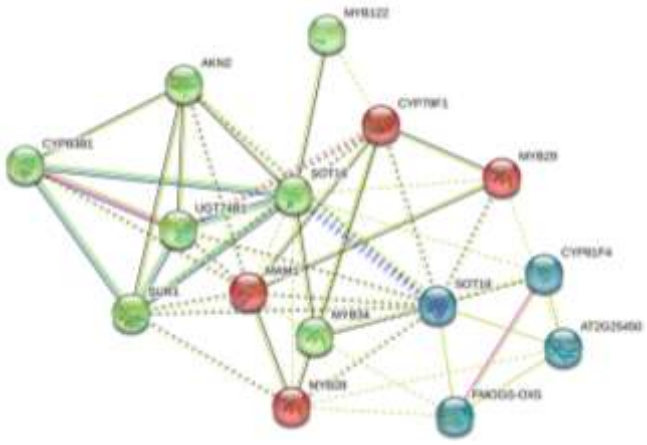



**Figure 38.** Protein–protein interaction network

By identifying the presence of CAEs (Cis-acting Elements) within the gene promoter regions linked to water stress responses in *Brassica oleracea* as showed in figure 39, we gain valuable insights into the potential regulatory mechanisms governing the plant's ability to withstand water stress. This information holds considerable significance in unraveling the

genetic basis of drought tolerance and may pave the way for devising strategies to further enhance water stress resilience in *Brassica oleracea* crops.

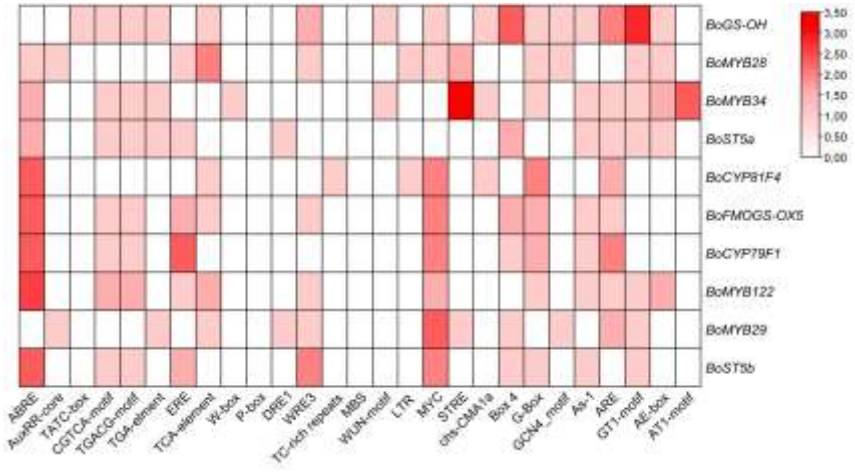



**Figure 39.** The cis-acting element (CAEs) were detected by the online tool PlantCARE

### 3.4 Discussion

In this comprehensive study, we delved into the intricate dynamics of GLS variation in *Brassica oleracea* under the influence of water stress, drawing illuminating parallels with existing scientific literature. One striking revelation was the contrasting GLS allocation between roots and leaves, with leaves consistently exhibiting significantly higher total GLS concentrations compared to roots under water stress conditions, aligning seamlessly with the observations made by Sønderby et al. (2010), emphasizing the role of organ-specific GLS synthesis. Furthermore, the investigation unveiled the profound influence of both genotype and irrigation regime on GLS content in both roots and leaves. Genotype-specific responses to water stress were evident, as some genotypes showcased the remarkable ability to maintain elevated GLS levels even when subjected to limited water availability, akin to the genotype diversity identified by Brown et al. (2003) in *Brassica napus*. Significantly, the research unveiled complex relationships among GLSs, providing a detailed depiction of their biosynthesis pathways. While some compounds exhibited synchronized biosynthesis pathways, suggesting coordinated regulation, others appeared to operate independently, aligning with the insights offered by Halkier and Gershenzon. (2006) regarding GLS regulation. These variations in GLS content carry profound implications for the plant's defense mechanisms and nutritional quality. They resonate strongly with Fahey et al. (2001) discourse on the pivotal role of GLSs in plant protection mechanisms and with Ku et al. (2011) exploration of breeding strategies aimed at enhancing nutritional attributes. Importantly, under water stress conditions, we observed shifts in correlations between GLSs, offering a glimpse into the plant's adaptive responses, a

phenomenon in line with Agerbirk et al. (2009) observations on Brassicaceae species. These collective findings underscore the intricacy of the interplay between genotype, environmental factors, and GLS variations, advancing the understanding of how plants respond to environmental challenges.

Among the selected accessions, the observations revealed limited variation in morphometric traits in response to drought stress. Nevertheless, a notable and significant increase was noted in the levels of the indolic GLS GBS (in BH1, BH2, BH3, CCP4, and CV3 accessions) and NGBS (in BR5 and CV4 accessions). The interrelation between GBS and NGBS hints at their possible conversion. This observation is consistent with the findings of Hornbacher et al. (2022), who highlighted the importance of glucobrassicin in mitigating the effects of water stress. Hornbacher's research also proposed the possibility that glucobrassicin could serve as a potential source of auxins for *Arabidopsis thaliana* when grown under drought conditions. In the study, sinigrin, glucobrassicin, and glucobrassicanapin were identified as the predominant GLSs in kale. This differs from the findings in other study, Kushad et al. (1999), where sinigrin was notably high at 10.4 μmol·g−1 D.W., while glucobrassicin was comparatively low at 1.2 μmol·g−1 D.W., and glucoraphanin levels were negligible. Notably, the examination of different broccoli accessions yielded significant variations in the content of indole GLSs, with glucobrassicin emerging as the predominant compound among the surveyed broccoli cultivars. This discovery stands in contrast to the present findings, which indicate the predominance of aliphatic GLSs. This variance underscores the divergent regulation of GLS pathways, a facet shaped by the specific crop and the gene expression dynamics involved. Regarding GLS concentration in roots, the study confirmed the GLS concentrations reported as control values by Li et al. (2021) and showed a substantial 41.4% increase under water stress conditions. Notably, Huang et al. (2022) documented an initial decline succeeded by an upswing in total GLS content in roots during the mustard plant's developmental stages Conversely, in mustard's life cycle, total GLS content in leaves displayed an initial surge followed by a decrease, peaking during the bolting stage. To delve further into the intricate relationship between plant organs and GLSs accumulation, a principal component analysis (PCA) was conducted. The outcomes of this analysis unveiled the distinct attributes of GLS in different plant organs. Notably, aliphatic GLSs emerged as the prevailing type in Brassica leaves, whereas roots exhibited a stronger correlation with indolic and aromatic GLSs. This pattern of results resonates with the findings of Huseby et al. (2013), who identified divergent

regulation of aliphatic and indolic MYB factors in Arabidopsis plants in response to light cycling.

Supplementary correlation analyses of GLS offered deeper insights into the distribution of these compounds across diverse organs and genotypes. This study underscores a significant observation: while there isn't a particular GLS uniquely tied to water stress resistance, a broad spectrum of GLS levels and profiles exists, showing considerable variations contingent on the genotype. When comparing the correlations of different GLSs observed in the roots of *Brassica oleracea* under normal and water stress conditions with those in the leaves of the same plant, several notable distinctions and similarities emerge. SIN and GBN (Roots vs. Leaves): Under normal conditions, SIN and GBN in both roots and leaves display a strong positive correlation, suggesting that these GLSs are closely linked in biosynthesis across plant parts. This similarity underscores their shared regulation regardless of the plant's condition. Regarding GER and GBN (Roots vs. Leaves): In roots, GER and GBN share a moderate positive correlation, whereas in leaves, there's no mention of a significant correlation between these two GLSs. This disparity suggests that the relationship between GER and GBN may be organ-dependent.

GBS and NGBS (Roots vs. Leaves): There is no specific mention of GBS and NGBS correlations in roots under normal conditions. However, in leaves, GBS and NGBS exhibit a moderate negative correlation. This difference implies that the relationship between these GLSs varies between roots and leaves, potentially influenced by the plant's condition. SIB and GST (Roots vs. Leaves): In both roots and leaves, SIB and GST show a strong positive correlation under normal conditions and during water stress. This consistency suggests that the association between SIB and GST remains robust across different plant parts and environmental conditions. GRA and GNA (Roots vs. Leaves): There's no mention of GRA and GNA correlations in roots. However, in leaves, GRA and GNA exhibit a moderately strong positive correlation under normal irrigation conditions. This difference suggests that the relationship between these GLSs may be specific to leaves and influenced by the plant's condition. The most important result from the comparison of GLS correlations in *Brassica oleracea* roots and leaves under normal and water stress conditions is the significant shift in the correlations between certain GLS compounds during water stress. Specifically, GER and GBS: The transition from a negative correlation to an extremely strong positive correlation between GER and GBS during drought stress is a particularly noteworthy finding. This suggests that these two compounds become highly synchronized in their changes under water stress, indicating a significant alteration in their relationship when the plant faces drought conditions. This shift in correlation may have

important implications for the plant's response to water stress and could potentially be a key adaptive mechanism. In the other hand, SIB and GST: Another critical result is the consistent strong positive correlation between SIB and GST in both roots and leaves, regardless of the condition (normal or drought stress). This suggests that the relationship between these two compounds is robust and not significantly affected by changes in water availability. This consistent association could be a crucial part of the plant's defense mechanisms or responses to stressors. The comparisons between roots and leaves of *Brassica oleracea* reveal that while some GLS correlations remain consistent across plant parts and conditions (e.g., SIB and GST), others show variability, suggesting that the interplay between GLSs can be influenced by both the plant's condition and the specific plant part under consideration. These variations may reflect the plant's adaptive responses to environmental challenges. Thus, the application of advanced metabolomics techniques, such as metabolomic profiling, facilitates the comprehensive assessment of secondary metabolites within plants under varying conditions. This analytical approach unveils patterns of co-occurrence or competition between GLSs, phenolic compounds, terpenoids, and other metabolites, providing a holistic view of the metabolic landscape. It's notable that the capacity for GLSs biosynthesis has served as a taxonomic indicator for classification systems predicated on crop evolution (Blaževic et al.,2020). The manipulation of GLS content has proven efficacious through diverse breeding and selection techniques within various *B. oleracea* crops. Likewise, divergent mass selection has emerged as a valuable tool in plant breeding, effectively generating distinct varietal groups within *B. oleracea* crops. These groups share a common genetic foundation yet exhibit notable diversity in terms of GLS levels and profiles.

The GSL biosynthetic pathway is divided into three main stages: amino acid side-chain extension, core structure formation, and side-chain modification. Each of these stages involves specific enzymes and genes responsible for different steps in GSL production. A comprehensive understanding of GSL biosynthesis requires insight into the regulation of these genes. Notably, MYB transcription factors play a pivotal role in governing GSL biosynthesis. Among the MYB TFs identified in research (MYB28, MYB29, MYB34, and MYB122), they act as master regulators, finely orchestrating the expression of genes involved in diverse aspects of GLS production, spanning from precursor molecules to the final GLS compounds. In essence, these MYB TFs function like conductors in an orchestra, coordinating the expression of multiple genes to harmoniously produce GLS compounds. What makes these MYB TFs particularly intriguing is their responsiveness to environmental cues. Numerous studies have underscored

their high sensitivity to changes in environmental conditions, including stressors such as drought, temperature fluctuations, and herbivore attacks. This responsiveness underscores the adaptability of plants in modulating GSL production in response to varying environmental challenges.

To gain a more comprehensive understanding of the intricate GSL biosynthetic pathway, it is crucial to delve into the specific roles of enzymes like FMO GS-OX1 and FMO GS-OX5 homologs, which are integral to side-chain modification—an essential step in GSL production. Their tissue-specific expression patterns suggest that GSL synthesis may vary across different plant parts and developmental stages. Moreover, examining the expression of genes responsible for aliphatic, indole, and aromatic GSL synthesis in various tissues reveals tissue-specific regulation of GSL production. For instance, the upregulation of genes encoding CYP81F1/2/4 in stalks and flower buds aligns with the higher indole GSL content in these tissues compared to leaves. Additionally, the expression patterns of these GSL-related genes provide a foundation for understanding GSL content variations in different plant parts. For instance, the heightened expression of genes encoding GS-OX5 correlates with increased GSL synthesis and accumulation in flower buds. These insights into the regulation of GSL biosynthesis align with the study of Wittstock and Halkier in 2000, which laid the foundation for understanding the intricate interplay between genes and environmental factors in shaping GSL production in *Brassica oleracea.*

### 3.5 Conclusion

Considering the increasing demand for *B. oleracea* products as healthy foods in local and national markets across the EU, it is imperative to obtain accurate information regarding the best cultivars to use. Moreover, there is a need to develop new cultivars with high yield, stress tolerance, and enhanced nutritional value to meet market demands and improve the overall resilience and productivity of *B. oleracea*. The results obtained in this study provide valuable insights into the responses of different genotypes of *B. oleracea* to drought conditions. Both plant morphometric traits and the quantity and composition of GLSs were found to vary significantly in response to water stress, with notable interactions between these factors. This suggests the presence of elite genetic material that can be utilized for organic breeding of *B. oleracea* crops.

The research unveiled significant fluctuations in GLS content in both roots and leaves in response to different irrigation regimes (IR) and genetic backgrounds (GE). Importantly, each

specific GLS compound, whether aliphatic, indolic, or aromatic, displayed its unique sensitivity to these interactions. Promising candidates for further breeding programs were identified among the analyzed accessions, including kales BH1, BH2, and BH3; broccoli BR5; cauliflowers CV3 and CV4; and CCPP4. These accessions exhibited low variation indices for several morphometric traits, indicating stable performance under drought conditions. Moreover, it is noteworthy that the leaves of these accessions contained higher levels of GLSs than the roots, which is particularly important for *B. oleracea* crops, where leaves are the primary product, such as kale and broccoli. This study also highlighted the significance of GLSs in enhancing the antioxidant capacity of *B. oleracea* plants to cope with water stress, consistent with previous research findings. GLSs play a crucial role in plant defense mechanisms during water scarcity.

These findings hold immense promise for practical applications in agriculture and nutrition. They open the door to tailored breeding programs aimed at selecting *Brassica oleracea* varieties with optimal GLS profiles, whether for enhanced plant defense, improved nutritional value, or sustainable livestock feed. Moreover, our study highlights the complexity of GLS regulation, acknowledging that environmental cues beyond irrigation, such as light, temperature, and biotic stresses, can further modulate these compound

# Conclusion & Perspectives

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The unwavering commitment to advancing the pre-breeding of *Brassica oleracea* in response to the specific requirements of organic farming has led to the initiation of three intricately interconnected research paths. These research lines have played a pivotal role in shedding light on various aspects of *Brassica oleracea's* responses to environmental challenges, with a particular emphasis on the formidable issue of drought stress. Collectively, these lines of inquiry lay the groundwork upon which the cultivation of more resilient, sustainable, and precisely tailored crop varieties that seamlessly align with the demands of organic agriculture reformulation is aspired to be built.

The exploration began with a comprehensive examination of the genetic diversity within the *Brassica oleracea* primary gene pool, encompassing its wild relatives as well. This phase of the research involved meticulous characterization efforts and the application of advanced molecular techniques, all aimed at unraveling the intricate morphological and genetic complexities inherent to this remarkably diverse species. The investigation not only documented remarkable phenotypic diversity within *B.oleracea* but also revealed discernible patterns of genetic variation and relatedness. This inquiry extended beyond cultivated varieties to encompass the wild relatives of *B.oleracea*, enriching the understanding of the broader genetic context of the species. By employing genotyping techniques, specifically utilizing 11 SSR markers to analyze 100 individuals spanning 12 distinct varieties, distinct groupings within the population were successfully delineated. This analysis underscored not only the prevalence of inter-population variability but also provided insights into the evolutionary history and genetic relationships within *B. oleracea.*

The newfound understanding of the genetic landscape of *B.oleracea*, encompassing both cultivated and wild varieties, serves as the solid foundation upon which we can build targeted trait mapping endeavors. These efforts are pivotal in the quest to develop robust and high-yielding cultivars capable of withstanding environmental stresses. Furthermore, the genetic diversity uncovered within *B.oleracea* and its wild relatives represents a treasure trove of potential traits that can be strategically harnessed to fortify these crops against a range of environmental challenges, thereby enhancing their overall performance and resilience.

In the subsequent phase of the research, exploration extended into the realm of *Brassica oleracea's* biochemical responses to water deficiency. The objective was to pinpoint

exceptional genetic materials within the species that showcase a remarkable capacity to endure and flourish under drought conditions. Through thorough investigation and carefully planned research, valuable insights were uncovered regarding how *Brassica oleracea* adapts to various environmental challenges, with a specific focus on the formidable obstacle posed by drought stress. Specific biochemical markers linked to water stress tolerance were successfully identified, showcasing substantial effects on essential morpho-physiological and biochemical factors that are pivotal for plant growth and development. Significantly, the identification of genotypes with effective antioxidant systems, including CR, CC, BH, CI, and BTR, underscores the potential for breeding programs to improve water stress tolerance. These markers serve as indicators of the plant's adaptability to limited water availability. This discovery holds immense promise for breeding programs, offering a solid foundation for the development of water-efficient and stress-resistant cultivars. Harnessing these elite genetic materials empowers agriculture to address the challenges posed by water scarcity and changing climatic conditions.

The third research line led us into the intriguing realm of GLS metabolism within *Brassica oleracea*. These secondary metabolites play a vital role in the plant's defense mechanisms, and we sought to understand how their production and functions are influenced by abiotic stress, particularly drought. The comprehensive evaluation of *B. oleracea* accessions, with a specific focus on GLSs, uncovered their significant contributions to the plant's resilience in the face of environmental challenges. Delving into the intricacies of how these compounds respond to water stress, light is shed on the underlying mechanisms that enable *B. oleracea* to thrive even in stress conditions. The insights gained from this research have far-reaching implications, especially in terms of developing healthier and more nutritious products. The intricate realm of GLS biosynthesis revealed itself as a multifaceted puzzle, inviting further exploration. The transcriptomic data driving GLS diversity holds the promise of unlocking phytochemicals and nutraceuticals for Brassica crops. As we tread this path, the regulation of GLS genes remains enigmatic, urging us to uncover the intricate network of biosynthetic genes and their regulatory mechanisms.

The exploration of these three research lines has significantly enriched the understanding of *B. oleracea* responses to drought stress. Genetic diversity and relationships within the species were examined, biochemical markers of water stress tolerance were identified, and the complex interplay between GLSs and abiotic stress was unraveled. These findings collectively contribute to the development of crop varieties that are not only resilient but also capable of providing

enhanced nutritional value. Looking ahead into the realm of *B. oleracea* research, a future filled with exciting potential unfolds. The perspectives that follow invite an intriguing and revolutionary expedition.

The practical applications of the genetic knowledge acquired can be further explored. Trait mapping and breeding efforts can be undertaken to utilize the identified genetic diversity for the identification of specific genes and alleles associated with desirable traits such as drought tolerance, disease resistance, and enhanced nutritional content. This strategic approach has the potential to expedite the development of improved *Brassica oleracea* cultivars that are finely tuned to the requirements of organic farming and sustainable agriculture.

Exploring Wild Relatives and Functional Genomics: Expanding genetic diversity analysis to encompass more wild relatives of *B. oleracea* represents a third avenue of research. These wild species may harbor unique and valuable traits that, when introgressed into cultivated varieties, can bolster resilience and adaptability to diverse environmental conditions. Additionally, delving into functional genomics is essential for deepening the understanding of *Brassica oleracea'*s responses to environmental stresses. By elucidating the roles of specific genes and regulatory elements in these responses, the path is paved for targeted genetic modifications that enhance stress tolerance and other desirable traits.

Integrating Multi-Omics Approaches: Embracing multi-omics approaches, which encompass genomics, transcriptomics, proteomics, and metabolomics, provides a comprehensive view of the molecular mechanisms governing stress responses. This holistic perspective can uncover intricate regulatory networks and potential intervention points, further enhancing the ability to develop resilient and high-yielding cultivars.

Breeding Climate-Smart Cultivars: A focus on breeding cultivars adaptable to changing climatic conditions is crucial. This includes addressing not only drought tolerance but also adaptability to heat stress and optimizing resource use efficiency.

In conclusion, the journey through the intricate landscape of *Brassica oleracea* responses to water stress serves as a cornerstone for the advancement of scientific inquiry. It illuminates the profound complexity of plant biology and presents an array of uncharted avenues for continued exploration and innovation within the realm of plant science.

The future of *Brassica oleracea* research shines with multifaceted dimensions, encompassing climate resilience, precision agriculture, nutritional enhancement, biodiversity

conservation, global collaboration, education, and policy advocacy. By steadfastly addressing these perspectives, the challenges posed by water stress are not only navigated but also contribute significantly to global food security and the advancement of our understanding of plant biology. Through the lens of rigorous scientific inquiry, a path is forged toward a more sustainable and prosperous society.

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# Annexes

**Table 18. List of the core collection of *Brassica oleracea*. L and wild species used in this study**

| COMMERCIAL NAME | NAME | CROP CODE | NUMBER | Origin | COLLECTION |
|---|---|---|---|---|---|
| BROCCOLI | *B. oleracea var italica* | BR356 | UNICT4956 | BAVICCHI SEMENTI GEO | UNICT |
| | *B. oleracea var italica* | BR 211 | UNICT4369 | Adrano | UNICT |
| | *B. oleracea var italica* | BR 354 | UNICT4939 | Adrano | UNICT |
| | *B. oleracea var italica* | BR 29 | UNICT568 | Adrano | UNICT |
| | *B. oleracea var italica* | BR 80 | UNICT613 | Furnari | UNICT |
| | *B. oleracea var italica* | BR 127 | UNICT656 | Favignana | UNICT |
| | *B. oleracea var italica* | BR 325 | UNICT4960 | RAMOSO CALABRESE | UNICT |
| | *B. oleracea var italica* | BR 363 | UNICT 5085 | MODICA CIURIETTO | UNICT |
| | *B. oleracea var italica* | BR362 | UNICT 5083 | friarolo | UNICT |
| | *B. oleracea var italica* | BR 359 | UNICT 5080 | mazzarolo | UNICT |
| | *B. oleracea var italica* | BR358 | UNICT 5079 | aprilino | UNICT |
| | *B. oleracea var italica* | BR 365 | UNICT 5088 | maiolino | UNICT |
| | *B. oleracea var italica* | BR 364 | UNICT 5087 | di giugno | UNICT |
| | *B. oleracea var italica* | BR 360 | UNICT 5081 | settembrino | UNICT |
| | *B. oleracea var italica* | BR 361 | UNICT 5082 | natalino | UNICT |
| | *B. oleracea var italica* | BR 96 | UNICT 628 | settembrino | UNICT |
| | *B. oleracea var italica* | BR 82 | UNICT 615 | settembrino | UNICT |
| | *B. oleracea var italica* | BR 370 | UNICT 5109 | CHINA | CHINA |
| | *B. oleracea var italica* | BR 369 | UNICT 5108 | CHINA | CHINA |
| Brussels sprouts | *B. oleracea var. gemmifera* | CZ | HRIGRU8302 | FRA | LIVERPOOL |
| | *B. oleracea var. gemmifera* | CZ | HRIGRU6817 | FRA | LIVERPOOL |
| | *B. oleracea var. gemmifera* | CZ | HRIGRU7027 | FRA | LIVERPOOL |
| | *B. oleracea var. gemmifera* | CZ | HRIGRU7026 | FRA | LIVERPOOL |
| | *B. oleracea var. gemmifera* | CZ | HRIGRU282 | FRA | LIVERPOOL |
| | *B. oleracea var. gemmifera* | CZ | HRIGRU417 | FRA | LIVERPOOL |
| | *B. oleracea var. gemmifera* | CZ | HRIGRU605 | FRA | LIVERPOOL |
| | *B. oleracea var. gemmifera* | CZ | HRIGRU544 | FRA | LIVERPOOL |
| | *B. oleracea var. gemmifera* | CZ | HRIGRU4605/6 | FRA | LIVERPOOL |
| | *B. oleracea var. gemmifera* | CZ | HRIGRU4494/6 | FRA | LIVERPOOL |
| cabbage | *B. oleracea var. capitata* | CC | CRI09H1800149 | SLO recieved 1995 | PRAGA |
| | *B. oleracea var capitata* | CC | HRIGRU5567 | NLD | LIVERPOOL |
| | *B. oleracea var capitata* | CC | UNICT4636 CC42 | SR | UNICT |
| CAULIFLOWER | *B. oleracea var botrytis* | CV192 | UNICT4449 | Modica | UNICT |
| | *B. oleracea var botrytis* | CV194 | UNICT4451 | Modica | UNICT |
| | *B. oleracea var botrytis* | CV 26 | UNICT 428 | NAPOLETANO NATALINO | UNICT |
| | *B. oleracea var botrytis* | CV246 | UNICT 5110 | CHINA | CHINA |
| | *B. oleracea var botrytis* | CV 250 | UNICT 5115 | CHINA | CHINA |
| | *B. oleracea var botrytis* | CV 248 | UNICT 5112 | TUNISIA | CHINA |

| | | | | | |
|---|---|---|---|---|---|
| | *B. oleracea var botrytis* | CV 238 | UNICT 5098 | TUNISIA | TUNISIA |
| | *B. oleracea var botrytis* | CV 239 | UNICT 5099 | TUNISIA | TUNISIA |
| | *B. oleracea var botrytis* | CV 240 | UNICT 5100 | TUNISIA | TUNISIA |
| | *B. oleracea var botrytis* | CV 241 | UNICT 5101 | TUNISIA | TUNISIA |
| | *B. oleracea var botrytis* | CV 242 | UNICT 5102 | TUNISIA | TUNISIA |
| | *B. oleracea var botrytis* | CV 243 | UNICT 5103 | TUNISIA | TUNISIA |
| | *B. oleracea var botrytis* | CV 244 | UNICT 5104 | TUNISIA | TUNISIA |
| | *B. oleracea var botrytis* | CV 245 | UNICT 5105 | TUNISIA | TUNISIA |
| | *B. oleracea var botrytis* | CV 249 | UNICT 5113 | CHINA | CHINA |
| | *B. oleracea var botrytis* | CV 247 | UNICT 5111 | CHINA | CHINA |
| CROSS | *B oleracea var INCROCIO* | BRXCV1 | UNICT5040 | Monsampolo del tronto | CREA |
| | *B oleracea var INCROCIO* | BRXCV2 | UNICT5041 | Monsampolo del tronto | CREA |
| | *B oleracea var INCROCIO* | BRXCV3 | UNICT5042 | Monsampolo del tronto | CREA |
| | *B oleracea var INCROCIO* | BRXCV4 | UNICT5043 | Monsampolo del tronto | CREA |
| | *B oleracea var INCROCIO* | BRXCV5 | UNICT5044 | Monsampolo del tronto | CREA |
| | *B oleracea var INCROCIO* | BRXCV6 | UNICT5045 | Monsampolo del tronto | CREA |
| | *B oleracea var INCROCIO* | BRXCV7 | UNICT5046 | Monsampolo del tronto | CREA |
| | *B oleracea var INCROCIO* | BRXCV8 | UNICT5047 | Monsampolo del tronto | CREA |
| KALE | *B. oleracea var acephala* | BH | HRIGRU6421 | | LIVERPOOL |
| | *B. oleracea var acephala* | BH | HRIGRU7546 | DEU | LIVERPOOL |
| | *B. oleracea var acephala* | BH 50 | UNICT3381 | Orto Gangi | UNICT |
| | *B. oleracea var acephala* | BH 14 | UNICT364 | | UNICT |
| | *B. oleracea var acephala* | BH 30 R | UNICT4481 | | UNICT |
| | *B. oleracea var acephala* | BH 10 | UNICT4538 | | UNICT |
| | *B. oleracea var acephala* | BH 1R | UNICT4591 | Salina | UNICT |
| | *B. oleracea var acephala* | BH 81 | UNICT 4448 | CAPIZZI | UNICT |
| | *B. oleracea var acephala* | BH 103 | UNICT 5123 | SCRAFANI BAGNI | UNICT |
| | *B. oleracea var.tronchuda* | BTR | HRIGRU4690 | PRT | LIVERPOOL |
| | *B. oleracea var sabauda* | CA | UNICT4633 CA8 | MANIACE | UNICT |
| kohl rabi | *B. oleracea var. gongylodes* | CR | CRI09H2200023 | CSK | PRAGA |
| | *B. oleracea var. gongylodes* | CR | CRI09H2200003 | CSK | PRAGA |
| | *B. oleracea var. gongylodes* | CR | HRIGRU12936 | DEU | LIVERPOOL |
| | *B. oleracea var. gongylodes* | CR | HRIGRU6211 | ITA | LIVERPOOL |
| | *B. oleracea var. gongylodes* | CR 34 | UNICT4447 CR 34 | Milazzo | UNICT |
| | *B. oleracea var. gongylodes* | CR45 | UNICT5038 | Acireale | UNICT |
| | *B. oleracea var. gongylodes* | CR47 | UNICT5040 | S.Maris La Scala | UNICT |
| | *B. oleracea var. gongylodes* | CR 50 | UNICT 5096 | TRUNZO BIANCO | UNICT |
| | *B. oleracea var. gongylodes* | CR 51 | UNICT 5097 | TRUNZO BIANCO | UNICT |
| | *B. oleracea var. gongylodes* | CR 52 | UNICT 5116 | PENNISI ACIREALE | UNICT |
| SELF POLINATED | *B oleracea var AUTOFECONDATA* | AUTO16 | UNICT5075 | Monsampolo del tronto | CREA |
| | *B oleracea var AUTOFECONDATA* | AUTO17 | UNICT5076 | Monsampolo del tronto | CREA |
| | *B oleracea var AUTOFECONDATA* | AUTO2 | UNICT5061 | Monsampolo del tronto | CREA |

| | | | | | |
|---|---|---|---|---|---|
| | *B oleracea var AUTOFECONDATA* | AUTO3 | UNICT5062 | Monsampolo del tronto | CREA |
| | *B oleracea var AUTOFECONDATA* | AUTO4 | UNICT5063 | Monsampolo del tronto | CREA |
| | *B oleracea var AUTOFECONDATA* | AUTO5 | UNICT5064 | Monsampolo del tronto | CREA |
| WILD | *B.drepanensis* | BD 4 | UNICT 4796 | ERICE | UNICT |
| | *B.rupestris* | BU 21 | UNICT 3687 | STILO CIMITERO | UNICT |
| | *B.rupestris* | BU 2 | UNICT3677 | SCLAFANI BAGNI | UNICT |
| | *B.rupestris* | BU 3 | UNICT 741 | GRATTERI | UNICT |
| | *B.rupestris* | BU 4 | UNICT 919 | CAPO SANT'ALESSIO | UNICT |
| | *B.rupestris* | BU 26 | UNICT 931 | CALTAVUTURNO | UNICT |
| | *B.rupestris* | BU 5 | UNICT 920 | ROCCELLA VALDEMONE | UNICT |
| | *B.villosa* | BV13 | UNICT 5035 | CALTABELLOTTA | UNICT |
| | *B.villosa* | BV 6 | UNICT 3229 | GALLO D'ORO | UNICT |
| | *B.villosa* | BV | UNICT 3944 | MARIANOPOLI | UNICT |
| | *B. incana* | BY 4 | UNICT 3419 | CAPO D'ORLANDO | UNICT |
| | *B. incana* | BY7 | UNICT 4158 | SORTINO -PANTALICA | UNICT |
| | *B. incana* | BY 6 | UNICT3513 | Agnone Bagni | UNICT |
| | *B. incana* | BY 15 | UNICT4803 | CASTELMOLA | UNICT |

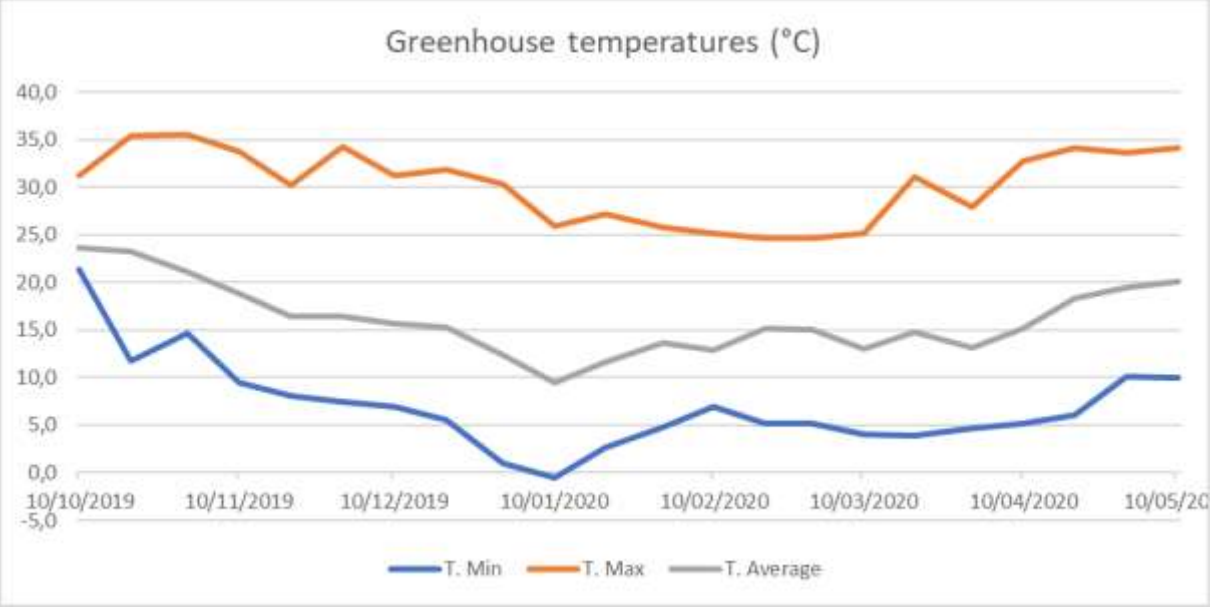


**Figure 40**. **Temperature registered during the trial in the greenhouse**

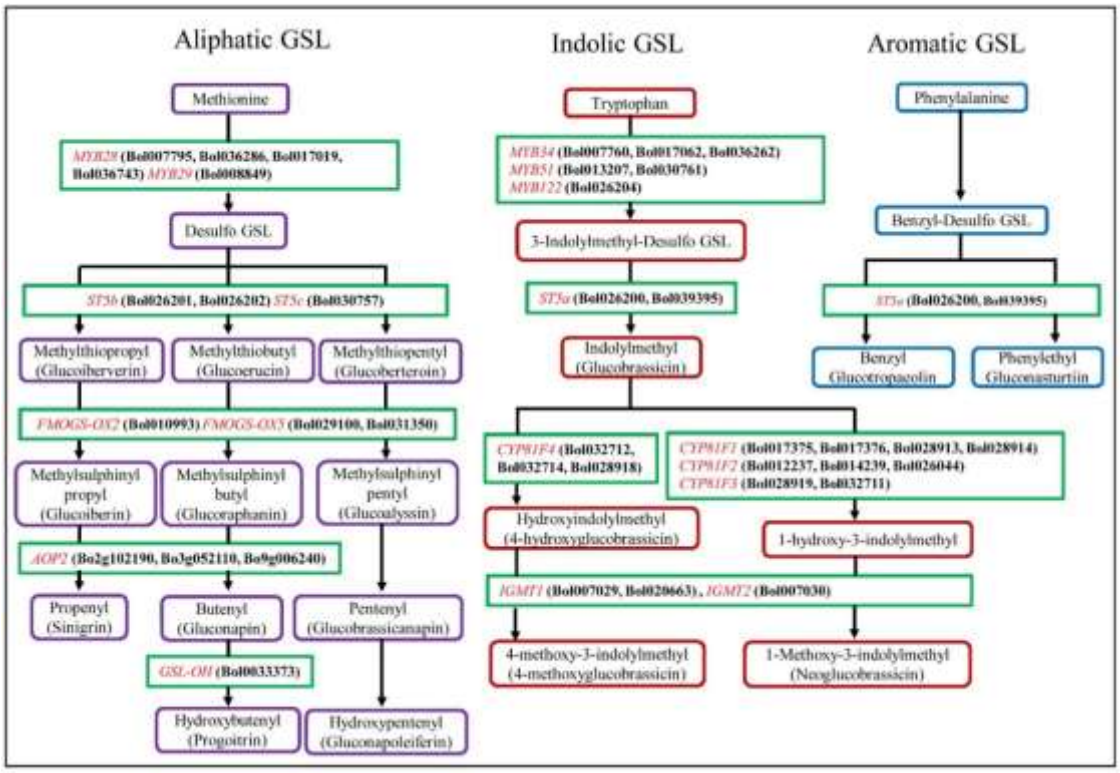


**Figure 41. The glucosinolate biosynthesis and related transcription factor genes (Green square box with red letters) analyzed in this work, with their positions in the aliphatic and indolic glucosinolate (GSL) biosynthesis pathways indicated.**

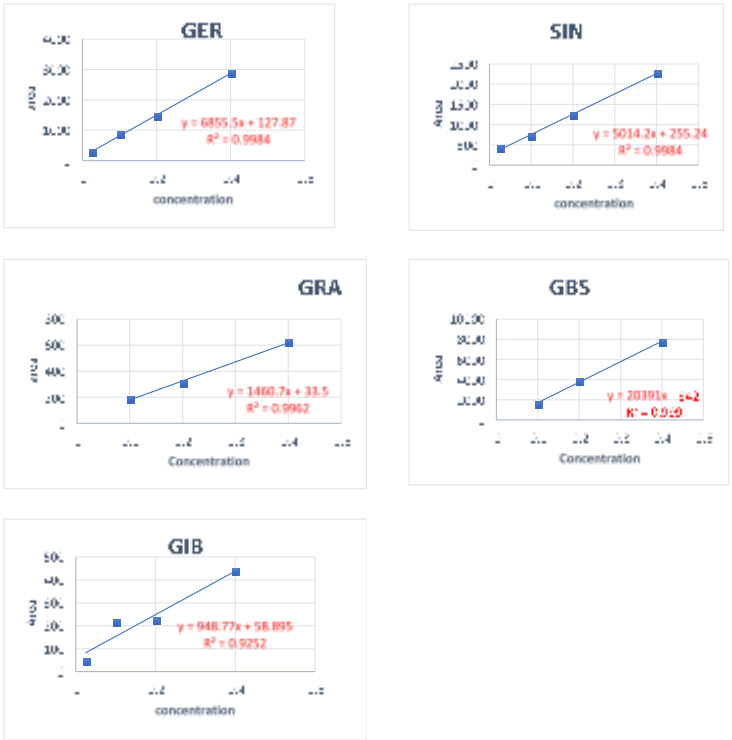


**Figure 42. Equations of the GLS standards used for the analysis**

**Table 19. Standards used in the quantification of GLSs (Retention time and equations)**

| | standards | abb | Retention time | Equation |
|---|---|---|---|---|
| **1** | Glucoiberin | GIB | 7,103 | |
| **2** | Singrin | SIN | 7,973 | y = 0.0043x |
| **3** | glucoraphanin | GRA | 9,265 | y = 0.0023x |
| **4** | sinalbin | SIB | 12,447 | y = 0.0022x |
| **5** | Gluconapin | GIN | 13,829 | y = 0.0018x |
| **6** | glucoerucin | GER | 19,385 | y = 0.0021x |
| **7** | glucobrassicanapin | GBAS | 20,346 | y = 0.0032x |
| **8** | glucobrassicin | GBRA | 22,298 | y = 0.0024x |
| **9** | gluconasturtin | GNAS | 26,219 | |
| **10** | glucoalyssin | GAL | 15 | y = 0.0023x |
| **11** | neoglucobrassicin | NGB | 24,4 | y = 0.0023x |

Résumé

L'agriculture biologique joue un rôle crucial dans la promotion d'un système agricole plus durable et respectueux de l'environnement. Elle se concentre sur l'utilisation de pratiques agricoles naturelles et respectueuses de l'écosystème, en évitant l'utilisation de produits chimiques synthétiques tels que les pesticides et les engrais chimiques. L'un des principaux objectifs de l'agriculture biologique est de maintenir la santé des sols, de préserver la biodiversité et de produire des aliments de haute qualité nutritionnelle.

Dans ce contexte, le développement de variétés de *Brassica oleracea* adaptées à l'agriculture biologique revêt une importance particulière. *Brassica oleracea* est une plante largement cultivée, comprenant différentes variétés de choux, qui sont couramment utilisées dans l'alimentation humaine. En développant des variétés spécifiques adaptées aux exigences de l'agriculture biologique, il est possible de maximiser les avantages de ce mode de production durable.

Les variétés de *Brassica oleracea* adaptées à l'agriculture biologique peuvent offrir plusieurs avantages :

Résistance aux maladies et aux ravageurs : Les cultures biologiques sont plus exposées aux attaques de maladies et de ravageurs en raison de l'absence de produits chimiques de synthèse pour les contrôler. Le développement de variétés résistantes aux maladies et aux ravageurs permet de réduire l'impact de ces problèmes et de minimiser les pertes de récolte.

Tolérance aux conditions environnementales difficiles : Les variétés de *Brassica oleracea* adaptées à l'agriculture biologique peuvent être sélectionnées pour leur capacité à résister aux conditions environnementales défavorables telles que les sécheresses, les sols pauvres en éléments nutritifs et les fluctuations de température. Cela permet de garantir une production stable et fiable même dans des conditions difficiles.

Qualité nutritionnelle améliorée : L'agriculture biologique met l'accent sur la production d'aliments de haute qualité nutritionnelle. En améliorant génétiquement les variétés de *Brassica oleracea,* il est possible d'augmenter les teneurs en nutriments essentiels tels que les vitamines, les minéraux et les antioxydants, ce qui contribue à une alimentation plus saine et plus équilibrée.

Adaptation aux pratiques de gestion biologique du sol : Les pratiques de gestion du sol en agriculture biologique, telles que la rotation des cultures, la fertilisation organique et la lutte biologique contre les ravageurs, peuvent être mieux intégrées en développant des variétés de *Brassica oleracea* adaptées à ces pratiques spécifiques. Cela permet d'optimiser les interactions entre la plante et le sol, favorisant ainsi la santé du sol et la durabilité globale du système agricole.

**Materiel et méthodes**

Plan expérimental : Condition de stress hydrique

Le plan expérimental visait à étudier les effets du stress hydrique sur les accessions de Brassica. Les accessions utilisées provenaient de la collection de Brassica du Département d'Agriculture, d'Alimentation et d'Environnement (Di3A) de l'Université de Catane (UNICT) en Italie. Les étapes de l'expérience sont détaillées ci-dessous :

Semis des graines : Les graines ont été semées dans des plateaux cellulaires en utilisant un substrat biologique (Terri Bio, "Agro-Chimica S.p.", Bolzano, Italie). Ce substrat a été choisi pour assurer des conditions de croissance optimales.

Environnement de culture : Les plateaux cellulaires ont été placés dans une serre froide située sur la ferme expérimentale de l'Université de Catane (Di3A) dans le sud de l'Italie. La serre était exposée à la lumière naturelle. Cette localisation géographique précise est 37°31', 37°31'10" N 15°04'18" E.

Transplantation des plantules : Après environ un mois de croissance, les plantules ont été repiquées individuellement dans des pots de 0,3 L remplis du même substrat utilisé pour le semis. Cette étape a permis de garantir des conditions de culture uniformes pour toutes les accessions.

Division des parcelles : Quatre semaines après la transplantation, les plantes ont été séparées en deux parcelles distinctes : l'irriguée (IRR) et la non-irriguée (NIR). La parcelle irriguée a été utilisée comme témoin, tandis que la parcelle non-irriguée a été soumise à un stress hydrique.

Caractérisation bio-morphologique : Les accessions de *Brassica oleracea* ont été caractérisées sur le plan bio-morphologique. Cette caractérisation a été réalisée en utilisant des descripteurs morphologiques internationaux définis par l'IBPGR (International Board for Plant

Genetic Resources) et l'UPOV (Union internationale pour la protection des obtentions végétales). Ces descripteurs standardisés permettent de comparer et de classer précisément les différentes variétés de *Brassica oleracea.*

Génotypage par SSR : Le génotypage par SSR (Simple Sequence Repeat) a été utilisé pour analyser la variation génétique au sein des accessions de *Brassica oleracea.* Les marqueurs SSR sont largement utilisés dans la recherche en amélioration des plantes car ils permettent une analyse précise et reproductible de la variation génétique. Dans cette étude, 12 paires d'amorces SSR liées à la teneur en glucosinolates ont été testées sur les différentes variétés de *Brassica oleracea*.

En suivant ce plan expérimental, il était possible d'évaluer les effets du stress hydrique sur les accessions de *Brassica oleracea* et de caractériser leur variation morphologique et génétique.

Validation des gènes impliqués dans la biosynthèse des glucosinolates par la technique de la réaction en chaîne par polymérase quantitative (qPCR) : Les gènes responsables de la biosynthèse des glucosinolates sont identifiés et confirmés par qPCR, une méthode permettant de quantifier l'expression génique.La corrélation entre les niveaux d'expression génique et le contenu en glucosinolates ou d'autres traits phénotypiques pertinents : Les niveaux d'expression des gènes sont comparés aux caractéristiques phénotypiques, tels que les niveaux de glucosinolates, pour établir des corrélations et comprendre comment les gènes influencent ces traits.

La sélection des parents les plus adaptés pour le croisement, en tenant compte des caractéristiques souhaitées et de la diversité génétique disponible : Les plantes parentales qui présentent les caractéristiques souhaitées, comme des niveaux élevés de glucosinolates ou d'autres traits bénéfiques, sont sélectionnées pour le croisement. La diversité génétique est également prise en compte pour maintenir la variabilité dans la population hybride.

La réalisation du croisement en transférant le pollen (manuellement) : Le pollen des plantes parentales sélectionnées est transféré manuellement pour réaliser le croisement contrôlé. Cela garantit la reproduction sexuée entre les plantes choisies et permet de combiner les caractéristiques génétiques souhaitées.

La collecte des graines résultant du croisement pour produire une population de plantes hybrides : Les fleurs fécondées sont laissées à maturité et les graines résultantes sont collectées.

Ces graines donneront naissance à une population de plantes hybrides qui porteront les caractéristiques génétiques combinées des plantes parentales.

Ce processus permet de développer une population de plantes hybrides possédant des caractéristiques spécifiques, telles que des niveaux élevés de glucosinolates, en utilisant des connaissances sur la génétique, l'expression génique et la sélection appropriée des parents.

L'analyse biochimique du *Brassica oleracea* a révélé une composition riche en polyphénols, glucosinolates, vitamines et minéraux. Ces composés confèrent à la plante des propriétés bénéfiques pour la santé humaine, notamment des propriétés antioxydantes, anticancéreuses, anti-inflammatoires et antimicrobiennes. Il est donc intéressant d'étudier le profil biochimique de cette plante sous des conditions de sécheresse afin de comprendre ses réponses métaboliques et d'identifier les mécanismes de défense et d'adaptation impliqués dans la réponse au stress hydrique.

Dans le cadre de cette étude, la quantification des glucosinolates a été réalisée par HPLC en utilisant des standards externes. Les résultats ont montré une présence prédominante de quantités élevées de glucosinolates dans les feuilles par rapport aux racines. Cela revêt une grande importance pour les cultures de *Brassica oleracea* qui fournissent des feuilles en tant que produits, tels que le chou frisé et le brocoli.

De plus, il a été observé que la valeur des glucosinolates augmentait de manière significative en raison des conditions de sécheresse. Ces résultats sont cohérents avec des études antérieures qui soulignent l'importance des glucosinolates pour augmenter le statut antioxydant de la plante et contrôler le stress hydrique. Ces constatations pourraient avoir des implications dans l'identification de différentes accessions qui pourraient être utilisées dans le cadre de programmes d'amélioration future visant à créer une diversité génétique dans le germplasm local de Brassica avec une valeur élevée pour un glucosinolate spécifique.

En résumé, l'étude du profil biochimique du *Brassica oleracea* sous des conditions de sécheresse offre une perspective intéressante pour mieux comprendre les réponses métaboliques des plantes au stress hydrique et pour identifier des variétés ou des accessions présentant des caractéristiques spécifiques bénéfiques. Cela pourrait contribuer à l'amélioration future des cultures de *Brassica oleracea* et à la promotion de ses propriétés bénéfiques pour la santé humaine.

Riassunto

L'agricoltura biologica svolge un ruolo cruciale nella promozione di un sistema agricolo più sostenibile e rispettoso dell'ambiente. Si concentra sull'utilizzo di pratiche agricole naturali e rispettose dell'ecosistema, evitando l'uso di prodotti chimici sintetici come pesticidi e fertilizzanti chimici. Uno dei principali obiettivi dell'agricoltura biologica è mantenere la salute del suolo, preservare la biodiversità e produrre alimenti di alta qualità nutrizionale.

In questo contesto, lo sviluppo di varietà di *Brassica oleracea* adatte all'agricoltura biologica riveste una particolare importanza. *Brassica oleracea* è una pianta ampiamente coltivata, che include diverse varietà di cavoli, comunemente utilizzate nell'alimentazione umana. Sviluppando varietà specifiche adatte alle esigenze dell'agricoltura biologica, è possibile massimizzare i vantaggi di questo metodo di produzione sostenibile.

Le varietà di *Brassica oleracea* adatte all'agricoltura biologica possono offrire diversi vantaggi:

Resistenza alle malattie e ai parassiti: Le colture biologiche sono più esposte agli attacchi di malattie e parassiti a causa dell'assenza di prodotti chimici di sintesi per il loro controllo. Lo sviluppo di varietà resistenti alle malattie e ai parassiti permette di ridurre l'impatto di questi problemi e di minimizzare le perdite di raccolto.

Tolleranza alle condizioni ambientali difficili: Le varietà di *Brassica oleracea* adatte all'agricoltura biologica possono essere selezionate per la loro capacità di resistere a condizioni ambientali sfavorevoli come siccità, suoli poveri di nutrienti e fluttuazioni di temperatura. Ciò garantisce una produzione stabile e affidabile anche in condizioni difficili.

Miglioramento della qualità nutrizionale: L'agricoltura biologica pone l'accento sulla produzione di alimenti di alta qualità nutrizionale. Attraverso il miglioramento genetico delle varietà di Brassica oleracea, è possibile aumentare i livelli di nutrienti essenziali come vitamine, minerali e antiossidanti, contribuendo a un'alimentazione più sana ed equilibrata.

Adattamento alle pratiche di gestione biologica del suolo: Le pratiche di gestione del suolo in agricoltura biologica, come la rotazione delle colture, la concimazione organica e il controllo biologico dei parassiti, possono essere meglio integrate sviluppando varietà di Brassica oleracea

adatte a tali pratiche specifiche. Ciò consente di ottimizzare le interazioni tra la pianta e il suolo, favorendo la salute del suolo e la sostenibilità complessiva del sistema agricolo.

Per quanto riguarda il metodo sperimentale, il piano di studio si concentra sugli effetti dello stress idrico sulle accessioni di Brassica. Le accessioni utilizzate provengono dalla collezione di Brassica del Dipartimento di Agricoltura, Alimentazione e Ambiente (Di3A) dell'Università di Catania (UNICT) in Italia. Le fasi dell'esperimento sono dettagliate come segue:

Semina dei semi : I semi sono stati seminati in vassoi cellulari utilizzando un substrato biologico (Terri Bio, "Agro-Chimica S.p.", Bolzano, Italia) selezionato per garantire condizioni di crescita ottimali.

Ambiente di coltura: I vassoi cellulari sono stati posizionati in una serra fredda situata presso la fattoria sperimentale dell'Università di Catania (Di3A) nel sud Italia. La serra era esposta alla luce naturale.

Trapianto delle piantine: Dopo circa un mese di crescita, le piantine sono state trapiantate singolarmente in vasi da 0,3 L riempiti con lo stesso substrato utilizzato per la semina. Questo passaggio ha garantito condizioni di coltura uniformi per tutte le accessioni.

Divisione delle parcellizzazioni: Quattro settimane dopo il trapianto, le piante sono state suddivise in due distinte parcellizzazioni: irrigata (IRR) e non irrigata (NIR). La parcellizzazione irrigata è stata utilizzata come controllo, mentre la parcellizzazione non irrigata è stata sottoposta a stress idrico.

Caratterizzazione biomorfologica: Le accessioni di *Brassica oleracea* sono state caratterizzate dal punto di vista biomorfologico utilizzando descrittori morfologici internazionali definiti da IBPGR (International Board for Plant Genetic Resources) e UPOV (Union internationale pour la protection des obtentions végétales). Questi descrittori standardizzati consentono di confrontare e classificare in modo preciso le diverse varietà di Brassica oleracea.

Genotipizzazione mediante SSR : La genotipizzazione mediante SSR (Simple Sequence Repeat) è stata utilizzata per analizzare la variazione genetica all'interno delle accessioni di *Brassica oleracea.* I marcatori SSR sono ampiamente utilizzati nella ricerca sull'ingegneria genetica delle piante perché consentono un'analisi precisa e riproducibile della variazione genetica. In questo studio, sono stati testati 12 coppie di primer SSR legati al contenuto di glucosinolati nelle diverse varietà di *Brassica olerace*

# Publications

- **Ben Ammar, H**.; Arena, D.; Treccarichi, S.; Di Bella, M.C.; Marghali, S.; Ficcadenti, N.; LoScalzo, R.; Branca, F. The Effect of Water Stress on the Glucosinolate Content and Profile: A Comparative Study on Roots and Leaves of *Brassica oleracea* L. Crops. Agronomy 2023, 13,579

- Treccarichi, S.; **Ben Ammar, H**.; Amari, M.; Calì, R.; Tribulato, A.; Branca, F. Molecular Markers for Detecting Inflorescence Size of *Brassica oleracea* L. Crops and *B. oleracea* Complex Species (n = 9) Useful for Breeding of Broccoli (*B. oleracea* var. *italica*) and Cauliflower (*B. oleracea* var. *botrytis*). Plants 2023, 12, 407.

- **Ben Ammar, H**.; Picchi, V.;Arena, D.; Treccarichi, S.; Bianchi, G.;Lo Scalzo, R.; Marghali, S.; Branca, F. Variation of Bio-Morphometric Traits and Antioxidant Compounds of *Brassica oleracea* L. Accessions in Relation to Drought Stress. Agronomy2022, 12, 2016.