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Article

Variation of Bio-Morphometric Traits and Antioxidant Compounds of *Brassica oleracea* L. Accessions in Relation to Drought Stress

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Abstract: Drought tolerance of *Brassica* crops can be genetically improved by establishing plant ideotypes with improved yield responses associated with agronomic traits and biochemical markers. The objective of this study was to compare 20 *Brassica oleracea* L. accessions grown under two different water treatments (100% and 35% reintegration of evapotranspiration by irrigation) to select potential tolerant genotypes for organic cultivation based on several agronomic and biochemical parameters measured in response to drought stress. Significant differences were registered for the genotype and the irrigation regime and for their interaction ($p < 0.0001$ ***). A principal component analysis was performed to summarize the correlations among the analyzed phytochemicals and the stressed and not stressed genotypes and highlighted the importance of the antioxidant compounds as stress biomarkers. The present results showed that drought significantly reduces growth parameters and increases the amount of ascorbic acid and polyphenols compared to the irrigated control. Additionally, the results show that antioxidant metabolism increased by drought in some genotypes while others maintained a good biomass production by increasing the value of growth parameters considered. Based on the average sum of ranks (ASR) of morpho-physiological and biochemical parameters, the genotypes CR, CC, and BH were determined to be the most drought tolerant, whereas CI5, BU, and CV1 were determined to be the most susceptible. Due to the potential of these genotypes, further molecular and cellular research will be carried out to identify the genetic marker associated with the water stress response.

Keywords: *Brassica oleracea*; deficit irrigation; photosynthetic pigments; polyphenols; ascorbic acid; glutathione; HPLC

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1. Introduction

In both natural and agricultural environments, plants are susceptible to a range of environmental challenges during their growth and development [1]. In the current period of climate change, water stress is one of the main risks to food safety [2]. The impacts of the drought on agriculture are exacerbated by the loss of water resources and the rise in global food demand [3]. Unfortunately, most crops grow and propagate under suboptimal conditions due to the scarcity of water, which is becoming more and more frequent due to global warming [4]. The lack of water affects photosynthesis, nutrient and water relations, and growth and eventually results in a considerable decrease in agricultural production [5]. Therefore, researching more about how plants may adapt to waterlogging is necessary and should continue to receive attention, particularly in arid and semi-arid regions. Thus,

understanding the consequences of drought stress in plants is essential for improving agricultural breeding techniques and anticipating what will happen to natural vegetation under dramatic climatic changes [6].

The performance of cultivated plants, as well as the growth and distribution of natural vegetation, are both affected by permanent or temporary water shortages [7]. Water stress has a significant negative impact on most physiological processes [8]. All plants that were exposed to a water shortage displayed substantial alterations in the shape of their roots and shoots. Both the total number of leaves and the size of each individual leaf decline during a drought. The assimilate supply and osmotic pressure frequently control how much the leaf expands [9]. Plant height and stem diameter decreased dramatically under water-limiting conditions; fresh weight and dry weight also decreased significantly [10,11].

Water deficiency affects a variety of plant functions, but one of the most common is the decrease in photosynthetic activity [12]. Chlorophyll (Chls) reduction is an indicator of oxidative stress and could be caused by photooxidation [13]. Both chlorophyll (Chls) and carotenoids (CAR) content, as well as Chls to CAR ratio, are often used to monitor the physiological state of plants during acclimation and adaptation to different stimuli [14,15]. Thus, abiotic stress increases the production of the Reactive Oxygen Species (ROS), such as superoxide radicals, hydrogen peroxide (H2O2), hydroxyl radicals (OH•), and singlet oxygen (1O2) which affect proteins and lipids content, resulting in cellular damage and plant death [16]. At high levels, ROS are toxic to cells, but at the same time, they can function as a signal transducer that triggers a local and systemic plant defense against stress [17].

Despite the detrimental effects of water scarcity on plant performance, plants could respond differently to various levels of water deficit [18]. For each stress, different tolerance levels and mechanisms can lead to different responses depending on the plant's growth stage [19]. Over the past decade, researchers have focused on the molecular processes controlling biotic and abiotic stress tolerance, emphasizing individual stress tolerance mechanisms [20]. Whenever a water deficit occurs, plants develop highly complex systems at the molecular, physiological, and environmental levels [21].

Indeed, water stress requires immediate acclimation through morphological changes in plants to adapt to their altered environment [22]. The secondary phenotypic changes seen in plants under water stress, whether or not the plants can adapt, are a result of metabolic changes [23]. Osmotic adjustment, osmoprotection, antioxidation, and scavenging defense mechanisms have been identified as interconnected strategies that plants have developed to resist stress [24]. Biochemical reactions are detected after a decrease in CO_2 availability in the mesophyll, being crucial for coping with drought [25]. The first and most important response of plants to water scarcity is the stomatal closure, which is activated to reduce water loss by transpiration. Stomatal closure may be a response to reduced leaf water potential or reduced atmospheric humidity [26]. It also determines a reduction of CO_2 fixation that, if it persists for a long period, finally impacts plant yield [27].

The fundamental abiotic stress tolerance is influenced by the metabolites that react to different stresses and can function as compatible solutes [28]. Plants have evolved mechanisms to control the production and scavenging of the ROS through enzymatic and non-enzymatic antioxidative processes [29]. Metabolite profiling is a commonly used method to determine the molecular responses of plants under abiotic stress [30–32]. Drought stress is expected to cause an increase in the biosynthesis of key antioxidant molecules such as ascorbic acid (AsA), one of the most abundant antioxidants in plants [33]. Moreover, dehydroascorbic acid (DHA) increases rapidly during abiotic stress and influences the equilibrium of the redox state between apoplast and cytoplasm, triggering plant response to adverse environmental conditions [34]. By controlling the redox status of plant cells, AsA combines the effects of different signaling pathways and controls the abiotic stress responses of plants [35]. As markers of response to abiotic stressors, both total ascorbic acids, as sum, the reduced (AsA) and oxidized forms (DHA) and their ratio could vary in relation to the ROS formation [36]. It was observed that plants with higher AsA levels and better regeneration rates of reduced AsA might be more resistant to such stressors [37].

The antioxidant metabolite strictly related to the regeneration of AsA is glutathione, a tripeptide with a thiol (-SH) functional group. This compound is present in both reduced (-SH) and oxidized form (S-S), and it actively participates in the reactions regenerating the reduced form of AsA through the Halliwell–Asada cycle [38]. Other metabolites with high antioxidant capacity include polyphenolic compounds, which are receiving increasing attention not only for their beneficial effects on human health but also for their protection against oxidative damage in stressed plants [39]. Polyphenols are directly involved in the response of plants to various types of stress since they contribute to healing by lignifying damaged areas and possess antimicrobial proprieties [40].

Given that antioxidant compounds produced by plants in response to abiotic stress are also potentially interesting for human health, understanding the dynamics and mechanisms involved in the biosynthesis and distribution of active compounds in edible plants such as *Brassica oleracea* with increased bioactive compounds content could therefore provide a pathway for developing improved plant varieties [41]. *B. oleraceae* crops and related wild species (n = 9) have received special attention among leafy vegetables [42] for their high phytochemical content, which includes high levels of vitamins, minerals, dietary fiber, glucosinolates, and phenolic compounds [43]. The leaves are characterized by a typical taste due to the presence of a wide array of sulfur compounds and are one of the ingredients of the Mediterranean diet [44]. Cauliflower and broccoli showed significant antioxidant activity and could be used as sources of antioxidants [45]. Cultivation under stress conditions can promote the production of these bioactive molecules associated with the antioxidant system and plant defense mechanisms. The concentration of bioactive compounds may greatly vary depending on species and genotypes since they may respond differently to stress [46].

The aim of this study is to investigate the effect of water stress on the morpho-agronomic parameters as well as biochemical traits on 20 genotypes of *Brassica oleracea* L. grown under normal and drought stress conditions. The analysis of the results led to the screening of the most tolerant genotypes of *B. oleracea* L. to water stress. Understanding the physiological and biochemical adaptations to variations in drought resistance in plants could be used as selection criteria for the development of drought-tolerant cultivars.

2. Material and Methods

2.1. Plant Material and Experimental Design

In this study, 20 accessions of *B. oleracea* and the related *B. oleracea* complex species (n = 9) (Supplementary Table S1) were evaluated. The seeds of each accession were placed in a cellular tray arranged in a cold greenhouse (in light natural conditions from 4.6 to 9.2 MJ.m^{-2}.d^{-1} and temperature between 15.4 ± and 5.8 ± °C) in Catania (37°31′10″ N 15°04′18″ E). The containers were filled with Brill soil (Geotec, Italy) and were irrigated according to ordinary techniques. The experiment design included three replicates for each cultivar in both treatments. The four-leaf-stage plantlets were transplanted in a certified organic greenhouse located in Santa Croce Camerina (36°51′13.3″ N 14° 29′32.0″ E, Ragusa, Italy) using single rows, with 1.0 m between the rows and 0.5 m between the plants along the rows, at crop density of 2 plants/m^2. The experiment was conducted by comparing plants grown under two different irrigation regimes (IR): 100% and 35% evapotranspiration (100% and 35% ETc) [47]. The total volumes used during the trial were 20.45 m^3 for 35% ETc and 51.65 m^3 for 100% ETc. The temperature was recorded daily (Supplementary Figure S1) using a hygro-thermometer (445702, Extech Instruments, Nashua, NH, USA). The treatments performed were against snails (Ferramol), aphids (Pyganic 2.5 mL/L), and *Pieris brassicae* (Bacillus 1.5 g/L) and were effectuated granular fertilization based on micro- and macro-elements. The harvest was performed in January 2020 at the commercial maturity stage of the plants. Leaves were rapidly frozen at −80 °C for 72 h. The freeze-dried material was powdered using an IKA-A10 mill (IKA-Werke GmbH & Co. KG, Staufen, Germany) and stored at −20 °C until analyses.

2.2. Bio-Morphometric Traits

The characterization of the plants was done using the international Descriptors IBPGR (International Board for Plant Genetic Resources) and UPOV (The International Union for the Protection of New Varieties of Plants) morphological descriptors. Height (cm), weight (g), stem diameter (cm), number of leaves (n), fresh biomass of leaves (g), and the % of dry matter were evaluated as morphometric traits.

2.3. Soluble Solids Content and SPAD Measurements

The soluble solids content (SSC, expressed in Brix degrees, °Bx) is an important index that is used to evaluate the quality and is mostly related to the sugar content of fruits and vegetables. The SSC was measured directly on plant juice with a digital refractometer (PAL-1, Atago, Tokyo, Japan). The SPAD index was measured on three fully developed leaves near the plant apex per plant using a portable chlorophyll meter SPAD-502 (Minolta Camera Co., Osaka, Japan).

2.4. Biochemical Traits

2.4.1. Determination of Photosynthetic Pigment

The concentration of chlorophyll *a* and *b* were determined according to the method of Lichtenthaler et al. (2001) [48]. An amount of (0.1 g) of freeze-dried powder from each sample (leaves) was homogenized in 2 mL EtOH/acetone 1:1, and 0.02% BHT (butylated hydroxytoluene) was added. The extract was centrifuged at 1200 rpm for 5 min, and the absorbance of the supernatant was measured after dilution with EtOH using a spectrophotometer with a 1 cm optical path length. Chlorophyll concentrations were then determined using the formulas proposed in the protocol mentioned above. The absorbance was measured at 649 nm and 664 nm for chlorophylls and 470 nm for carotenoids using a spectrophotometer. Ethanol was used as a blank. Chlorophyll *a*, chlorophyll *b*, and total chlorophyll were calculated on an exponential basis using the following equations:

$$\text{Chlorophyll a } (\mu g/mL) = [13.36(A664) - 5.19(A649)]$$

$$\text{Chlorophyll b } (\mu g/mL) = [27.43(A649) - 8.12(A664)]$$

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

In the above-mentioned equations, Ca and Cb are the concentrations of Chlorophylls a and b. The final measure units for chlorophyll and total carotenoids have been mg/100 g of freeze-dried material.

2.4.2. Ascorbic Acid Analysis

Ascorbic acid was analyzed following the method described by [49]. An aliquot of 50 mg of freeze-dried leaves was treated with 1 mL of cold 3% metaphosphoric acid. The resulting suspension was shaken for 1 min and then centrifugated at 12,000 rpm for 5 min. For each extract, the supernatant (100 µL) was diluted with 900 µL of 0.02 M ortho-phosphoric acid. The HPLC analysis was performed using an HPLC Agilent 1200 series system (Agilent, Santa Clara, CA, USA) equipped with a diode array detector (DAD). Separations were performed on LICHROSPHERE-RP C18 4 × 250 mm, column maintained at 30 °C; the isocratic elution was performed using orthophosphoric acid 0.02 M as mobile phase, at a flow rate of 0.5 mL/min. and a UV detector set at 254 nm. The volume of injected samples was 10 µL and the retention time of ascorbic acid (AsA) under these conditions was 5.96 min. A reduction reagent, tris-2-carboxy-ethyl phosphine (TCEP), 0.1M dissolved in 0.1M HCl was added to the extract and allowed to react for 10 min at room temperature to convert DHA into AsA to determine the total ascorbic acid, while the basal AsA concentration was obtained from the extract without the addition of TCEP [50]. For quantitative analysis, calibration curves were constructed by diluting with 0.02 M metaphosphoric acid 3% stock solutions of AsA at known concentrations to obtain a

linear equation (y = 90923x, R^2 = 0.999) by plotting the obtained peak areas against the concentrations in mg/100 mL. Concentrations were expressed in µmol/g DW.

2.4.3. Glutathione Determination

Reduced glutathione (GSH) and oxidized glutathione (GSSG) were detected by HPLC using a coulometric electrochemical detector (ESA mod. 6210, Chelmsford, MA, USA) according to the method of [51], as previously described in [52]. Isocratic elution was performed using 25 mM monobasic sodium phosphate with 0.5 mM heptane sulfonic acid (ion-pairing agent) and 2.5% acetonitrile. A flow rate of 0.6 mL/min was used with a Zorbax 250-4 mm C18 column. The four-array electrode system was 1 = + 300.2 = + 450.3 = + 600.4 = +900 mV. Electrodes 1 and 2 served as screening electrodes to oxidize potentially interfering compounds. GSH was detected at electrodes 3 and 4 (retention times 7.5 min), and GSSG was monitored at electrode 4 (retention time 9.5 min). Quantification was performed using a calibration curve of a standard mixture containing GSH and GSSG (range 0.001–0.004 mg/mL).

2.4.4. Total Phenolic Content (TPC)

Total phenolic content was determined using the Folin–Ciocalteu method. The Folin–Ciocalteu index (FCI) was calculated on methanolic extracts as described by Di bella et al. (2020) [53], with slight modifications. Sixty milligrams of lyophilized material were homogenized in 1.5 mL of 80% (*v*/*v*) methanol, then centrifuged at 15,000 rpm for 10 min at 4 °C. An aliquot of 0.2 mL of the supernatant was mixed with 0.5 mL of Folin–Ciocalteu reagent and carefully mixed. After 3 minutes at room temperature, 1 mL of 7.5% sodium carbonate was added. Each tube was vortexed for 20 s, then allowed to stand for 60 min in the dark at room temperature for color development. Then, the absorption was measured at 730 nm against a blank with all reagents, except sample or standard solutions. The amount of total phenolics is expressed as gallic acid equivalents (GAE, mg gallic acid/g sample) through a calibration curve of gallic acid, obtained with solutions at known concentrations. The TPC calculation equation is expressed in mg/100 g DW, following the formula: ((C × DF × mg)/g) × 100.

Where: C is the concentration of the sample; DF is the dilution factor of 25; mg indicates milligrams of the initial sample; and g are the grams of the sample used.

2.5. Statistical Analysis

For each analysis, the results are represented by the mean ± standard deviation (SD) of the replicates. Two-way ANOVA with genotypes and water stress as variation factors followed by Tukey's multiple comparisons test was performed using GraphPad Prism version 8.0 (GraphPad Software, Inc., San Diego, CA, USA). The statistical significance was considered for ***p***-values < 0.05. The correlations among morphological variables and biochemical compounds were evaluated by computing the Pearson correlation coefficient. Finally, the multivariate analysis PCA (principal compound analysis) (and cluster analysis) was computed to highlight and summarize the differences among samples using the XLSTAT2018 software (Addinsoft, Paris, France).

Relative change (RC) due to stress was also determined for each trait using the following formula:

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

Additionally, iPASTIC [54], an online toolset, calculated the stress tolerance index (STI) to screen better-performing genotypes under water stress conditions, and the genotypes with the lowest average sum of rankings (ASR) were considered the most tolerant.

3. Results

3.1. Bio Morphometric Traits

Based on the morphometric parameters, the accessions studied were evaluated for their response to water stress treatment. The data showed a clear effect of drought stress and indicated considerable variability among the genotypes (Tables 1 and 2). In this study, we observed that the water stress significantly ($p < 0.001$) affected the growth parameters. It affected the weight and height of plants in most of the genotypes tested 31.3% and 10.80%, respectively, except for the accession BR5 (*Brassica oleracea* var. *italica*) (−52.3% and −31.8%, respectively) and for the wild species BU (*Brassica rupestris*) (−9.7% and −35.7%, respectively) (Table 2). Compared to the control, the stem diameter decreased in stressed plants (%var = 14.91%). However, an increase in this parameter was observed for some accessions: BR4, BU, and CI1 (−42.7%, −33.4%, and −26.5%, respectively).

Moreover, the obtained results showed that the water stress had a significant ($p < 0.05$) effect on the number of leaves per plant. An increase in the number of leaves was observed in some accessions BR5 (*Brassica oleracea* var. *italica*) and CI1 (*Brassica oleracea* var. *italica x botrytis*) with a variation of −54.3% and −42.9%, respectively. For the SPAD, the interaction between the factors showed no significant difference ($p = 0.05$).

In addition, the drought stress increases the SSC (%var = −5.9%). However, a different response was observed in the 20 accessions studied; the accession BU (*Brassica rupestris*) showed a significant decrease in SSC content of 38.2% while three accessions (CI2, CV2, CI5) showed an increase in SSC following drought stress %var= −46.2%, −35.3%, and −33.3%, respectively (Table 2).

Brassica plants grown with a reduced water supply (35%ETc) showed a significant decrease ($p < 0.05$) in their shoot fresh weight (FW) in comparison to the control plants. However, an increase in the percentage of reduction of FW for the wild species BU was observed (%var= −21.9%). The decrease in the total dry matter could be due to a sharp decline in plant growth and physiological parameters during water stress. Accessions BR2 (*Brassica oleracea* var. *italica*), BTR (*Brassica oleracea tronchuda*), and CI4 (*Brassica oleracea* var. *italica x botrytis*) could be considered drought-susceptible since they had the highest percentage of weight and height reductions in stressed plants, as well as the highest percentage of leaves and stem length reductions.

3.2. Biochemical Analysis

3.2.1. Photosynthetic Pigment

Water stress reduced the levels of photosynthetic pigments Chla, Chlb, and total carotenoids (CAR) in the leaves of most of the accessions analyzed (13 of 20), although there were significant variations in the level of the reduction among the accessions ($p < 0.0001$ ***) (Table 3). In fact, Chla, Chlb, and Carotenoids ranged in concentration in the control circumstances from 101.5 to 955.5; 15.6 to 310; and from 12.8 to 148,00 mg/100g DW, respectively. In contrast, under water stress, *Brassica oleracea* genotypes responded to stress in different ways and displayed a significant shift in their amounts with RC = 46.6%, 42.63%, and 16.04%, respectively (Table 3).

Leaf pigments were significantly and gradually reduced under drought stress except for three accessions, i.e., BH (*Brassica oleracea* var. *acephala*), BR5 (*Brassica oleracea* var. *italica*), and CV3 (*Brassica oleracea* var. *botrytis*), which increased the amount of chlorophyll Chla under drought stress. The accessions CC (*B. oleracea* var. *capitata)* showed the lowest contents, that is, 102, 28, and 129 mg/100g DW for Chla, Chlb, and CAR, respectively. The highest reductions due to drought stress were observed for the accessions BR1, CR, CI1, CI2, and CV2, with a percentage of reductions greater than 70% compared to controls. In 5 out of 20 accessions, i.e., CC, CI5, CI7, BR3, and CV3, photosynthetic pigments content did not change significantly in water-stressed plants compared to controls. Finally, accessions BH and BR5 showed a significant increase in pigment content three-fold (in BH) and two-fold (in BR5) (Figure 1A).

Table 1. Variation of morphological traits (Mean ± SD) studied in the 20 accessions of *Brassica oleracea* subspecies.

Accessions	Conditions	PW(g)	PH (cm)	SD (cm)	N°L	FW (mg)	%DM	SPAD	SSC
		MEAN ± SD	MEAN ± SD	MEAN ± SD	MEAN ± SD	MEAN ± SD	MEAN ± SD	MEAN ± SD	MEAN ± SD
BH	Control	1367.4 ± 642.6 f–i	114.0 ± 64.0 a	18.8 ± 0.3cde	17.0 ± 4.0 c–f	85.9 ± 24.1 e	26.5 ± 0.9 abc	60.4 ± 18.9 abc	5.7 ± 0.8 a–e
	Stress	673.0 ± 194.9 hi	97.5 ± 12.5 bcd	19.7 ± 1.6 c–f	14.0 ± 2.0 i	30.1 ± 2.5 n	17.0 ± 3.4 c–f	50.2 ± 7.4 c	5.8 ± 0.4 b–g
BR1	Control	1758 ± 33.4 e–i	75.6 ± 13.4 abc	26.5 ± 4.7 a–d	23.0 ± 2.0 a–e	60.6 ± 90.5 e	17.7 ± 2.3 efg	67.9 ± 0.9 abc	6.8 ± 0.3 abc
	Stress	1012 ± 226.6 f–i	66.1 ± 19.1 efg	20.4 ± 5.4 b–f	18.0 ± 3.0 ghi	50.6 ± 4.8 lm	15.1 ± 0.9 c–f	65.2 ± 7.9 abc	6.2 ± 1.0 a–g
BR2	Control	2586.4 ± 176.2 c–h	131.8 ± 0.3 a	32.8 ± 4.1 abc	22.0 ± 1.0 a–e	909.5 ± 2.5 abc	10.32 ± 4.2 g	53.2 ± 9.9 abc	5.8 ± 0.3 a–e
	Stress	990.0 ± 6.0 f–i	78.0 ± 4.0 d–g	18.1 ± 0.7 def	26.0 ± 2.0 b–g	319.6 ± 2.6 a	10.9 ± 0.7 def	65.0 ± 3.9 abc	6.5 ± 1.0 a–f
BR3	Control	2732.5 ± 187.3 b–g	95.0 ± 10.4 abc	34.4 ± 5.1 ab	27.0 ± 8.5 abc	1505.1 ± 5.0 a	11.9 ± 1.7 d–h	70.4 ± 10.5 abc	7.3 ± 0.8 a
	Stress	1330 ± 223.9 e–i	81.3 ± 13.4 c–f	22.4 ± 2.4 b–e	19.0 ± 1.0 e–i	157.6 ± 57.9 f	16.6 ± 4.7 c–f	76.0 ± 13.1 ab	8.2 ± 1.0 a
BR4	Control	1208.1 ± 470.0 f–i	83.0 ± 15.1 abc	15.3 ± 1.7 de	19.0 ± 4 b–e	483.1 ± 4.4 b–e	11.6 ± 0.7 cd	75.8 ± 7.5 ab	7.3 ± 1.3 a
	Stress	1421.8 ± 109.5 d–i	59.5 ± 0.5 fg	21.8 ± 0.1 b–e	27 ± 3.2 b–e	305.2 ± 1.4 b	13.1 ± 0.0 c–f	65.7 ± 7.9 abc	6.2 ± 0.8 a–g
BR5	Control	1052.4 ± 280.4 g–i	85.0 ± 11.0 abc	28.8 ± 16.3 a–d	16.0 ± 2.0 ef	197.1 ± 0.0 cde	26.1 ± 0.7 abc	67.4 ± 9.5 abc	6.2 ± 0.8 a–d
	Stress	1603.7 ± 184.1 d–h	112 ± 15.0 ab	22.4 ± 1.6 b–e	35.0 ± 3.0 a	57.7 ± 0.2 kl	15.3 ± 2.4 c–f	77.6 ± 4.2 a	7.9 ± 1.1 ab
BTR	Control	2134.3 ± 384.3 d–i	115.0 ± 10.0 ab	27.9 ± 0.1 a–d	21.0 ± 1.0 a–e	255 ± 0.0 b–e	18.2 ± 1.9 c–f	47.8 ± 4.6 c	3.5 ± 0.5 e
	Stress	918.5 ± 1.6 f–i	64.5 ± 1.5 fg	29.7 ± 6.5 a–d	14.0 ± 2.0 i	59.6 ± 0.1 kl	8.7 ± 0.7 f	58.5 ± 2.4 abc	3.0 ± 0.0 i
BU	Control	738.6 ± 4.45 hi	70.0 ± 25.0 bc	22.7 ± 5.2 b–e	17.0 ± 1.0 c–f	123.6 ± 0.0 de	19.9 ± 3.7 cd	52.5 ± 6.7 bc	5.7 ± 0.8 a–e
	Stress	810.4 ± 400.7 g–i	95.0 ± 5.0 bcd	30.3 ± 5.9 abc	17 ±2.0 hi	150.7 ± 30.7 f	14.5 ± 2.6 bcd	67 ± 13.0 abc	3.5 ± 0.5 hi
CC	Control	2970.7 ± 221.3 b–f	90.3 ± 1.3 abc	31.8 ± 7.9 abc	26.0 ± 3.0 a–d	118.3 ± 0.0 de	32.2 ± 5.8 a	49.2 ± 1.3 bc	4.7 ± 0.3 cde
	Stress	2155 ± 192.5 c–e	81.8 ± 0.5 c–f	25.5 ± 0.5 a–d	25.0 ± 3.0 b–g	64.6 ± 0.2 jk	15.4 ± 1.2 a	66.2 ± 3.8 abc	4.2 ± 0.3 ghi
CI1	Control	5226 ± 707.4 a	105.7 ± 12.7 ab	22.3 ± 2.9 b–e	21.0 ± 7.0 a–e	302.3 ± 0.0 b–e	20.9 ± 1.3 bcd	50.7 ± 7.6 bc	5.3 ± 0.6 a–e
	Stress	3790.2 ± 240.2 a	108.5 ± 3.5 abc	28.2 ± 0.1 a–d	30.0 ± 1.0 abc	85.45 ± 5.15 hi	19.8 ± 1.4 bc	51.8 ± 4.5 bc	6.5 ± 0.5 a–f
CI2	Control	2930.2 ± 990.0 b–g	92.0 ± 27.9 abc	30.6 ± 5.4a–d	22.0 ± 1.0 a–e	215 ± 28.0 a–d	14.8 ± 4.0 d–h	58.6 ± 1.9 abc	4.8 ± 0.3 b–e
	Stress	2593.1 ± 874.9 bc	93.8 ± 16.8 b–e	24.5 ± 8.1 a–d	24.0 ± 2.0 b–g	77.2 ± 7.9 i	20.34 ± 1.9 c–f	48.2 ± 13.9 c	7.1 ± 1.6 a–e

Table 1. *Cont.*

Accessions	Conditions	PW(g)	PH (cm)	SD (cm)	N°L	FW (mg)	%DM	SPAD	SSC
		MEAN ± SD	MEAN ± SD	MEAN ± SD	MEAN ± SD	MEAN ± SD	MEAN ± SD	MEAN ± SD	MEAN ± SD
CI3	Control	1928 ± 196.4 e–i	118.5 ± 5.5 ab	30.6 ± 1.0 a–d	31.0 ± 3.0 a	316.3 ± 18.3 b–e	14.4 ± 1.8 d–h	66.2 ± 0.7abc	5.3 ± 0.6 a–e
	Stress	1660.2 ± 391.0 c–g	94.0 ± 5.3 bcd	21.8 ± 3.3 b–e	28.0 ± 4.0 a–d	40.9 ± 7.9 mn	3.4 ± 0.8 c–f	61.9 ± 7.5 abc	5.3 ± 0.5 e–h
CI4	Control	4544.3 ± 1271.9 ab	124 ± 5.3 ab	30.1 ± 5.4 a–d	29.0 ± 3.0 ab	427.8 ± 0.3 b–e	17.7 ± 3.1 c–g	57.5 ± 7.4 abc	6.8 ± 1.6 abc
	Stress	1817.9 ± 423.6 c–f	101.7 ± 12.6 bcd	23.9 ± 3.2 bcd	26.0 ± 2.0 b–f	74.4 ± 24.4 ij	3.8 ± 1.8 c–f	55.2 ± 5.1 abc	6.6 ± 0.4 a–f
CI5	Control	3858.2 ± 52.5 a–d	118.5 ± 8.5 ab	27.5 ± 1.9 a–d	23.0 ± 4.0 a–e	393.6 ± 193.4 b–e	9.4 ± 1.8 fgh	80.6 ± 8.3a	5.8 ± 0.75 a–e
	Stress	2197.8 ± 66.2 c–e	99.5 ± 10.5 bcd	24.9 ± 2.5 a–d	31.0 ± 1.0 ab	96 ± 0.0 h	21.8 ± 1.9 ef	71.5 ± 3.2 abc	7.7 ± 0.3a–d
CI6	Control	4463.2 ± 413.1 a–c	118.5 ± 4.5 ab	31.5 ± 2.8 abc	26.0 ± 3.0 a–e	257.3 ± 24.9 b–e	17.1 ± 3.8 h	71.6 ± 15.5 abc	6.1 ± 0.5 a–d
	Stress	3686.3 ± 53.7 a	130.9 ± 2.7 a	36.2 ± 7.9 a	27.0 ± 3.0 b–f	135.6 ± 15.6 g	8.3 ± 3.0 f	58.6 ± 3.6 abc	5.5 ± 0.5 d–h
CI7	Control	3539 ± 1439 a–e	127.6 ± 26.7 ab	40.8 ± 1.0 a	28.0 ± 2.0 ab	324.9 ± 24.9 b–e	11.9 ± 2.8 d–h	67.5 ± 3.9 abc	4.3 ± 0.8 de
	Stress	3498.4 ± 2.0 ab	133.5 ± 1.5 a	32.3 ± 3.9 ab	22.0 ± 2.0 d–h	169.8 ± 51.8 e	11.4 ± 0.0 c–f	58.7 ± 16.4 abc	5.7 ± 0.3 c–h
CR	Control	651.6 ± 100.5 i	36.5 ± 5.5 c	10.43 ± 0.7 e	7.0 ± 2.0 f	78.15 ± 7.6 e	29.5 ± 4.5 ab	54.8 ± 6.1 abc	4.0 ± 0.5 de
	Stress	541.0 ± 191.0 i	28 ± 1.0 h	8.3 ± 0.3 f	6.0 ± 2.0 j	74.9 ± 15.1 ij	25.8 ± 4.2ab	49.6 ± 8.2 c	4.5 ± 0.0 f–i
CV1	Control	5010.2 ± 866 a	107.3 ± 7.5 ab	29.23 ± 0.3 a–d	29.0 ± 1.0 ab	923.6 ± 16.5 ab	16.4 ± 1.8 d–h	58.6 ± 3.8 abc	7.2 ± 1.3 ab
	Stress	2360.5 ± 266.1 cd	94.25 ± 5.8 bcd	18.1 ± 4.6 def	19.0 ± 5.0 f–i	183.45 ± 1.7 d	12.0 ± 3.8 c–f	59.0 ± 8.9 abc	7.8 ± 0.3 abc
CV2	Control	5281.1 ± 592.2 a	93.0 ± 7.0 abc	32.0 ± 0.9 abc	29.0 ± 2.0 ab	538.1 ± 11.9 b–e	8.6 ± 2.7 gh	54.4 ± 6.6 abc	5.7 ± 0.8 a–e
	Stress	3957.1 ± 440.8 a	92.75 ± 2.75 b–e	28.2 ± 0.06 a–d	23.0 ± 3.0 c–h	231.7 ± 11.9 c	10.7 ± 0.0 f	54 ± 5.9 abc	7.7 ± 0.8 a–d
CV3	Control	775.5 ± 276.3 hi	76.5 ± 23.5 abc	26.1 ± 2.6 a–d	16.0 ± 3 def	259.1 ± 69.1 b–e	18.8 ± 4.3 cde	61.3 ± 16.8 abc	7.0 ± 0.5 abc
	Stress	606.2 ± 157.2 i	51.5 ± 0.5 gh	11.3 ± 0.1 ef	16.0 ± 1.0 hi	51.0 ± 1.3 lm	19.3 ± 3.0 cde	55.7 ± 6.3 abc	6.3 ± 0.8 a–g

PW: plant weight; PH: plant height; SD: stem diameter; N°L: number of leaves; % DM: dry matter; SSC: Soluble Solids Content (° BRIX). Values are reported as mean ± standard (n = 3). Different letters indicate significant differences among accessions undergoing the same treatment according to Tukey test ($p < 0.05$).

Table 2. Percentage of variance and statistical significance of morphological parameters (%variation = ((control-stress)/control × 100).

Accessions	PW(g)		PH (cm)		SD (cm)		N°L		FW (mg)		%DM		SPAD		SSC (°Bx)	
	% Var	*p* Value	% Var	*p* Value	% Var	*p* Value	% Var	*p* Value	% Var	*p* Value	% Var	*p* Value	% Var	*p* Value	% Var	*p* Value
BH	50.8	ns	14.5	ns	−4.79	ns	17.6	ns	50.86	ns	35.80	**	16.80	ns	−2.60	ns
BR1	42.4	ns	12.6	ns	23.3	ns	20.3	ns	91.70	***	14.60	ns	3.90	ns	8.60	ns
BR2	61.7	***	40.8	****	44.9	**	−16.7	ns	64.90	***	−5.30	ns	22.30	ns	11.40	ns
BR3	51.3	***	14.4	ns	35.1	*	29.3	*	89.50	***	39.10	ns	−8.10	ns	11.40	ns
BR4	17.7	ns	28.3	ns	−42.5	ns	−37.9	ns	36.80	ns	13.00	ns	13.30	ns	15.90	ns
BR5	−52.3	ns	−31.8	*	22.3	ns	−54.3	****	70.70	ns	41.30	***	15.20	ns	28.10	ns
BTR	56.9	**	43.9	***	−6.3	ns	31.7	ns	76.60	ns	51.90	**	22.40	ns	14.30	ns
BU	−9.7	ns	−35.7	ns	−33.4	ns	0	ns	−21.93	ns	27.14	ns	27.62	ns	38.60	*
CC	27.5	*	9.4	ns	19.6	ns	6.3	ns	45.60	ns	52.00	***	34.60	ns	10.00	ns
CI1	27.5	***	−2.6	ns	−26.5	ns	−42.9	**	71.30	*	5.10	ns	−2.20	ns	21.90	ns
CI2	11.5	ns	−2	ns	19.8	ns	−10.6	ns	64.20	ns	37.10	ns	17.80	ns	46.20	**
CI3	13.9	ns	20.7	ns	28.6	ns	9.7	ns	87.10	**	76.50	***	6.40	ns	0.00	ns
CI4	60	***	18	ns	20.6	ns	10.2	ns	82.60	***	78.60	***	3.90	ns	3.90	ns
CI5	43	***	16	ns	9.2	ns	−36.2	*	82.50	***	56.80	***	11.30	ns	33.30	*
CI6	17.4	ns	−10.5	ns	−14.8	ns	−1.9	ns	47.30	***	51.60	**	18.20	ns	9.80	ns
CI7	1.1	ns	−4.7	ns	21	ns	21.2	ns	47.70	***	4.90	ns	13.00	ns	30.80	ns
CR	17	ns	23.3	ns	20.6	ns	21.7	ns	4.20	ns	12.60	ns	9.50	ns	12.50	ns
CV1	52.9	***	12.2	ns	38.2	ns	34.5	**	80.10	***	26.40	ns	−0.60	ns	−8.10	ns
CV2	25.1	**	0.3	ns	11.9	ns	20.7	ns	57.00	***	24.10	ns	0.80	ns	35.30	*
CV3	21.8	ns	32.7	ns	56.7	**	2.1	ns	80.30	ns	−2.60	ns	9.20	ns	9.50	ns

PW: plant weight; PH: plant height; SD: stem diameter; N°L: number of leaves; % DM: dry matter; SSC: Soluble Solids Content (° BRIX). For the effect of the treatment, ns, *, **, and *** indicate respectively that the effect is not significant or significant at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Table 3. Analysis of variance (ANOVA) and summary statistics for measured morphological and physio-biochemical traits of 20 *Brassica oleracea* accessions evaluated under control and stress.

Traits	G	IR	G X IR	Control				Stress				RC (%)
				Min	Max	MEAN ± SD	CV (%)	Min	Max	MEAN ± SD	CV (%)	
PW	<0.0001 ***	<0.0001 ***	<0.0001 ***	651.60	5281.00	2738 ± 1578	59.58	540.80	3957.00	1881 ± 1121	60.10	31.30
PH	0.001 **	<0.0001 ***	0.0003 ***	36.50	131.80	98.89± 23.7	23.97	28.00	133.50	88.21 ± 25.6	29.03	10.80
SD	<0.0001 ***	<0.0001 ***	<0.0001 ***	10.40	40.80	27.5 ± 6,92	25.20	8.28	36.20	23.4 ± 6.67	28.50	14.91
N°L	<0.0001 ***	0.72	<0.0001 ***	7.67	31.00	22.6 ± 5.93	26.30	6.00	35.00	22.4 ± 6.94	31.00	0.88
DM	<0.0001 ***	<0.0001 ***	0.72	8.25	32.20	15.6 ± 6.53	42.00	3.40	26.50	16.3 ± 6.44	39.50	−4.49
SPAD	<0.0001 ***	0.74	0.05	47.80	80.60	61.3 ± 9.22	15.00	48.20	77.60	60.8 ± 8.46	13.90	0.82
SSC	<0.0001 ***	0.016 *	<0.0001 ***	3.50	7.33	5.76 ± 1.11	19.30	3.00	8.17	6.1 ± 1.46	23.90	−5.90
Chla	<0.0001 ***	<0.0001 ***	<0.0001 ***	101.50	955.50	453.3 ± 258.6	57.06	48.91	515.30	243.4 ± 142.5	58.53	46.30
Chlb	<0.0001 ***	<0.0001 ***	<0.0001 ***	15.60	310.00	137 ± 94.1	68.70	4.44	213.00	78.6 ± 67.7	86.20	42.63
Tchl	<0.0001 ***	<0.0001 ***	<0.0001 ***	129.00	1261.00	590 ± 347	58.80	53.40	728.00	322 ± 206	64.00	45.42
Caro	<0.0001 ***	0.0122 *	<0.0001 ***	12.80	148.00	66.7± 40.4	60.60	6.49	439.00	56± 93.7	167.00	16.04
Tchl/caro	<0.0001 ***	0.31	0.0036 **	1.50	11.10	7.34 ± 2.44	33.30	0.13	17.10	7.78 ± 3.58	46.00	−5.99
TPC	<0.0001 ***	<0.0001 ***	<0.0001 ***	177.00	710.00	403 ± 152	37.80	259.00	1594.00	656 ± 310	47.30	−62.78
GSH	<0.0001 ***	0.88	<0.0001 ***	0.02	0.75	0.207 ± 0.25	121.00	0.02	1.53	0.205 ± 0.327	160.00	0.97
GSSG	<0.0001 ***	0.0403 *	<0.0001 ***	0.32	1.56	0.691 ± 0.323	46.70	0.33	1.26	0.72 ± 0.271	37.70	−4.20
AsA	<0.0001 ***	<0.0001 ***	<0.0001 ***	0.02	1.33	0.388 ± 0.423	109.00	0.00	1.59	0.342 ± 0.5	146.00	11.86
Tot AsA	<0.0001 ***	<0.0001 ***	<0.0001 ***	0.03	1.70	0.509 ± 0.495	97.30	0.02	1.79	0.467 ± 0.585	125.00	8.25

PW: plant weight; PH: plant height; SD: stem diameter; N°L: number of leaves; % DM: dry matter; SSC: Soluble Solids Content (° BRIX); Chla: chlorophyll a; Chlb: chlorophyll b; TChl: total chlorophyll (a + b); Caro: carotenoids; TPC/ Total phenolic compounds; GSH: Reduced glutathione; GSSH: oxidized glutathione; AsA: ascorbic acid; Tot AsA: DHA + AsA. The interaction of the factors *, **, and *** indicate that the Tukey test is significant at $p < 0.05$, $p < 0.01$, and $p < 0.001$ respectively.

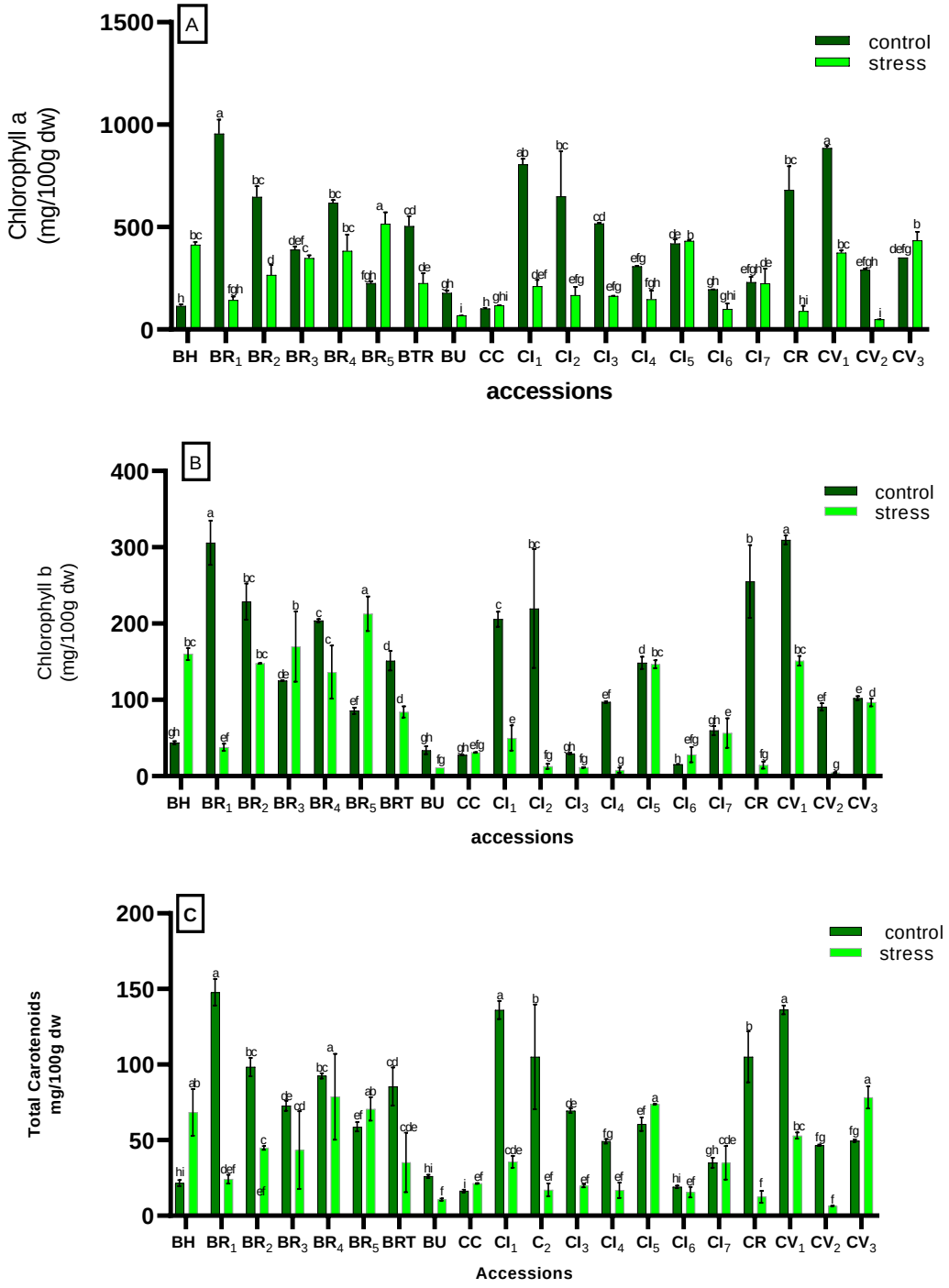

Figure 1. Variation on photosynthetic pigment content in the 20 *Brassica oleracea* accessions studied in relation to water stress treatment (**A**) chlorophyll a (Chla); (**B**) chlorophyll b (Chlb); **(C)** total carotenoids (Caro); and Error bars indicate SE (n = 3). For each accession, different letters indicate significant differences between accessions subjected to the same treatment according to Tukey's test ($\alpha = 0.05$).

3.2.2. Total Phenolic Compound (TPC)

In our study, the TPC were determined. The results are shown in Figure 2A. TPC were higher in drought conditions for all *Brassica* accessions studied, with significant differences among genotypes ($p < 0.001$) (Table 3). In the control conditions, the concentration ranged from 177 to 710 (mg GAE g^{-1} DW), and from 259 to 1594 (mg GAE g^{-1} DW) under water stress conditions, with %var= −62.78%. Interestingly, under water stress, CV1 (*Brassica*

oleracea var. *botrytis*) showed the highest TPC with 1594.1 ± 108.5 mg GAE g^{-1} DW, which was more than double that compared to controls, while the lowest value was observed in wild genotype BU (*Brassica rupestris*), corresponding to 259.4 ± 13.1 mg GAE g^{-1} DW. The accessions CR (*Brassica oleracea* var. *gongylodes*) and CI5 (*Brassica oleracea incrocio*) showed the highest increase in TPC amount under water stress (%var = −376% and −361.6%, respectively), followed by BR1 (*Brassica oleracea* var. *italica*) with a percentage of variation equal to −188.7%, which indicated a moderate tolerance to water stress.

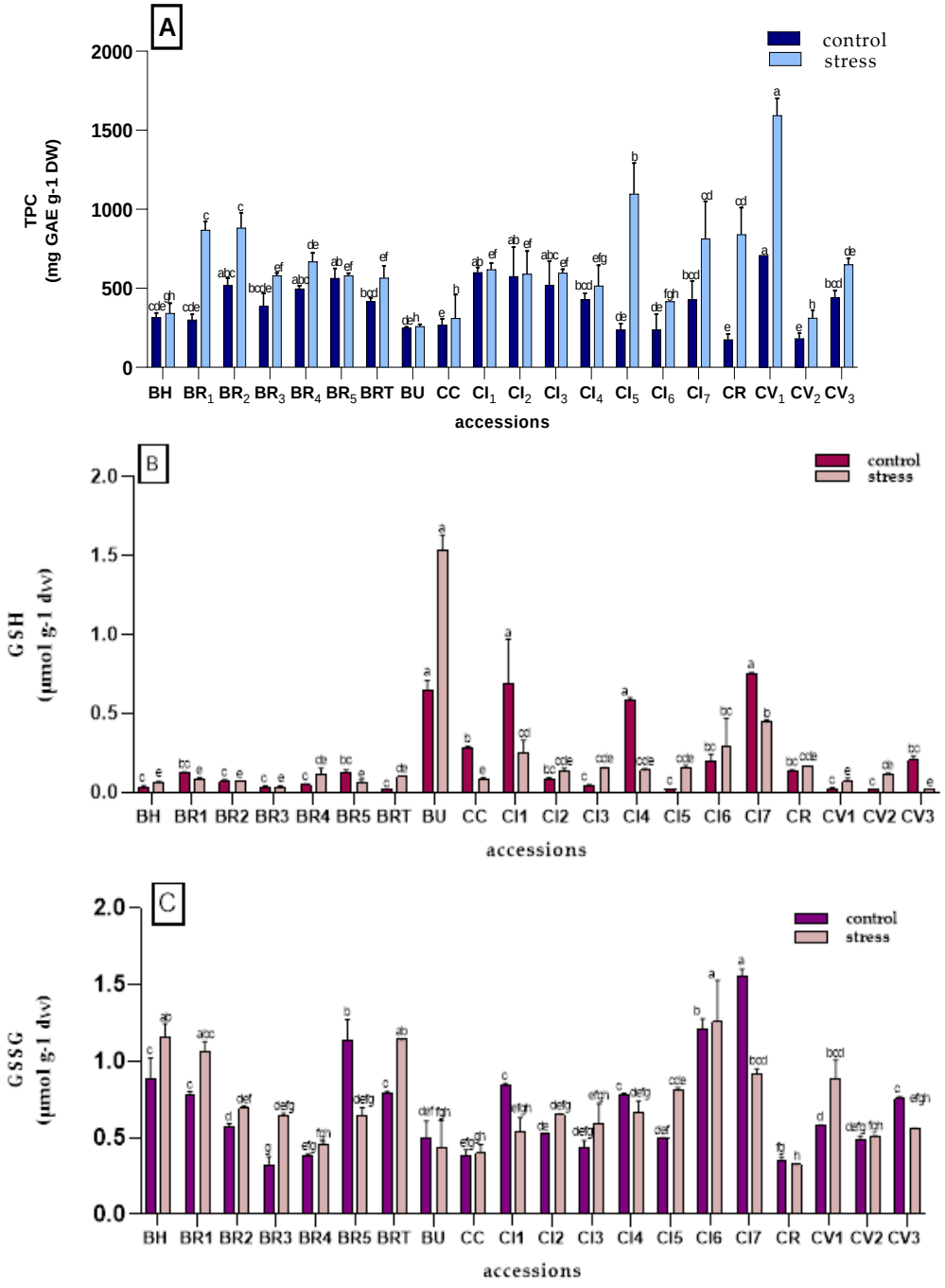

Figure 2. *Cont.*

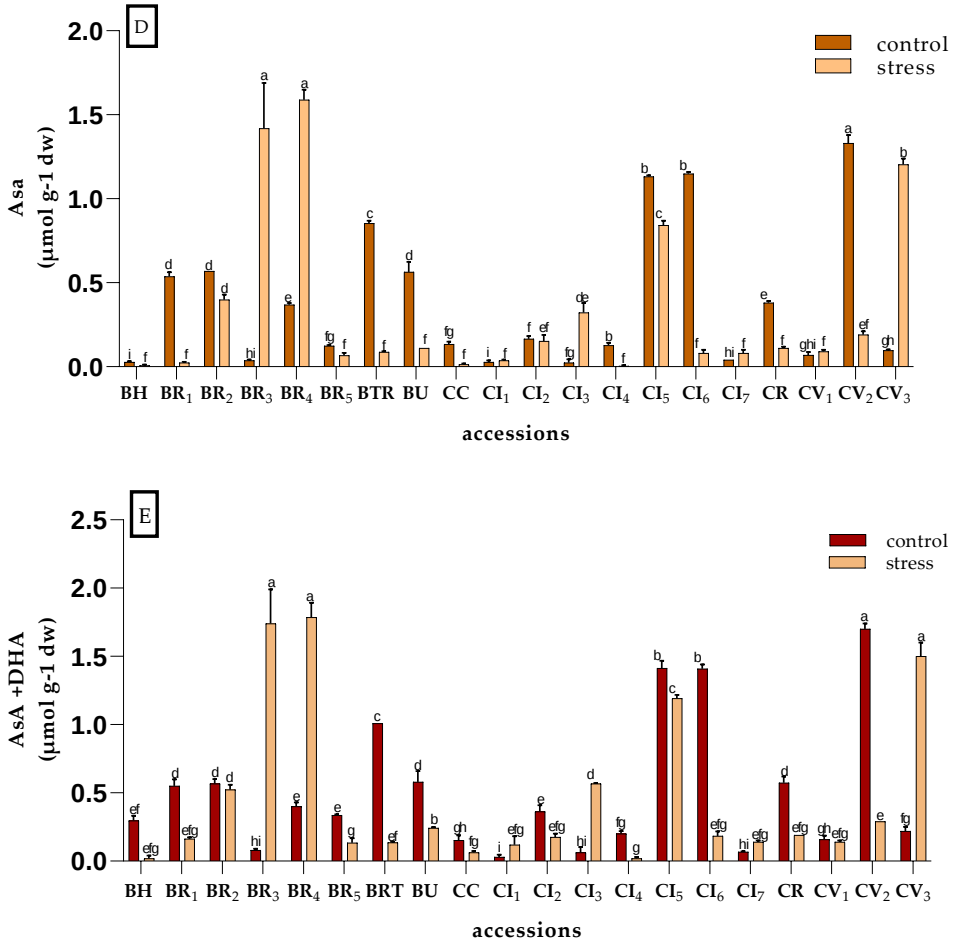

Figure 2. Influence of genotype and drought treatment on the antioxidant compounds in the 20 accessions studied. (**A**) Total phenolic compounds (TPC); (**B**) Reduced Glutathione (GSH); (**C**) Oxidized glutathione (GSSG); (**D**) Reduced Ascorbic acid (AsA); (**E**) Total ascorbic (AsA + DHA). Data are reported as mean ± S.E. Different letters indicate significant differences among accessions undergoing the same treatment according to Tukey test ($p < 0.05$).

3.2.3. Ascorbic Acid and Glutathione

The concentration of both AsA and DHA in leaves, as well as GSH and GSSG are shown in Figure 2B–E, and mean data are reported in Table 3. In the control plants, AsA content varied between 0.22 and 1.70 µmol g^{-1} DW, with minimum and maximum for CI3 and CV2, respectively. As shown in Figure 2C, AsA content decreased in half for the accessions studied (10 out 20) in relation to water stress. Interestingly, CI3, BR3, BR4, and CV3 strongly increased their AsA content from about three to more than thirty-fold compared to controls (Figure 2D).

In the drought-stress trial, the total vitamin C content ranged from 0.02 µmol g^{-1} DW to 1.74 µmol g^{-1} DW, for the accessions BH and BR3, respectively. The highest variation was observed in BR3, with an increase from 0.08 to 1.74 µmol g^{-1} DW.

From the comparison of the results in Figure 2B, it appears that the GSH content in Brassica leaves is different among genotypes. In the control trial, GSH content ranged between 0.02 and 0.75 µmol g^{-1} DW for CI5 and CI7, respectively. Almost half of the accessions studied (9 out of 20) showed an increase in GSH after drought stress, from 1.5 to about 7-fold higher. The wild species BU distinguished with the highest GSH content in stress conditions, which increased from 0.65 to 1.35 µmol g^{-1} DW. On the other hand, CI1, CI4, and CI7 showed a significant decrease in GSH in the water stress trial with %var= 64.29%, 76.67%, and 41.34%, respectively.

In the control conditions, the GSSG ranged from 0.32 µmol g^{-1} DW for the accession BR3 and 1.6 µmol g^{-1} DW for the accession CI7. While in the drought stress trial, the lowest amount was recorded in the accession CR (0.3 µmol g^{-1} DW), and the highest was for CI6 (1.33 µmol g^{-1} DW).

3.3. Principal Component Analysis and Heat Maps of Correlations

The PCA analysis of the biochemical composition of *Brassica oleracea*, shown in Figure 3, was performed to establish a preliminary understanding of the general differences within and between subspecies in relation to water stress. The first two components extracted by the PCA accounted together for 54% of the variability. PC1 explained 31.43% of the variability and divided GSH, GSSG, and caro/chl ratio with negative values and from the other phytochemical parameters with positive values. PC2 explained the 22.57% of the variability. In stressed conditions, BR3, BR4, and CV3 are associated with AsA, CV1 to polyphenols, BR2 and CV5 to carotenoids, and most of the other genotypes to both glutathione forms. This indicated the significant effect of the drought stress treatment on *Brassica* crop metabolism. The accessions CC and BH appeared in the same quadrant in control and stressed conditions, which suggested that these genotypes showed the same performance in both normal and drought stressed conditions and may be considered water stress tolerant. The biplot (Figure 3) clearly shows that cultivars respond differently to drought stress.

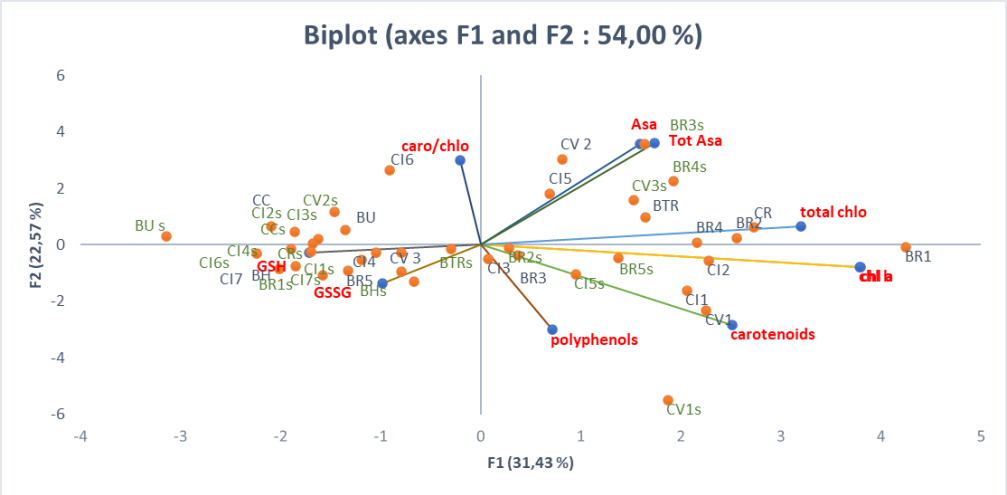

Figure 3. Two-dimensional principal component analysis (2D-PCA) illustrating the biochemical compounds used as biomarkers of water stress. The color green indicates the stressed crops.

Pearson's correlation coefficients (r) were performed based on the mean value to measure the association between the morphological and biochemical traits under control and stress conditions separately to understand better the impact of the drought stress on the traits studied. The correlations among the variables examined in this study were calculated and shown as a heat map in Figure 4a,b. In control conditions, a strong correlation was evidenced between carotenoids and chlorophylls (r = 0.9 $p < 0.05$), while in drought conditions (r = −0.058). In both control and drought conditions, polyphenols and chla, chlb were positively correlated. In drought conditions, polyphenols and carotenoids were positively and significantly correlated (r = 0.792, $p < 0.05$). Moreover, the total ascorbic acid correlated positively with Chla and Chlb (r = 0.483, $p = 0.05$ and r = 0.501. $p = 0.05$, respectively), while the correlation was not significant under control conditions. A negative correlation was observed between the TPC and AsA in control (r= −0.587, $p < 0.05$).

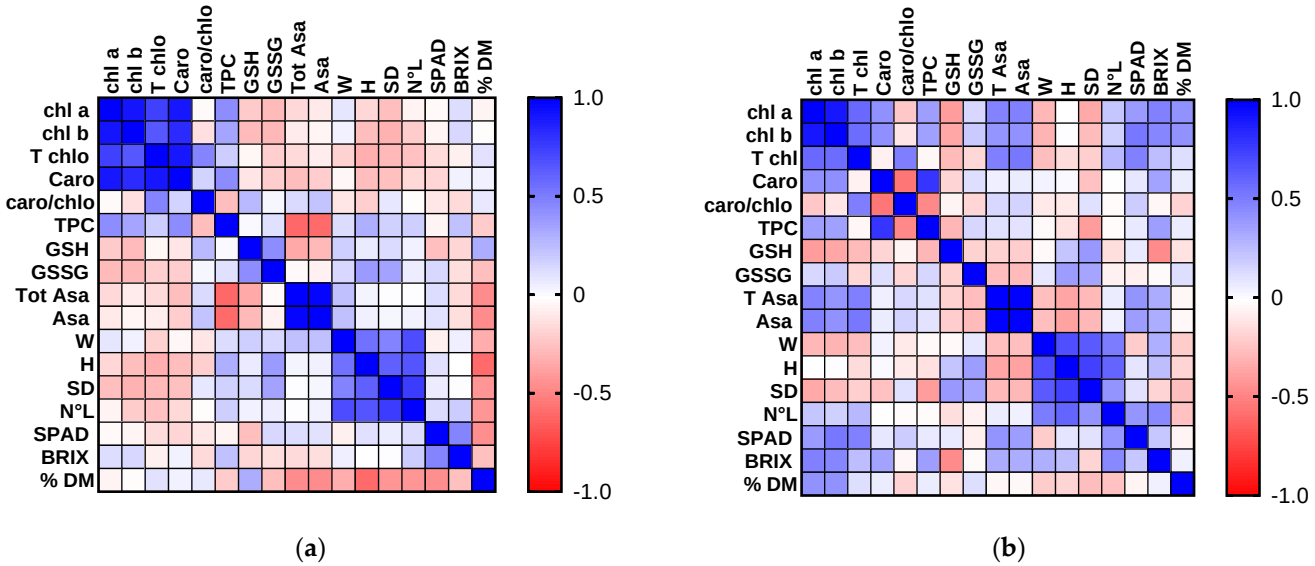

Figure 4. Heat maps of Pearson's correlation between morphological and biochemical traits in (**a**) control conditions; (**b**) drought conditions; Chla: chlorophyll a; Chl b: chlorophyll b; TChl: total chlorophyll (a + b); Caro: carotenoids; TPC: Total phenolic compounds; GSH: Reduced glutathione; GSSH: oxidized glutathione; AsA: ascorbic acid; Tot AsA: DHA + AsA; W: plant weight; H: plant height; SD: stem diameter; N°L: number of leaves; % DM: dry matter; SSC: Soluble Solids Content (° BRIX).

3.4. Screening of Brassica Genotypes using Stress Tolerance Index (STI)

Using iPastic, an online toolbox created by Pour et al. [54], drought stress indices were calculated based on dry matter. According to the STI bar graph (Figure 5), the accessions CR(3.14), CC(2.05), BH(1.86), CI1(1.71), BR5(1.65), CV3(1.50), CI2(1.25), and BU(1.19) are classified as drought-tolerant genotypes, while BR1(1.1), CI5(0.85), BR3(0.82), CV1(0.81) are medium tolerant, the rest are classified susceptible. Therefore, the average sum of rank (ASR) was calculated using all the drought stress indices (Supplementary Table S2), and genotypes having low ASR values were identified as drought tolerant and high ASR as sensitive. Based on ASR, the accessions CR (*B. oleracea* var. *gongylodes*), CC (*B. oleracea* var. *capitata*), and BH (*B. oleracea* var. *acephala*) have low ASR values and are categorized as the best drought-tolerant, and CI5 (*B. oleracea* var. *botrytis* x *italica*), BU (*Brassica rupesteris*), and CV1 (*B. oleracea* var. *botrytis*) as sensitive genotypes with high ASR value.

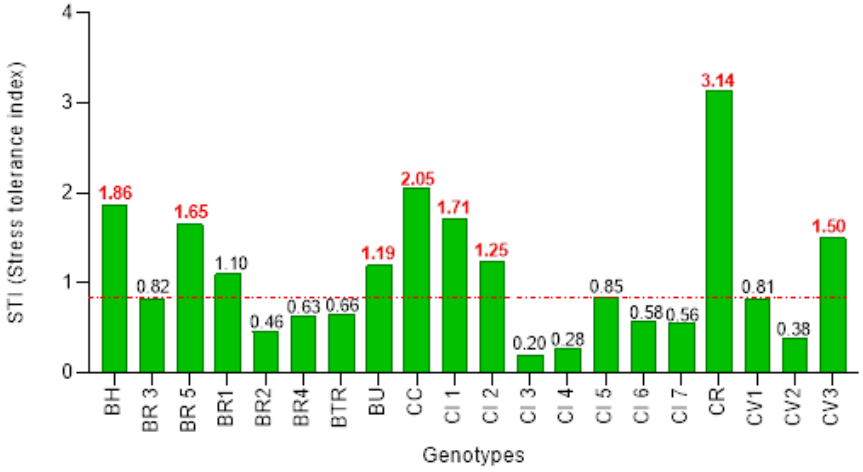

Figure 5. Stress tolerance index (STI) of 20 *Brassica oleracea* genotypes. The horizontal red line indicated the median value (0.83). The red font values above the vertical bar are the highest STI which indicated the most drought tolerant genotypes.

4. Discussion

One of the greatest challenges in agronomy is to develop stress-tolerant varieties while maintaining high productivity. The use of reliable drought tolerance markers in the selection of tolerant genotypes is an important technique. Plant response to drought can be assessed by morphological, physiological, and biochemical traits. Screening these traits in genetically several materials could be a useful method to find drought-tolerant genotypes. Stress treatments applied during the vegetative growth phase under greenhouse conditions are low cost and easy to carry out, and the level of stress imposed can be regulated, making them ideal for selecting effective stress markers in plants.

The results of this study show that drought stress drastically affects biomass, physiological performance, and secondary metabolite concentrations. Our findings are consistent with those of Barickman et al. (2020) [55] on kale, who conclude that production is highly influenced by irrigation time and water application, as both affected growth and nutrient quality. Based on the results of the current study, the water stress has a negative impact on morphological traits weight (31,30%), height (10.8%), stem diameter (14.91%), and total chlorophyll (45.42%), which are in accordance with the results published by Channaoui et al. (2017) [56] and El sabagh et al. (2019) [57] who reported that the irrigation intervals have a significant impact on canola (*Brassica napus*) growth, yield, and quality traits.

In addition to the growth responses of *Brassica oleracea* crops to water stress, biochemical variations were examined to gain a better understanding of the impact of drought stress. The leaves of *Brassica oleracea* were considered an excellent source of carotenoids and chlorophylls [58]. Chlorophyll concentration is one of the most used indicators of drought stress. It is well known that drought stress causes significant damage to photosynthetic pigments by accelerating chlorophyll degradation [59]. Both chlorophyll a and b are susceptible to drought [60]. In our study, the effect of water stress on chlorophyll *a* and *b* was highly significant ($p < 0.0001$ ***) (Table 3). Our results are in accordance with those of Farnheim and Kopsell (2009) [61], who find that there are significant effects of genotype on Chla and Chlb content of broccoli. In drought conditions, the Chla concentration (243.4 mg/100 g) was found to be higher than Chl b (78.6 mg/100g DW). These findings agree with Ahmad et al. (2018) [62], who reported that, under water stress, chlorophyll a was three times higher than chlorophyll b. When comparing genotypes, higher chlorophyll content was observed in two genotypes (BH and BR5) and maybe is associated with greater stress resistance. Lefsrud et al. (2007) [63] reported that the highest pigment amounts occurred with leaves between 1 and 3 weeks of age, which can explain the variation in the present study, as the analysis was carried out at the maturity stage.

According to the literature, chlorophyll reduction is a sign of oxidative stress and can be triggered by photooxidation of pigments and chlorophyll degradation. Active oxygen species-induced damage to chloroplasts causes this decline in chlorophyll during drought stress [64]. Such a decline in photosynthesis causes plants to absorb more light energy than can be consumed by photosynthetic carbon fixation. This excess energy has the potential to trigger an increase in reactive oxygen species production (ROS) [65]. However, in the study by Issarakraisila et al. (2007) [66], the authors observed that water stress increased leaf dry matter nitrogen concentration by more than 60% and double chlorophyll concentration. The leaf color of plants that suffered from water stress was dark green compared to the leaf color of well-watered plants, which was consistent with the increase in chlorophyll. Kalaji et al. (2016) [67] concluded that the changes in physical and optical properties of leaves caused by external factors might lead to deviations from the expected linear relationship between chlorophyll meter records and absolute chlorophyll content. For this reason, we did not find a high positive correlation between the SPAD value and chlorophyll content ($r = 0.49$).

Carotenoids and chlorophylls are functionally related and share common components in their biosynthesis. In our study, their changes were quite similar and positively correlated, with a general decrease after drought stress in most of the genotypes. This result is in accordance with what is generally reported, which is a decrease in photosynthetic pigments

following drought stress [68]. However, the amount of carotenoid in the leaves of leafy vegetables is affected by several factors, including species, variety, cultivar, maturity, and environmental growth factors such as light, temperature, and soil qualities [69]. For this reason, a certain number of genotypes may have shown an opposite behavior, i.e., higher pigment concentrations in stressed plants.

Metabolic profiling could be effective for identifying the molecular features linked to drought resistance, which would be useful for plant breeding [70]. Several studies agree that crops grown at intermediate temperatures, high light intensity, longer days, and dry conditions (or low rainfalls) have higher phytochemical concentrations [71].

Brassicaceae polyphenols composition has been extensively investigated, and several studies evaluated the concentrations of various phytochemicals in organic and conventional fruits and vegetables [72,73]. Our results are consistent with those of the study by Heimler et al. (2006) [74] in which the total phenolic content ranged from 4.30 to 13.80 (mg gallic acid g^{-1} DW). Sousa et al. (2005) [75] compared the quantities of phenolic compounds in leaves of *Brassica oleracea* var. *tronchuda* cabbage grown under organic and conventional conditions and concluded that the organically produced leaves had higher levels, most likely since mineral fertilizers and pesticides affect the production of phenolic compounds. Kaulmann et al. (2014) [76] found that the total phenolic compounds (TPC) differ significantly among different *Brassica* cultivars. In fact, there was large variability between the TPC of different Brassica cultivars, with the lowest concentrations for white Brassica (5.4–61.5 mg GAE /100 g FW) and the highest concentrations for red and green Brassica varieties (13–139 mg GAE /100 g FW). Total phenolic contents might vary by up to 200 percent between different broccoli cultivars [76]. Regarding the effect of drought on polyphenols, contrasting results are found in the literature [77,78]. In our study, polyphenols were generally increased following drought stress, and in some genotypes, the increase corresponded to two- three-folds compared to controls.

One of the first metabolic responses to biotic and abiotic stressors is the production of reactive oxygen species (ROS) [79]. Vitamin C content in *Brassica* plants varies widely between and within species and among species. Additionally, the variability of each chemical between or among subspecies is important because it can be used to estimate the maximum concentration of each compound that can be achieved by genetic manipulation. From a quantitative point of view, according to Singh et al. (2007) [80], kale has the highest vitamin C concentration (82.14 mg /100 g). Moreover, the ascorbic acid content in fresh products was 94.18 mg /100g and 107 mg /100 g in the investigation of [81]. Brussels sprouts (76–192 mg/100 g FW) and kale (92–186 mg/100 g FW) appear to have the highest vitamin C concentration among *Brassica* genotypes, followed by broccoli (34–146 mg/100 g FW) and cauliflower (17–81 mg/100 g FW) and white cabbage (19–47 mg/100 g FW). Vitamin C levels varied over 4-fold in broccoli and cauliflower, 2.5-fold in white cabbage, and twice in kale. White cabbage is the poorest source of vitamin C among *Brassica* vegetables in general [82]. In the present study, the genotypes belonging to *Brassica oleracea* L. var. *botritys* and var. *italica* showed different behaviors since three genotypes (CI5, CI6, and CV2) distinguished for the highest vitamin C content for the controls, but they significantly decreased under drought stress. Three genotypes (BR3, BR4, and CV3) greatly increased their vitamin C content following drought stress. This different behavior is in accordance with previous literature data where contrasting responses were observed in other plant systems [83,84]. Thus, many factors influence vitamin C content in *Brassica* crops, including cultivar, harvest date, growing conditions, soil quality, and postharvest storage conditions [85]. Considering the results of [86], AsA appears to be severely affected by rapid oxidation to DHA under unsuitable growing conditions.

According to Raseetha et al. (2013) [87], broccoli was found to be a good source of ascorbic acid and glutathione. Both natural plant antioxidants provide various health benefits when consumed. The content of total ascorbic acid and total glutathione in broccoli florets was 5.18 and 0.70 µmol/g DW, respectively. Regarding glutathione content, we observed generally low amounts of GSH following drought stress. Interestingly, the geno-

type BU distinguished among the genotypes since it strongly increased the GSH content following drought; moreover, this species had greater morphological plasticity in response to water stress. This finding confirms the importance of considering wild *Brassica* genotypes for their high content in phytochemicals and the potential tolerance to abiotic stress [88].

Stress-induced growth inhibition is a common response of both stress-sensitive and stress-tolerant species as resources are diverted from biomass accumulation to activation of stress defense mechanisms. A truly drought-tolerant plant can be defined when no changes in production indexes occur under stress [89]. Since it distinguishes between genotypes that are drought-tolerant and those that are susceptible to it, STI is the most common drought stress indicator for determining drought tolerance genotypes. The most drought-tolerant *Brassica oleracea* genotypes were identified in this research using STI calculations based on the dry matter under drought stress conditions. Among the 20 genotypes, our results revealed a wide range of STI values, from 0.20 to 3.14. Belay et al. (2021) [90] have reported that STI is the most effective way to identify genotypes for drought tolerance and discovered STI values (0.08–0.75) that were comparable to our results. The genetic heterogeneity of the study materials employed, the stage and length of drought stress application and the variance in STI value could all be contributing factors.

5. Conclusions

In the present work, the response to water stress of 20 *Brassica oleracea* genotypes was analyzed through the measurement of both bio-morphometric and biochemical traits. Our results showed that the genotypes were significantly affected by water stress, although a high genetic variability among genotypes was evident. Generally, drought had the greatest impact on some morphophysiological parameters, such as plant weight, number of leaves, fresh biomass, and dry weight. On the other hand, water stress also affected the biochemical traits, particularly pigment content and antioxidant compounds. Albeit the genotypes showed different sensitivity to drought, some genotypes, i.e., CR, CC, BH, CI, and BTR, distinguished for their best performance under stress. These genotypes can thus be considered tolerant to drought.

Although there are still significant gaps in our understanding of the molecular and physiological pathways underlying plant responses to drought, these preliminary results shed light on the identification of *Brassica oleracea* genotypes that may better tolerate water scarcity conditions and may represent an interesting genetic material to be taken into consideration by breeders for the selection of genotypes resistant to water scarcity.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12092016/s1, Supplementary Table S1 (doc): List of Brassica oleracea genotypes used in this study. Supplementary Figure S1 (doc): Maximum, minimum and average temperature of the greenhouse. Supplementary Table S2 (Xlsx): Stress tolerance index.

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