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Effect of Three Water Regimes on the Physiological and Anatomical Structure of Stem and Leaves of Different Citrus Rootstocks with Distinct Degrees of Tolerance to Drought Stress

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Abstract: Citrus is grown globally throughout the subtropics and semi-arid to humid tropics. Abiotic stresses such as soil water deficit negatively affect plant growth, physiology, biochemistry, and anatomy. Herein, we investigated the effect(s) of three water regimes (control, moderate drought, and severe drought) on the physiological and anatomical structure of 10 different citrus rootstocks with different degrees of tolerance to drought stress. Brazilian sour orange and Gadha dahi performed well by avoiding desiccation and maintaining plant growth, plant water status, and biochemical characters, while Rangpur Poona nucellar (*C. limonia*) and Sunki × bentake were the most sensitive rootstocks at all stress conditions. At severe water stress, the highest root length (24.33 ± 0.58), shoot length (17.00 ± 1.00), root moisture content (57.67 ± 1.53), shoot moisture content (64.59 ± 1.71), and plant water potential (−1.57 ± 0.03) was observed in tolerant genotype, Brazilian sour orange. Likewise, chlorophyll *a* (2.70 ± 0.06), chlorophyll *b* (0.87 ± 0.06) and carotenoids (0.69 ± 0.08) were higher in the same genotype. The lowest H_2O_2 content (77.00 ± 1.00) and highest proline content (0.51 ± 0.06) were also recorded by Brazilian sour orange. The tolerance mechanism of tolerant genotypes was elucidated by modification in anatomical structures. Stem anatomy at severe drought, 27.5% increase in epidermal cell thickness, 25.4% in vascular bundle length, 30.5% in xylem thickness, 27.7% in the phloem cell area, 8% in the pith cell area, and 43.4% in cortical thickness were also observed in tolerant genotypes. Likewise, leaf anatomy showed an increase of 27.9% in epidermal cell thickness, 11.4% in vascular bundle length, 21% in xylem thickness, and 15% in phloem cell area in tolerant genotypes compared with sensitive ones. These modifications in tolerant genotypes enabled them to maintain steady nutrient transport while reducing the risk of embolisms, increasing water-flow resistance, and constant transport of nutrients across.

Keywords: drought stress; citrus; oxidative stress; proline; photosynthesis; water potential; vascular bundle modifications

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1. Introduction

Citrus fruit crops in the family Rutaceae have the largest fruit industry globally [1,2]. Rootstock choice and selection for the citrus scion variety are the most valuable decisions for growers for the implication of better yield and quality with other valuable characters. Citrus rootstock controls the physiological, biochemical, morphological, and genetic characteristics of scion cultivars grafted on selected rootstocks through the rootstock scion interaction pathway [3]. Fruit juice quality and tree productivity of scion cultivars are also affected by rootstock characters. Rootstock has a significant impact on nutrition, horticultural and pathological traits of citrus cultivars, growth, vigor, stress resistance, and fruit quality of the scion [4–7]. Rootstock controls translocation of water and nutrients from the soil and distributes among different up ground plant organs, which were also disturbed negatively by the impact of stresses [8,9].

Citrus plants affected by several biotic (fungal, viral, and bacterial diseases) and abiotic (water deficit, heat, flooding, and salinity) stresses exhibit wide-ranging losses in citrus production [10,11]. Cultivars of genus *Citrus* are grown in a climate of wide range due to evergreens and perennial tree crops [12]. The capacity to tolerate the unfavorable climatic conditions of the citrus crop is high but the estimated yield loss due to environmental abiotic stresses in citrus is up to 82% [13]. Climate change conditioning causes a global rise in temperature and limited the availability of freshwater for the crop. Meanwhile, crop consumption is increasing rapidly resulting in reduced levels of groundwater ultimately limiting productivity [14]. Global warming due to climate change caused frequent extreme water deficit conditions, an important component for global agriculture [15,16]. Abiotic stresses are interlinked with each other in which soil water deficit/drought is a serious environmental factor, which frequently limits the growth and productivity of important crop species [17]. Supply in water restriction can severely limit plant cell division, plant growth, fruit development, and fruit production [18,19]. Soil water deficit for long-term events causes permanent negative changes in the plant which can be in response to previous stress. Water deficit conditions can be developed at different phenological stages which can change physiological and molecular processes of the plant [20].

Plant responses to water stress are mediated by changes in root growth pattern and stomatal closure at moderate stress resulting in reduced CO_2 intake, impairing photosynthesis, and loss in production [21,22]. Extreme water stress conditions alter the physiological and morphological processes of the plant. The molecular and biochemical machinery is also affected under stress conditions. Reactive oxygen species (ROS), i.e., hydrogen peroxide, superoxide, singlet oxygen, and hydroxyl radical are excessively produced and accumulate in plant cells at water deficit conditions. Photoinhibition or photooxidation caused by ROS accumulation leads to uncontrolled photosynthetic components oxidation [23,24]. Water deficit/drought damages photosynthetic apparatus and photosynthetic pigments (chlorophyll *a*, chlorophyll *b*, and carotenoids) which also reduced the photosynthetic rate [25,26]. Photosynthetic pigments are linked to stress tolerance in plants [27,28]. Resistant cultivars had a higher amount of chlorophyll contents and carotenoids when exposed to water deficit conditions [29]. Water deficit conditions decrease contents of total chlorophyll *a* and *b* contents [30]. Water deficit conditions damage thylakoid lamella and reduce active oxygen species' chlorophyll contents [31]. The chlorophyll content is linked inversely with the severity and duration of water deficit conditions [32]. After oxidative stress, accumulation of proline acts as an adaptive stress response. The intracellular levels of proline can increase by >100-fold during stress [33]. Proline accumulation acts as a compatible osmolyte to buffer cytosolic pH and to balance cell redox status. Proline can also function as a molecular chaperone stabilizing the structure of proteins and as a ROS scavenger [24,34].

Plant anatomical structure is also disturbed when the plant is subjected to water deficit which affects the regulation of water through the vascular bundles. The effect of drought stress firstly occurs on the cell structure [35,36]. Increased hydraulic resistance and decreased growth are directly associated with xylem structure [37]. Anatomical changes in citrus rootstocks under abiotic stress influence their ability to survive. In drought

conditions, the xylem vessel becomes emboli and dysfunctional. Therefore, plants with narrow and higher number of vessels are considered to be more drought tolerant [38]. The plant cells that face an environment with a shortage of water have generally shown an increase in vessel tissue, thick cell wall, reduction in cell size, and most severe condition cell wall and the cell membrane becomes ruptured [39,40]. The anatomical changes may occur to protect the plants under stress conditions. Multiple changes occur in response to water stress such as alteration of xylem phloem ratio, wall structure, lumen size, and lumen area that resist environmental stress on the plant [41]. The sensitivity of rootstock against drought stress is directly related to vessel dimension [42,43]. The vessel density of root and stem decreases with tree height as vessel diameter increases. The rootstock growth ability is dramatically affected by several xylem traits, xylem phloem ratio, vessel size, and vessel density [43,44]. Thus, traits play an important role in maintaining hydraulic conductance of root and stem and leaves [37,45]. Vessel number and size is the key factor to maintaining hydraulic conductance [46].

Strategies for drought tolerance are highly relevant in the case of rootstock selection and multiplication for ensuring continuous productivity. Rootstocks with a higher root growth ratio, better water use efficiency, higher root hydraulic conductivity, and lower osmatic conductance can withstand drought conditions while maintaining higher growth levels and mass accumulation [47,48]. After a severe drought, the recovery of tolerant rootstocks is much better than other rootstocks. Therefore, screening of drought-tolerant rootstocks is of utmost importance. For drought stress prevention, the plant increases water uptake efficiency either by increasing root density or deepening roots [26].

Climate changes shift the weather conditions by a high degree of unpredictability; water shortage in the soil and plant by a continuous increase in daily average temperature every year are inevitable, which intimidate overall globe agriculture industry stability by the negative effect on plant health and production consumer demand. Survival of the citrus industry against the water deficit needs to evaluate a tolerant/resistant citrus rootstock against the climate change scenario. The objective of the current study was to evaluate 10 citrus rootstocks against drought, based on visual changes, water potential, morphological and biochemical characters. After initial screening, the two most sensitive and tolerant genotypes were used to study the anatomical differences in leaf and stem to elucidate the drought tolerance mechanism in tolerant genotypes.

2. Materials and Methods

2.1. Plant Materials, Experimental Site, and Growth Conditions

Six months old citrus rootstocks potted plants were taken as experimental material. Ten genetically diverse citrus rootstocks were examined (details are given in Table 1). A potted plant experiment was executed at the Institute of Horticultural Science, University of Agriculture Faisalabad, Pakistan. Seeds of selected rootstock were taken from citrus rootstocks progeny block, HIS, UAF, Pakistan.

Table 1. Characteristics of citrus rootstocks used in this study.

Rootstock	Botanical Name	Citrus Category	Leaf Shape	Parentage/Origin
Gabbuchini	*Citrus aurantium* L.	Sour orange hybrid	Unifoliate	*C. aurantium* 'Bittersweet' × *C. sinensis*
Gada dahi	*Citrus maxima* Burm. Merrill/ *Citrus grandis* L. Osbeck/ *Citrus decumana* L.	Pummelo hybrid	Unifoliate	Subcontinent (Indo-Pak), seed selection
Sour orange	*Citrus aurantium* L.	Sour orange	Unifoliate	Subcontinent (Indo-Pak)
Keen sour orange	*Citrus aurantium* L.	Sour orange	Unifoliate	Selection/root sprout
Brazilian sour orange	*Citrus aurantium* L.	Sour orange	Unifoliate	Open-pollinated seed selection
Rough lemon	*Citrus jambhiri*	Lemon	Unifoliate	Open-pollinated seed selection
Sunki × bentake,	*Citrus* spp.	Unknown	Trifoliate	*Citrus sunki* × bentake hybrid

Table 1. *Cont.*

Rootstock	Botanical Name	Citrus Category	Leaf Shape	Parentage/Origin
X639	*Citroncirus* spp.	Mandarin × Poncirus	Trifoliate	Cleopatra mandarin × *Poncirus trifoliata* hybrid
Kirrumakki nucellar	*Citrus limonia* Osbeck	Lime	Unifoliate	Unknow/Subcontinent (Indo-Pak)
Rangpur Poona nucellar	*Citrus limonia* Osbeck	Lime	Unifoliate	Unknow/Subcontinent (Indo-Pak)

The seeds were sown in transparent grow bags (height 12″ and width 6″) and placed in a growth chamber (Model: BST/PGC-175; Bionics Scientific Technologies (p) ltd., Delhi, India) at 32 ± 2 °C, relative humidity oscillating between 80 and 95% and 12–14 h of light. Potted media contained sand, silt, and clay (1:1:1). These plants were placed in the chamber for six months before the treatment application and during growth, pots were regularly irrigated with tap water (75% field capacity) and fertilized weekly with nutrient solution (1.0 g L^{-1} $Ca(NO_3)_2$, 0.4 g L^{-1} KNO_3, 0.6 g L^{-1} $MgSO_4$ and 0.4 g L^{-1} $NH_4H_2PO_4$ (MAP)).

2.2. Water Regimes and Treatments

Citrus rootstocks were subjected to water deficit treatment applications after six months of growth in the controlled growth condition. Plants were exposed to three different groups: controlled condition, moderate water deficit (moderate drought), and severe water deficit (severe drought). Each treatment group consists of 10 citrus rootstocks with three replications. Control plants (sufficient water and optimal temperature ~32 °C) were irrigated once every two days (75% field capacity). Water deficit treatments were moderate (50% field capacity) and severe (25% field capacity) deficit conditions. Water was applied with 2 days interval for control and treated plants. The controlled and water deficit exposed genotypes were kept at 32 ± 2 °C for day and night temperature in a controlled growth room. Thereby, three experimental groups were established as control, moderate water deficit, and severe water deficit conditions.

2.3. Morphological and Biomass Measurements

Leaves were visually observed for leaf necrosis or chlorosis on rootstock seedlings after 15 days of stress applications. Plant height (cm) and root length (cm) were measured with help of a scale after uprooting the plants at the end of the experiment (15 DAS). Leaf water potential was determined to collect the healthy leaf samples at dawn (Shafqat et al., 2021) by using a pressure chamber (Model 3000, Soil moisture Equipment, Santa Barbara, CA, USA). Root and shoot fresh biomass were weighed on electric balance (TS-200 Digital Electronic Scale), oven-dried (70 °C) for 48 h, and weighed again separately [48]. Shoot and root moisture content were calculated using Equation (1):

$$\text{Moisture content } (\%) = \frac{\text{Fresh weight} - \text{dry weight}}{\text{Fresh weight}} \times 100 \quad (1)$$

2.4. Biochemical and Stress-Associated Biomarker Measurements

2.4.1. Leaf Photosynthetic Pigments Contents

Leaf sample of 0.5 g was cut into small pieces and homogenized by adding 80% acetone (v/v). The extract was transferred to a 15 mL tube [48]. Test tubes were placed in the dark to avoid light for 24 h and filtered through filter paper. The absorbance was determined at 663 nm for chlorophyll a, 647 nm for chlorophyll b, and 470 nm for carotenoid. The Chl *a*, Chl *b*, and carotenoids contents were determined using Equations (2)–(4), respectively.

$$\text{Chl } a \text{ (mg/g fresh weight)} = \frac{(12.7\text{OD}663 - 2.69\ \text{OD}645) \times \text{V}}{1000 \times \text{W}} \quad (2)$$

$$\text{Chl } b \text{ (mg/g fresh weight)} = \frac{(22.9\text{OD}645 - 4.68\ \text{OD}663) \times \text{V}}{1000 \times \text{W}} \quad (3)$$

$$\text{Carotenoids (mg/g fresh weight)} = \frac{\text{O.D } 480 + 0.114\ (\text{O.D } 663) - 0.638\ (\text{O.D } 645)}{\text{Em} \times 100} \quad (4)$$

where V = volume of the sample, W = weight of fresh tissue, Em = 2500.

2.4.2. Determination of Proline

Proline contents were assessed by following the acid ninhydrin method [49]. Fresh 0.5 g leaf material was extracted using 10 mL of 3% sulfosalicylic acid (Panreac, Barcelona, Spain) for 30 min. Centrifugation at 2000 *g* for 20 min at 4 °C was done. The 2 mL of filtered aqueous extract was mixed with acid ninhydrin reagent (2 mL), and glacial acetic acid (2 mL), and heated at 100 °C for 1 h. The reaction mixture after cooling was segregated against toluene (4 mL) and the absorbance of the organic phase was recorded at 520 nm using a spectrophotometer (IRMECO UV/VIS Model U2020). The resulting values were related with a standard curve plotted using known amounts of proline (Sigma, St Louis, MO, USA).

2.4.3. Determination of Hydrogen Peroxide (H_2O_2)

The leaf tissues of 0.1 g were ground in 1 mL of trichloroacetic acid solution (0.1%) within an ice bath [50]. After preparation in Eppendorf, the samples were centrifuged at 9719× *g* for 15 min. The supernatant of 500 µL was transferred into a new tube having a mixture of 1 M KI (1000 µL) and 10 mM potassium phosphate buffer (500 µL). Absorbance was read at 390 nm in a UV-1900 spectrophotometer (Eppendorf BioSpectrometer® basic). H_2O_2 content was calculated as µmol g^{-1} DW by comparing the absorbance values against the standard curve of commercial H_2O_2.

2.5. Stem and Leaf Anatomical Evaluation

2.5.1. Plant Material and Experiment

Two highly tolerant (Brazilian sour orange and Gadha dahi) and highly sensitive (Rangpur Poona nucellar and Sunki × bentake) genotypes from the first screening study were selected to screen based on the leaf and stem anatomical study. These selected rootstocks were exposed to the same treatments for 15 days then leaf and stem samples were collected for anatomical studies.

2.5.2. Preservation, Sectioning, Staining, and Mounting

Stem and leaf samples (2 cm) were preserved in a formalin acetic alcohol (FAA) solution containing 5% formalin, 10% acetic acid, 50% ethanol, and 35% distilled water. Thereafter, the preserved material was subsequently transferred to an acetic alcohol solution (acetic acid 25% v/v, ethanol 75%) for the long-term preservation of samples. A free-hand sectioning technique was used to prepare permanent slides of stem and leaf transverse sections cut with the razor blade, and some fine sections were carefully picked up on wash glass for staining and dehydration through a series of washings with ethanol (30%, 50%, and then 70% for 15 min each). For staining, the lignified tissues (xylem vessels and sclerenchyma) were transferred to safranin (1.0 g dissolved in 100 mL, 70% alcohol) for 20 min, dehydrated in 90% alcohol for 5 min, and stained with fast green (1.0 g dissolved in 90% ethanol) for one minute. Finally, the tissues were washed three times with absolute alcohol and then transferred to xylene for cleaning the contrast. The sections were mounted in Canada balsam by putting a drop of resin on a slide and placing the sections on the slides and photographed with a digital camera attached to a compound microscope.

2.5.3. Anatomical Traits

The stem and leaf cross-sectional area (mm^2) was measured under a compound light microscope (Olympus MX63, Japan) by recording the maximum length and width of the root sections. Cells present in plants epidermis were measured with the help of ruler

or cm scales, and the length of the epidermal cell was multiplied by 41.5 to obtain a value in micrometers. Vascular bundle length was calculated by measuring xylem and phloem which were multiplied by 41.5 to obtain the value in µm. Xylem thickness was calculated by the width of the xylem region. Metaxylem area, phloem cell area, pith cell area, and the cortical area were calculated in μm^2 by measuring the length and width of each trait. Cortical thickness was obtained by measuring the total width of the cortical region in micrometers. By using Equation (5), the areas of the different cells and tissues were calculated (which was modified from the area of the circle).

$$\text{Area} = \frac{\text{Maximum length} \times \text{Maximum width}}{28} \times 22 \quad (5)$$

2.6. Statistical Analysis

Throughout this study, all experiments were laid out using a full factorial split-plot design arranged in randomized complete blocks using rootstocks as main plots and water regimes in the subplots. All experiments were carried out using at least three biological replicates for each treatment. The analysis of variance (ANOVA) was used to test the significant differences among different drought levels (p_{Drought}), rootstocks ($p_{\text{Rootstocks}}$), and their interaction ($p_{\text{Drought} \times \text{rootstocks}}$). Tukey's honestly significant difference (HSD) test was used for post-hoc analysis ($p_{\text{Drought} \times \text{rootstocks}} < 0.05$). Moreover, the data matrix of all dependent variables was used to perform the principal component analysis (PCA). Finally, means of all dependent variables were used for two-way hierarchical cluster analysis (HCA). Similarities and variations between treatments were presented as a heat map.

3. Results

3.1. Drought Negatively Affects the Roots and Shoots Length

Both root and shoot length were significantly affected by varying drought levels ($p_{\text{Drought}} < 0.0001$) and rootstocks ($p_{\text{Rootstock}} < 0.0001$) (Table 2). Briefly, the root length of different citrus rootstocks was significantly reduced when plants were stressed with different levels of drought. Although control Brazilian sour orange had the highest root length (27.67 ± 0.58 cm; $p_{\text{Drought} \times \text{Rootstock}} < 0.0001$), shortest root lengths were recorded by Sunki × bentake when stressed with moderate (10.33 ± 0.58 cm) or severe drought (9.33 ± 0.58 cm) and Rangpur Poona nucellar rootstock under severe drought conditions (10.33 ± 0.58 cm) (Table 2). On the other hand, while there were no significant differences in shoot length of all tested rootstock, except Gabbuchini, under normal growth conditions, shoot lengths were significantly reduced under various drought levels. Rangpur Poona nucellar rootstock had the shortest shoots (5.33 ± 0.58 cm), followed by Sunki × bentake (6.00 ± 1.00 cm) when stressed under severe drought ($p_{\text{Drought} \times \text{Rootstock}} < 0.0001$) (Table 2).

3.2. Drought Stress Disrupts the Water Relations of Citrus Rootstocks

As strong wilt phenotype was visually observed on citrus seedlings after 15 days post applications (DPA; Figure 1), the water relations (water potential, root moisture content, and shoot moisture content) were assessed (Table 2). For instance, water potential was reduced significantly by water-deficient ($p_{\text{Drought}} < 0.0001$) and rootstocks ($p_{\text{Rootstock}} = 0.0018$). Kirrumakki nucellar rootstock had the highest water potential (−0.11 ± 0.66 Mpa), whereas, Keen sour orange, Rough lemon, Brazilian sour orange, Sunki × bentake, X639, Kirrumakki nucellar, and Rangpur Poona nucellar rootstocks had the lowest water potential when severely stressed with water deficiency, without significant differences between them ($p_{\text{Drought} \times \text{Rootstock}} = 0.0250$; Table 2). Likewise, the moisture content of both root and shoot was significantly reduced by drought levels ($p_{\text{Drought}} < 0.0001$) and rootstocks ($p_{\text{Rootstock}} < 0.0001$) (Table 2). Normal irrigated Brazilian sour orange had the highest moisture content of root (75.67 ± 0.59%) and shoot (84.75 ± 0.65%), whereas Rangpur Poona nucellar rootstock had the lowest moisture content of root (36.33 ± 0.58%) and shoot (40.69 ± 0.65%) under severe drought conditions (Table 2).

Table 2. Effect of different water regimes on the root length (cm), shoot length (cm), and water relations of 10 differentcitrus rootstocks with distinct degrees of tolerance to drought stress.

	Rootstock	Root Length (cm)	Shoot Length (cm)	Water Potential (Mpa)	Moisture Content	
					Root (%)	Shoot (%)
Control	Gabbuchini	25.33 ± 0.58 ab	21.00 ± 1.00 b	−0.32 ± 0.02 a–e	66.00 ± 1.00 cde	73.92 ± 1.12
	Gada dahi	24.33 ± 0.58 bc	24.33 ± 0.58 a	−0.23 ± 0.02 a–d	69.00 ± 1.00 b	77.28 ± 1.12 b
	Sour orange	24.33 ± 0.58 bc	23.67 ± 0.58 a	−0.27 ± 0.06 a–e	64.67 ± 0.58 e	72.43 ± 0.65 e
	Keen sour orange	25.33 ± 0.58 ab	25.33 ± 0.58 a	−0.19 ± 0.02 abc	67.33 ± 0.58 bcd	75.41 ± 0.65 bcd
	Rough lemon	26.00 ± 1.00 ab	25.00 ± 1.00 a	−0.19 ± 0.03 abc	64.67 ± 0.58 e	72.43 ± 0.65 e
	Brazilian sour orange	27.67 ± 0.58 a	25.00 ± 1.00 a	−0.23 ± 0.02 a–d	75.67 ± 0.58 a	84.75 ± 0.65 a
	Sunki × bentake	12.33 ± 0.58 jkl	24.67 ± 0.58 a	−0.28 ± 0.05 a–e	65.33 ± 0.58 de	73.17 ± 0.65 de
	X639	24.33 ± 0.58 bc	24.33 ± 0.58 a	−0.26 ± 0.06 a–e	67.00 ± 1.00 b–e	75.04 ± 1.12 b–e
	Kirrumakki nucellar	24.00 ± 1.00 bc	24.00 ± 1.00 a	−0.23 ± 0.02 a–d	68.00 ± 1.00 bc	76.16 ± 1.12 bc
	Rangpur poona nucellar	24.67 ± 0.58 bc	24.67 ± 0.58 a	−0.23 ± 0.02 a–d	66.00 ± 1.00 cde	73.92 ± 1.12 cde
Moderate drought	Gabbuchini	15.67 ± 0.58 ghi	12.00 ± 1.00 ghi	−0.60 ± 0.05 a–f	55.67 ± 0.58 fg	62.35 ± 0.65 fg
	Gada dahi	22.67 ± 0.58 cd	18.33 ± 0.58 c	−0.15 ± 0.58 ab	64.67 ± 0.58 e	72.43 ± 0.65 e
	Sour orange	19.00 ± 1.00 ef	15.67 ± 0.58 de	−0.61 ± 0.06 a–f	54.00 ± 1.00 g	60.48 ± 1.12 g
	Keen sour orange	21.00 ± 1.00 de	18.33 ± 0.58 c	−0.62 ± 0.14 a–f	58.00 ± 1.00 f	64.96 ± 1.12 f
	Rough lemon	17.33 ± 0.58 fg	14.33 ± 0.58 efg	−0.68 ± 0.03 b–f	54.33 ± 0.58 g	60.85 ± 0.65 g
	Brazilian sour orange	25.67 ± 0.58 ab	19.00 ± 1.00 bc	−0.70 ± 0.03 c–f	65.00 ± 1.00 de	72.80 ± 1.12 de
	Sunki × bentake	10.33 ± 0.58 lm	14.67 ± 0.58 def	−0.66 ± 0.04 b–f	50.33 ± 0.58 h	56.37 ± 0.65 h
	X639	16.33 ± 1.53 gh	13.00 ± 1.00 fgh	−0.74 ± 0.06 def	54.33 ± 0.58 g	60.85 ± 0.65 g
	Kirrumakki nucellar	15.33 ± 0.58 ghi	12.00 ± 1.00 ghi	−0.11 ± 0.66 a	54.00 ± 1.00 g	60.48 ± 1.12 g
	Rangpur poona nucellar	14.67 ± 0.58 hij	11.00 ± 1.00 hij	−0.78 ± 0.03 efg	48.67 ± 0.58 hi	54.51 ± 0.65 hi
Severe drought	Gabbuchini	11.67 ± 1.15 klm	08.33 ± 0.58 kl	−1.30 ± 0.06 gh	46.33 ± 0.58 ij	51.89 ± 0.65 ij
	Gada dahi	19.33 ± 0.58 ef	14.67 ± 0.58 def	−1.14 ± 0.01 fgh	54.67 ± 0.58 g	61.23 ± 0.65 g
	Sour orange	14.67 ± 0.58 hij	11.67 ± 0.58 hi	−1.30 ± 0.05 gh	46.67 ± 0.58 ij	52.27 ± 0.65 ij
	Keen sour orange	17.33 ± 0.58 fg	13.00 ± 1.00 fgh	−1.39 ± 0.07 h	50.00 ± 1.00 h	56.00 ± 1.12 h
	Rough lemon	13.67 ± 0.58 ijk	10.33 ± 0.58 ijk	−1.39 ± 0.04 h	45.00 ± 1.00 jk	50.40 ± 1.12 jk
	Brazilian sour orange	24.33 ± 0.58 bc	17.00 ± 1.00 cd	−1.57 ± 0.03 h	57.67 ± 1.53 f	64.59 ± 1.71 f
	Sunki × bentake	09.33 ± 0.58 lm	06.00 ± 1.00 lm	−1.39 ± 0.03 h	39.67 ± 0.58 l	44.43 ± 0.65 l
	X639	14.00 ± 1.00 hijk	08.33 ± 0.58 kl	−1.36 ± 0.06 h	43.33 ± 0.58 k	48.53 ± 0.65 k
	Kirrumakki nucellar	13.33 ± 0.58 ijk	08.67 ± 0.58 jk	−1.38 ± 0.03 h	45.00 ± 1.00 jk	50.40 ± 1.12 jk
	Rangpur poona nucellar	10.33 ± 0.58 lm	05.33 ± 0.58 m	−1.40 ± 0.03 h	36.33 ± 0.58 m	40.69 ± 0.65 m
	***p*-value**					
	$p_{Drought}$	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	$p_{Rootstock}$	<0.0001	<0.0001	=0.0018	<0.0001	<0.0001
	$p_{Drought \times Rootstock}$	<0.0001	<0.0001	=0.0250	<0.0001	<0.0001

Data presented are means ± standard deviation (mean ± SD) of three replicates. Different letters indicate statistically significant differences among treatments, while "ns" signifies no significant differences between them according to Tukey's honestly significant difference test ($p < 0.05$).

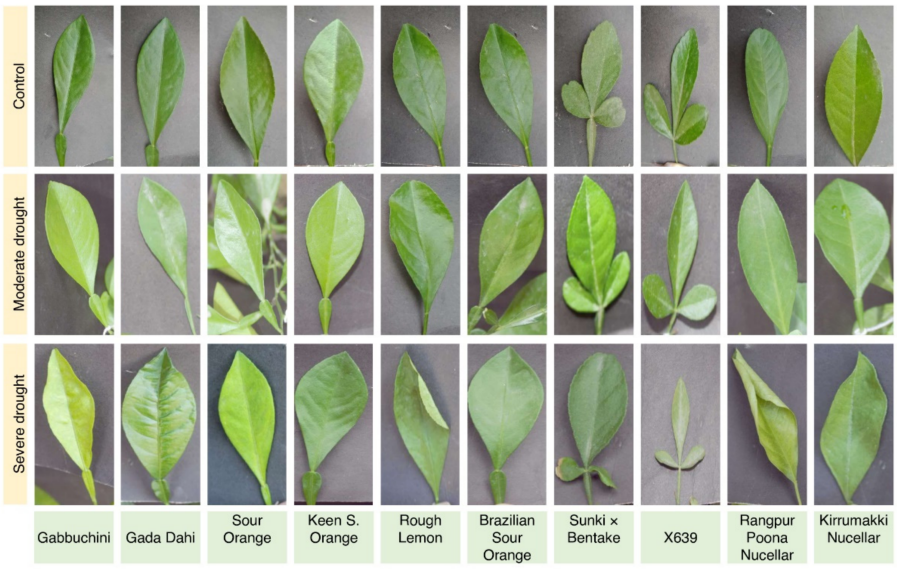

Figure 1. Effect of different water regimes on the leaf phenotype of 10 different citrus rootstocks with distinct degrees of tolerance to drought stress.

3.3. Water Deficiency Interrupts the Photosynthetic Pigments of Citrus Rootstocks

Drought stress considerably lessened chlorophyll *a*, chlorophyll *b*, and carotenoids content of different rootstocks ($p_{Drought} < 0.0001$; Table 3). Normal irrigated Brazilian sour orange had the highest chlorophyll *a* content (3.74 ± 0.12 mg g^{-1} FW), while Rangpur Poona nucellar rootstock had the lowest chlorophyll *a* content (1.57 ± 0.04% mg g^{-1} FW; $p_{Drought \times Rootstock} < 0.0001$). Furthermore, there was no significant difference in chlorophyll *b* content among all tested rootstocks, except Gabbuchini and Gada dahi, under normal irrigation conditions. However, chlorophyll *b* content was significantly decreased under water deficiency conditions (Table 3). Likewise, there were no significant differences in carotenoids content of all studied citrus rootstocks, except Gabbuchini, under normal irrigation conditions. Nevertheless, carotenoids content was significantly reduced when citrus rootstocks were stressed with different drought levels. It is worth mentioning that Rangpur Poona nucellar rootstock had the lowest carotenoids content (0.14 ± 0.03 mg g^{-1} FW), followed by Sunki × bentake (0.23 ± 0.02 mg g^{-1} FW) under severe drought conditions (Table 3).

Table 3. Effect of different water regimes on the photosynthetic pigments, H_2O_2, and proline content of 10 different citrus rootstocks with distinct degrees of tolerance to drought stress.

	Rootstock	Chlorophyll *a* (mg g^{-1} FW)	Chlorophyll *b* (mg g^{-1} FW)	Carotenoids (mg g^{-1} FW)	H_2O_2 (µmol g^{-1} FW)	Proline (µmol g^{-1} FW)
Control	Gabbuchini	3.52 ± 0.03 b	1.43 ± 0.06 abc	1.05 ± 0.04 ab	43.00 ± 2.65 d	0.33 ± 0.03 c–h
	Gada dahi	3.54 ± 0.01 ab	1.47 ± 0.06 ab	1.13 ± 0.04 a	44.33 ± 5.03 d	0.35 ± 0.01 c–g
	Sour orange	3.53 ± 0.02 b	1.53 ± 0.06 a	1.18 ± 0.04 a	42.33 ± 1.15 d	0.38 ± 0.02 c–f
	Keen sour orange	3.57 ± 0.05 ab	1.50 ± 0.10 a	1.15 ± 0.08 a	46.00 ± 2.00 d	0.29 ± 0.00 fgh
	Rough lemon	3.60 ± 0.08 ab	1.50 ± 0.10 a	1.15 ± 0.08 a	45.33 ± 2.52 d	0.32 ± 0.03 c–h
	Brazilian sour orange	3.74 ± 0.12 a	1.57 ± 0.06 a	1.26 ± 0.12 a	45.00 ± 2.65 d	0.38 ± 0.02 c–f
	Sunki × bentake	3.50 ± 0.05 b	1.53 ± 0.06 a	1.18 ± 0.04 a	44.67 ± 0.58 d	0.25 ± 0.01 ghi
	X639	3.51 ± 0.06 b	1.57 ± 0.06 a	1.21 ± 0.04 a	39.67 ± 6.03 d	0.26 ± 0.00 ghi
	Kirrumakki nucellar	3.57 ± 0.08 ab	1.57 ± 0.06 a	1.26 ± 0.04 a	42.67 ± 1.53 d	0.41 ± 0.01 bcd
	Rangpur poona nucellar	3.68 ± 0.04 ab	1.53 ± 0.06 a	1.18 ± 0.04 a	44.67 ± 2.08 d	0.17 ± 0.02 i–l
Moderate drought	Gabbuchini	2.95 ± 0.04 de	1.23 ± 0.06 cde	0.77 ± 0.08 c–f	64.33 ± 2.08 c	0.18 ± 0.03 i–l
	Gada dahi	3.10 ± 0.01 cde	1.27 ± 0.06 bcd	0.85 ± 0.05 bcd	68.33 ± 3.06 bc	0.29 ± 0.01 fgh
	Sour orange	2.99 ± 0.04 cde	1.03 ± 0.12 efg	0.79 ± 0.09 cde	64.67 ± 1.53 c	0.26 ± 0.04 ghi
	Keen sour orange	3.12 ± 0.02 cde	1.23 ± 0.06 cde	0.82 ± 0.12 cde	62.67 ± 2.08 c	0.23 ± 0.06 hij
	Rough lemon	3.14 ± 0.03 cd	0.93 ± 0.15 fgh	0.72 ± 0.12 c–f	62.00 ± 1.00 c	0.25 ± 0.05 ghi
	Brazilian sour orange	3.19 ± 0.04 c	1.37 ± 0.06 abc	0.87 ± 0.04 bcd	66.00 ± 2.65 c	0.31 ± 0.02 d–h
	Sunki × bentake	2.66 ± 0.05 g	0.80 ± 0.10 hi	0.61 ± 0.05 e–h	66.00 ± 1.73 c	0.14 ± 0.05 jkl
	X639	3.11 ± 0.03 cde	1.13 ± 0.06 def	0.79 ± 0.09 cde	60.67 ± 1.53 c	0.25 ± 0.02 ghi
	Kirrumakki nucellar	2.92 ± 0.03 ef	1.13 ± 0.06 def	0.90 ± 0.12 bc	64.67 ± 1.53 c	0.41 ± 0.00 bcd
	Rangpur poona nucellar	2.54 ± 0.02 gh	0.73 ± 0.06 h–k	0.57 ± 0.07 f–i	64.67 ± 2.52 c	0.08 ± 0.06 l
Severe drought	Gabbuchini	2.20 ± 0.03 jk	0.77 ± 0.06 hij	0.44 ± 0.04 c–f	83.00 ± 2.00 a	0.41 ± 0.01 abc
	Gada dahi	2.45 ± 0.04 hi	0.83 ± 0.06 ghi	0.66 ± 0.01 d–g	83.33 ± 2.08 a	0.40 ± 0.00 cde
	Sour orange	2.31 ± 0.03 ij	0.63 ± 0.06 i–l	0.41 ± 0.04 hij	84.00 ± 2.65 a	0.38 ± 0.06 c–f
	Keen sour orange	2.24 ± 0.06 ijk	0.63 ± 0.06 i–l	0.49 ± 0.04 ghi	82.33 ± 3.06 a	0.34 ± 0.03 c–g
	Rough lemon	2.71 ± 0.25 fg	0.57 ± 0.06 jkl	0.38 ± 0.08 ij	84.00 ± 1.00 a	0.29 ± 0.04 fgh
	Brazilian sour orange	2.70 ± 0.06 g	0.87 ± 0.06 gh	0.69 ± 0.08 c–g	77.00 ± 1.00 ab	0.51 ± 0.06 a
	Sunki × bentake	1.87 ± 0.04 l	0.47 ± 0.06 l	0.23 ± 0.02 jk	85.67 ± 4.16 a	0.18 ± 0.01 ijk
	X639	2.10 ± 0.06 k	0.57 ± 0.06 jkl	0.36 ± 0.04 ij	81.00 ± 6.08 a	0.30 ± 0.03 e–h
	Kirrumakki nucellar	2.10 ± 0.04 k	0.53 ± 0.06 kl	0.41 ± 0.04 hij	82.33 ± 2.31 a	0.51 ± 0.01 ab
	Rangpur poona nucellar	1.57 ± 0.04 m	0.43 ± 0.06 l	0.14 ± 0.03 k	82.00 ± 1.73 a	0.10 ± 0.00 kl
	***p*-value**					
	$p_{Drought}$	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	$p_{Rootstock}$	<0.0001	<0.0001	<0.0001	=0.0261	<0.0001
	$p_{Drought \times Rootstock}$	<0.0001	<0.0001	<0.0001	=0.0978	<0.0001

Data presented are means ± standard deviation (mean ± SD) of three replicates. Different letters indicate statistically significant differences among treatments, while "ns" signifies no significant differences between them according to Tukey's honestly significant difference test ($p < 0.05$).

3.4. Drought Stress Induced the Accumulation of Stress-Associated Biomarkers in Citrus Rootstocks

Two major stress-associated biomarkers, including H_2O_2 and endogenous proline content, were assessed (Table 3). Generally, both drought levels ($p_{Drought} < 0.0001$) and rootstocks ($p_{Rootstock} = 0.0261$) induced the H_2O_2 accumulation. Plants under drought

stress conditions (moderate or severe) had significantly higher H_2O_2 levels, compared with non-stressed rootstocks. It is worth mentioning that all tested rootstocks had their highest H_2O_2 levels under severe drought conditions with no noticeable difference between them (Table 3). Similarly, endogenous proline content was significantly affected by water-deficient ($p_{Drought} < 0.0001$) and citrus rootstock ($p_{Rootstock} < 0.0001$), and its levels were boosted with raising the severity of drought stress. Brazilian sour orange had the highest proline levels (0.51 ± 0.06 µmol g^{-1} FW) under severe drought conditions. Nevertheless, there was no significant difference in proline content of different citrus rootstocks under regular irrigation conditions (Table 3).

3.5. Principal Component Analysis (PCA) and Two-Way Hierarchical Cluster Analysis (HCA) Revealed the Differences among Water Treatments and Citrus Rootstocks

To better understand the water deficiency and citrus rootstocks interactions, principal component analysis (PCA) and two-way hierarchical cluster analysis (HCA) were carried out (Figure 2). PCA-associated scatterplot revealed a clear separation among water deficit treatment (control, moderate drought, and severe drought), as well as all studied rootstocks with respect to PC1 (approximately 81.28%) and PC2 (about 11.94%) (Figure 2A). Moreover, the PCA-associated loading plot showed that while root length, root moisture content, shoot moisture content, shoot length, carotenoids, chlorophyll a, chlorophyll b, and water potential were positively correlated with non-stressed control plants, H_2O_2, and proline content were positively associated with water deficit treatments (Figure 2B).

In harmony with PCA findings, the HCA and its associated heatmap revealed the differences among water-deficient treatments (Figure 2C). For example, HCA-associated dendrogram among rootstocks revealed that all studied rootstocks were clustered separately into three distinct clusters. Cluster (a) included all non-stressed rootstocks, Cluster (b) included all moderate drought-stressed rootstocks except Rangpur Poona nucellar and Sunki × bentake which were clustered with severely stressed rootstocks within Cluster (c). Additionally, the HCA-associated dendrogram among investigated variables revealed that they were clustered into two separate clusters. Cluster 'I' included root length, root moisture content, shoot moisture content, shoot length, carotenoids, chlorophyll a, chlorophyll b, and water potential which all were higher in regularly irrigated non-stressed control rootstocks. On the other hand, Cluster 'II' included only H_2O_2 and proline contents which were higher in citrus rootstocks grown under severe deficient water stress (Figure 2C).

3.6. Water Deficiency Alters the Anatomical Structure of Citrus Rootstocks

To better understand the mechanism of drought resistance in citrus, the effect of different drought levels on the anatomical structure of stem and leaves of two highly tolerant (Brazilian sour orange and Gadha dahi) and two highly sensitive genotypes (Rangpur Poona nucellar and Sunki × bentake) from the first screening study was investigated. As we mentioned above, under drought stress conditions, the moisture content of both roots and shoots was reduced, resulting in yellow, curled, wilted leaves, and some other adverse wilt-associated symptoms (Figure 1). Briefly, under severe drought stress, the length of epidermal cells and vascular bundles was reduced, as well as the thickness of the xylem and cortical was thinner.

3.6.1. Effect of Drought Stress on Stem Anatomy of Citrus Rootstocks

Microscopic observation of stem cross-section showed that the highly tolerant genotypes (Brazilian sour orange and Gadha dahi) had some features such as water-filled cells, small cell gaps, tight and round cells under normal water conditions, as well as under drought stress conditions (Figure 3).

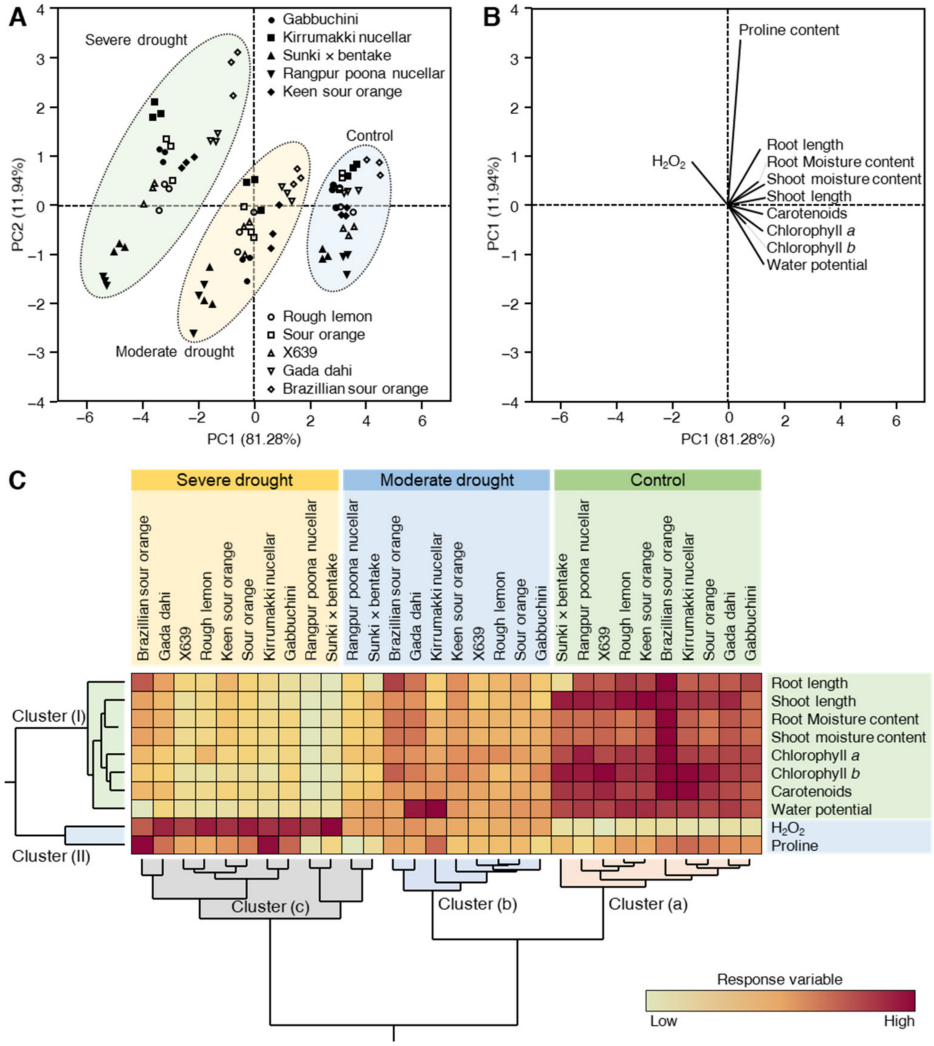

Figure 2. Principal component analysis (PCA) and two-way hierarchical cluster analysis (HCA) of individual morphological and physiological parameters were assessed in 10citrus rootstocks with distinct degrees of tolerance to drought stress under three water regimes. (**A**) PCA-associated scatterplots, (**B**) PCA-associated loading plots, and (**C**) two-way HCA. Variations in the dependent variables among studied treatments are visualized as a heat map. Rows correspond to dependent variables, whereas columns correspond to different treatments. Low numerical values are light-yellow-colored, while high numerical values are colored dark red (see the scale at the right bottom corner of the heat map).

However, both drought levels and rootstocks ($p_{Drought} < 0.0001$ and $p_{Rootstock} = 0.0002$, respectively) significantly affected the length of the epidermal cell and vascular bundle (Figure 4A,B, respectively), xylem thickness (Figure 4C), area of metaxylem cell (Figure 4D), phloem cell (Figure 4E), pith cell (Figure 4F), pith thickness (Figure 4G), and cortical cell (Figure 4H), and cortical thickness (Figure 4I). Under all water conditions, the anatomical structures of the highly tolerant genotypes Brazilian sour orange and Gadha dahi performed better than two highly sensitive genotypes Rangpur Poona nucellar and Sunki × bentake. For instance, under regular irrigation, both Brazilian sour orange and Gadha dahi rootstocks had the highest epidermal cell length ($p_{Drought \times Rootstock} = 0.0255$), vascular bundle length ($p_{Drought \times Rootstock} < 0.0001$), xylem thickness ($p_{Drought \times Rootstock} = 0.488$), metaxylem cell area ($p_{Drought \times Rootstock} = 0.0140$), phloem cell area ($p_{Drought \times Rootstock} = 0.0346$), pith cell area ($p_{Drought \times Rootstock} = 0.0316$), pith thickness area ($p_{Drought \times Rootstock} = 0.0112$), cortical cell area ($p_{Drought \times Rootstock} = 0.0180$), and cortical thickness ($p_{Drought \times Rootstock} = 0.0319$) with no significant differences between them. Although, both moderate and severe drought

stresses significantly reduced most, if not all, of these anatomical attributes, both highly tolerant genotypes did not show noticeable changes under drought stress. Nevertheless, the mesophyll cells of drought-sensitive Rangpur Poona nucellar and Sunki × bentake genotypes were slightly deformed and had shorter epidermal cell and vascular bundle length, as well as narrower metaxylem, phloem, pith, and cortical area (Figure 4).

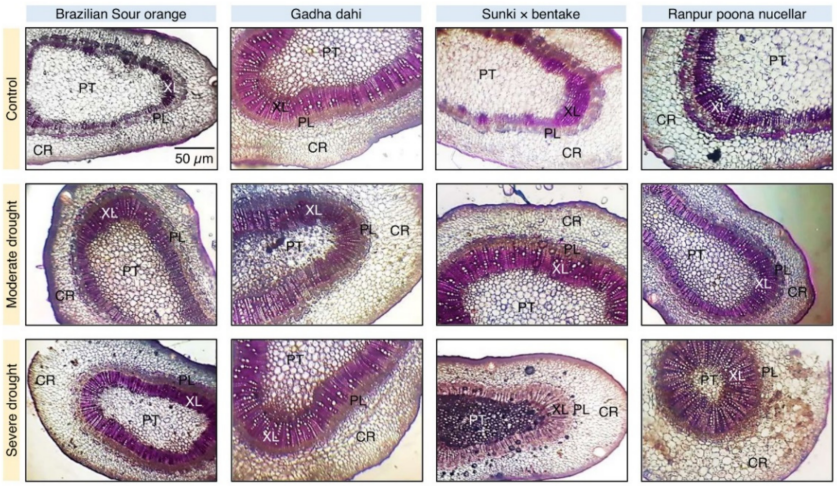

Figure 3. Stem transverse section of two highly tolerant (Brazilian sour orange and Gadha dahi) and two highly sensitive genotypes (Rangpur Poona nucellar and Sunki × bentake). PT: pith; CR: cortex; XL: xylem; PL: phloem.

3.6.2. PCA and Two-Way HCA Divulged the Variations in Stem Anatomy of Different Citrus Rootstocks

Briefly, the PCA-associated scatter plot showed a clear separation among water deficit treatment (control, moderate drought, and severe drought), as well as all studied rootstocks with respect to PC1 (approximately 87.29%) and PC2 (about 8.41%) (Figure 5A). It is worth mentioning that the drought-tolerant rootstocks Brazilian sour orange and Gadha dahi were grouped close to each other and separately from the two sensitive genotypes Rangpur Poona nucellar and Sunki × bentake under normal irrigation and moderate drought, but not severe drought conditions. Furthermore, the PCA-associated loading plot showed that all studied stem anatomical features were positively associated with normal water application (Figure 5B). In agreement with PCA results, the HCA and its associated heatmap uncovered the differences among different rootstocks under water-deficient treatments (Figure 5C). For example, HCA-associated dendrogram among rootstocks revealed that all studied rootstocks separated into two distinct clusters. Cluster (a) included all non-stressed rootstocks and the highly tolerant (Brazilian sour orange and Gadha dahi), grown under moderate drought, whereas cluster (b) included all severely stressed rootstocks and the two highly sensitive genotypes (Rangpur Poona nucellar and Sunki × bentake) that were moderately stressed with drought (Figure 5C).

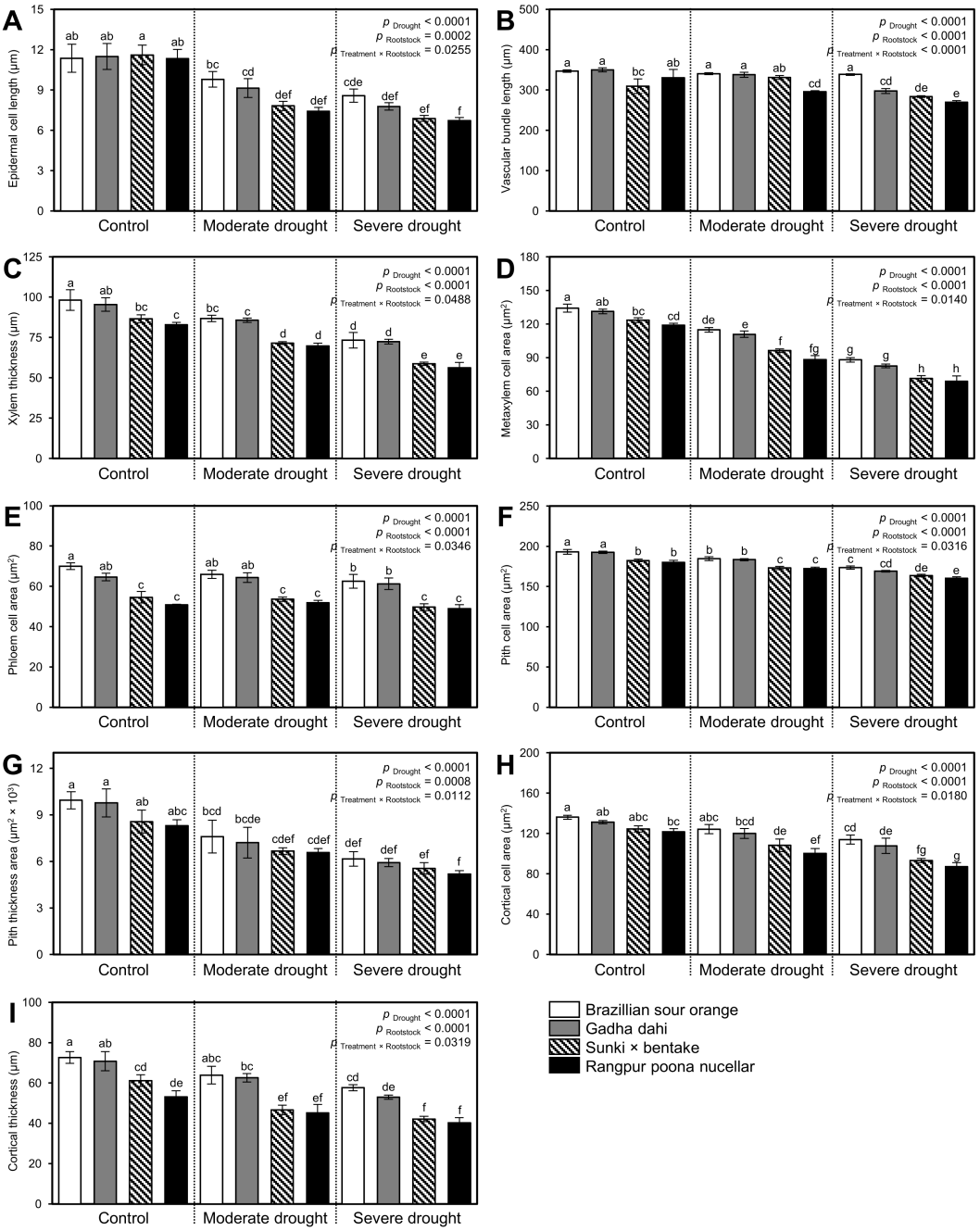

Figure 4. Effect of different water regimes on the stem anatomical features of two highly tolerant (Brazilian sour orange and Gadha dahi) and two highly sensitive genotypes (Rangpur Poona nucellar and Sunki × bentake). (**A**) Epidermal cell length (µm), (**B**) Vascular bundle length (µm), (**C**) Xylem thickness (µm), (**D**) Metaxylem cell area (µm^2), (**E**) Phloem cell area (µm^2), (**F**) Pith cell area (µm^2), (**G**) Pith thickness area (µm^2 × 10^3), (**H**) Cortical cell area (µm^2), and (**I**) Cortical thickness (µm). Data presented are means ± standard deviation (mean ± SD) of three biological replicates. Different letters indicate statistically significant differences among treatments, while "ns" signifies no significant differences between them according to Tukey's honestly significant difference test ($p < 0.05$).

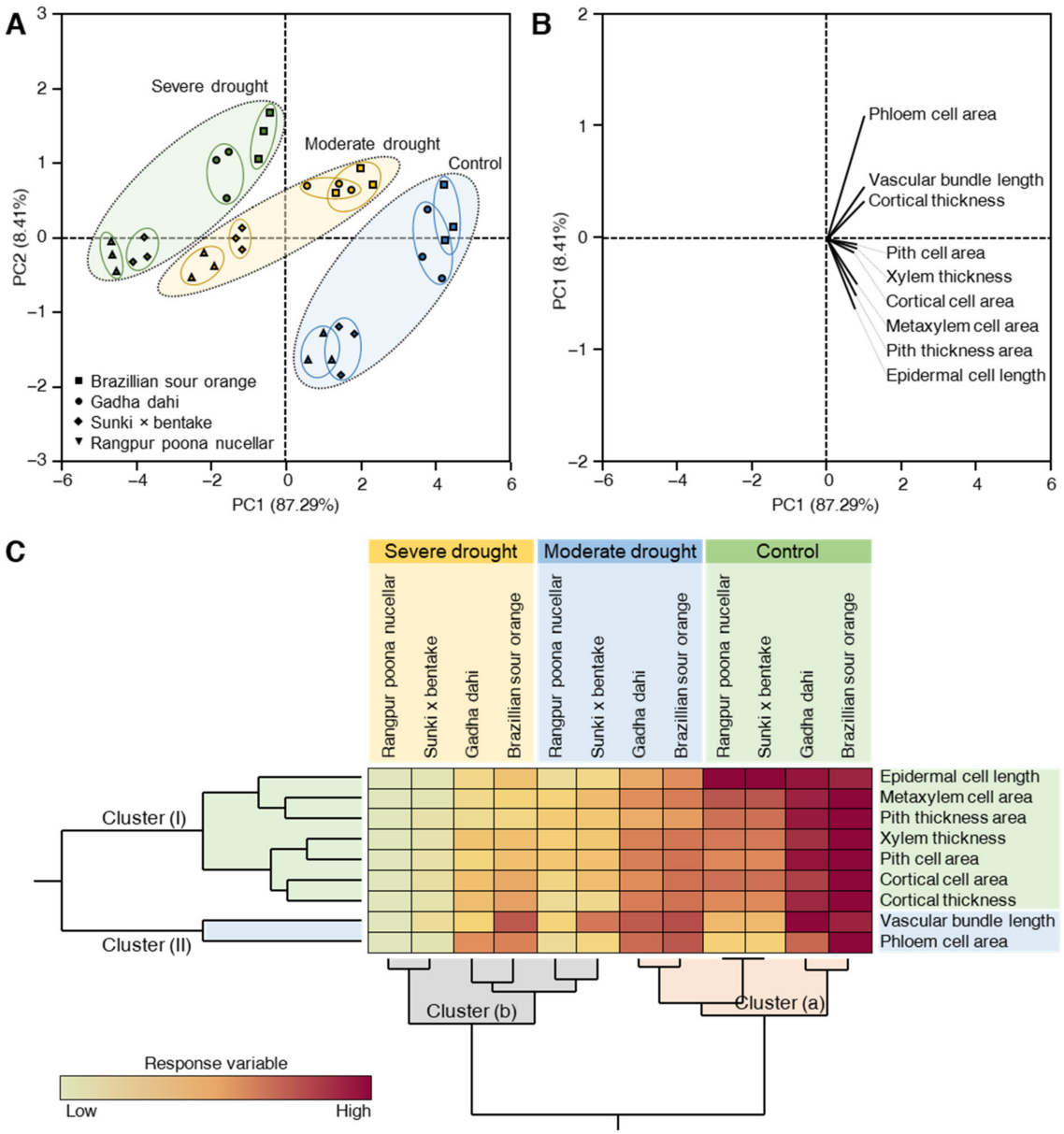

Figure 5. Principal component analysis (PCA) and two-way hierarchical cluster analysis (HCA) of individual stem anatomical features of two highly tolerant (Brazilian sour orange and Gadha dahi) and two highly sensitive genotypes (Rangpur Poona nucellar and Sunki × bentake) under three water regimes. (**A**) PCA-associated scatter plots, (**B**) PCA-associated loading plots, and (**C**) two-way HCA. Variations in the dependent variables among studied treatments are visualized as a heat map. Rows correspond to dependent variables, whereas columns correspond to different treatments. Low numerical values are light-yellow-colored, while high numerical values are colored dark red (see the scale at the right bottom corner of the heat map).

3.6.3. Effect of Drought Stress on Leaf Tissue Structure of Citrus Rootstocks

Microscopic observation of citrus leaves cross-section showed that it had an asymmetric heterogeneous structure that was characterized by two unequal palisade parenchyma (Figure 6).

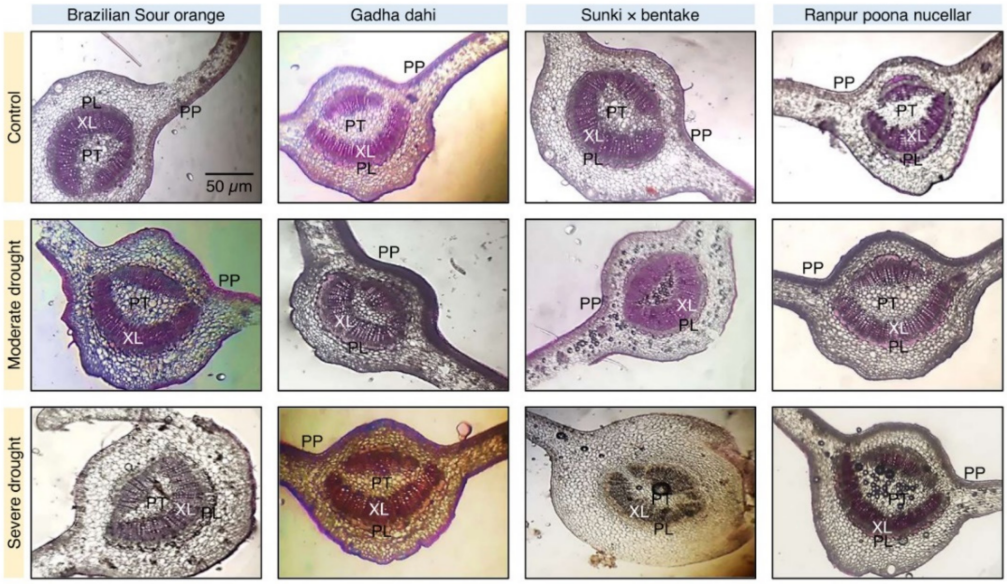

Figure 6. Leaf transverse section of two highly tolerant (Brazilian sour orange and Gadha dahi) and two highly sensitive genotypes (Rangpur Poona nucellar and Sunki × bentake). PT: pith; CR: cortex; XL: xylem; PL: phloem; PP: palisade parenchyma.

Like stem anatomy, citrus leaf anatomical attributes were significantly altered by both drought levels and rootstocks. However, the anatomical changes in leaf tissue structure were less significant in highly tolerant rootstocks (Brazilian sour orange and Gadha dahi) than sensitive genotypes (Rangpur Poona nucellar and Sunki × bentake). Interestingly, differences in all studied anatomical features of citrus leaves under different water regimes were cultivar-dependent. These features included epidermal cell length ($p_{\text{Drought} \times \text{Rootstock}}$ = 0.0315; Figure 7A), vascular bundle length ($p_{\text{Drought} \times \text{Rootstock}}$ = 0.0009; Figure 7B), xylem thickness ($p_{\text{Drought} \times \text{Rootstock}}$ = 0.0251; Figure 7C), metaxylem cell area ($p_{\text{Drought} \times \text{Rootstock}}$ = 0.0349; Figure 7D), phloem cell area ($p_{\text{Drought} \times \text{Rootstock}}$ = 0.0197; Figure 7E), pith cell area ($p_{\text{Drought} \times \text{Rootstock}}$ = 0.0163; Figure 7F), pith thickness area ($p_{\text{Drought} \times \text{Rootstock}}$ = 0.0335; Figure 7G), cortical cell area ($p_{\text{Drought} \times \text{Rootstock}}$ = 0.0250; Figure 7H), and cortical thickness ($p_{\text{Drought} \times \text{Rootstock}}$ = 0.0357; Figure 7I). Under all tested water regimes, highly tolerant rootstocks Brazilian sour orange and Gadha dahi had thicker epidermal and vascular bundle, as well as wider pith and cortical areas compared with sensitive genotypes (Rangpur Poona nucellar and Sunki × bentake). Additionally, severe drought stress significantly reduced the thickness of all leaf tissues, particularly in sensitive genotypes (Figure 7).

3.6.4. PCA and Two-Way HCA Revealed the Differences in Leaf Tissue Structure of Different Citrus Rootstocks

In brief, the PCA-associated scatter plot showed a clear separation among water deficit treatment (control, moderate drought, and severe drought), as well as all studied rootstocks with respect to PC1 (approximately 93.24%) and PC2 (about 3.95%) (Figure 8A). It is worth mentioning that the drought-tolerant rootstocks Brazilian sour orange and Gadha dahi were grouped close to each other and separately from the two sensitive genotypes Rangpur Poona nucellar and Sunki × bentake under all investigated water regimes. Moreover, the PCA-associated loading plot showed that all studied anatomical features of citrus leaves were positively associated with normal water application (Figure 8B). Like PCA, the HCA and its associated heatmap revealed the differences among different rootstocks under water-deficient treatments (Figure 8C). For example, HCA-associated dendrogram among rootstocks revealed that all studied rootstocks separated into two distinct clusters. Cluster (a) included all regularly irrigated genotypes and the two highly tolerant rootstocks (Brazilian sour orange and Gadha dahi) that were grown under moderate drought. On the other hand, cluster (b) included all severely stressed genotypes and the two sensitive

rootstocks (Rangpur Poona nucellar and Sunki × bentake) that were moderately stressed with drought (Figure 8C).

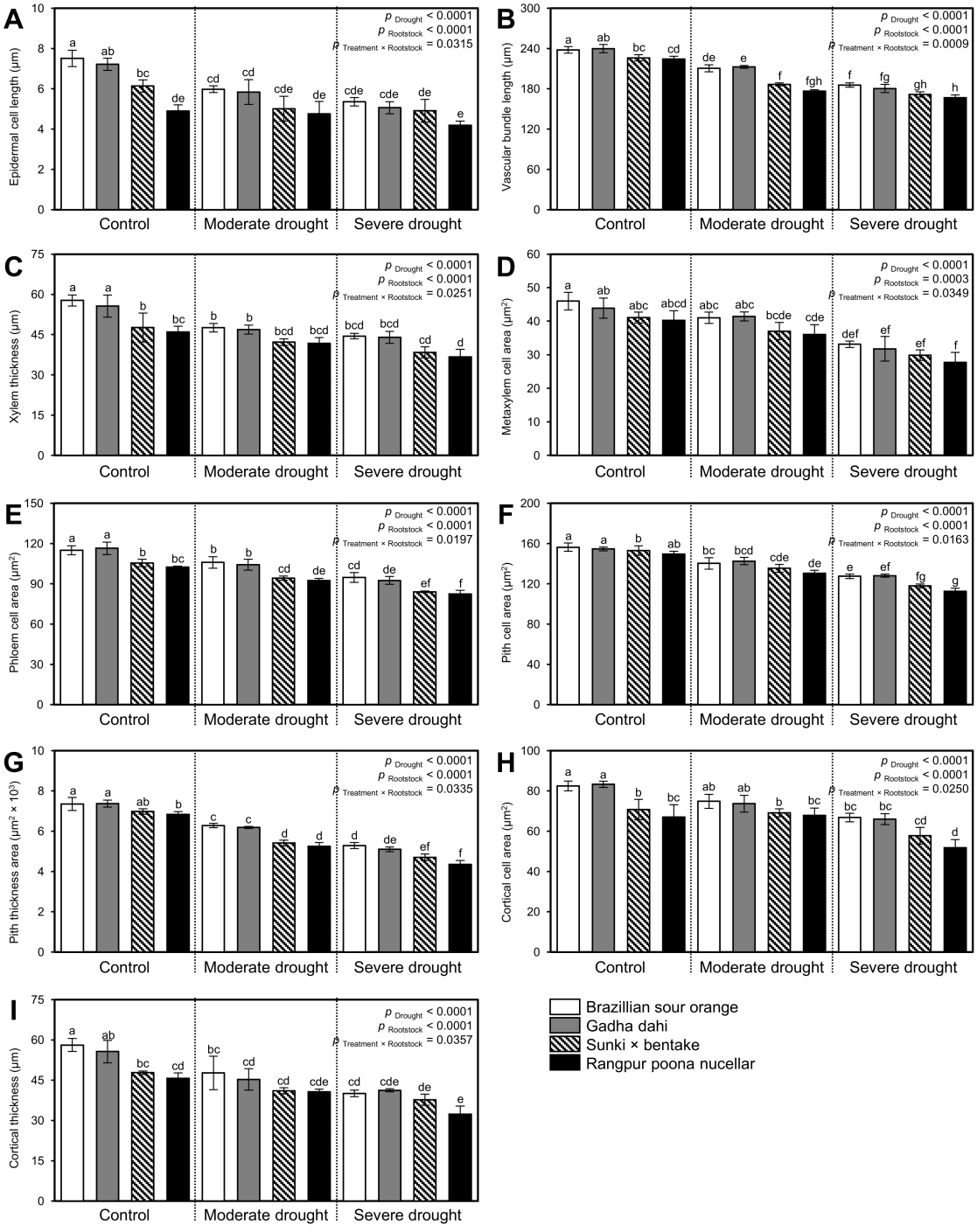

Figure 7. Effect of different water regimes on the leaf anatomical features of different two highly tolerant (Brazilian sour orange and Gadha dahi) and two highly sensitive genotypes (Rangpur Poona nucellar and Sunki × bentake). (**A**) Epidermal cell length (µm), (**B**) Vascular bundle length (µm), (**C**) Xylem thickness (µm), (**D**) Metaxylem cell area (µm^2), (**E**) Phloem cell area (µm^2), (**F**) Pith cell area (µm^2), (**G**) Pith thickness area (µm^2 × 10^3), (**H**) Cortical cell area (µm^2), and (**I**) Cortical thickness (µm). Data presented are means ± standard deviation (mean ± SD) of three biological replicates. Different letters indicate statistically significant differences among treatments, while "ns" signifies no significant differences between them according to Tukey's honestly significant difference test ($p < 0.05$).

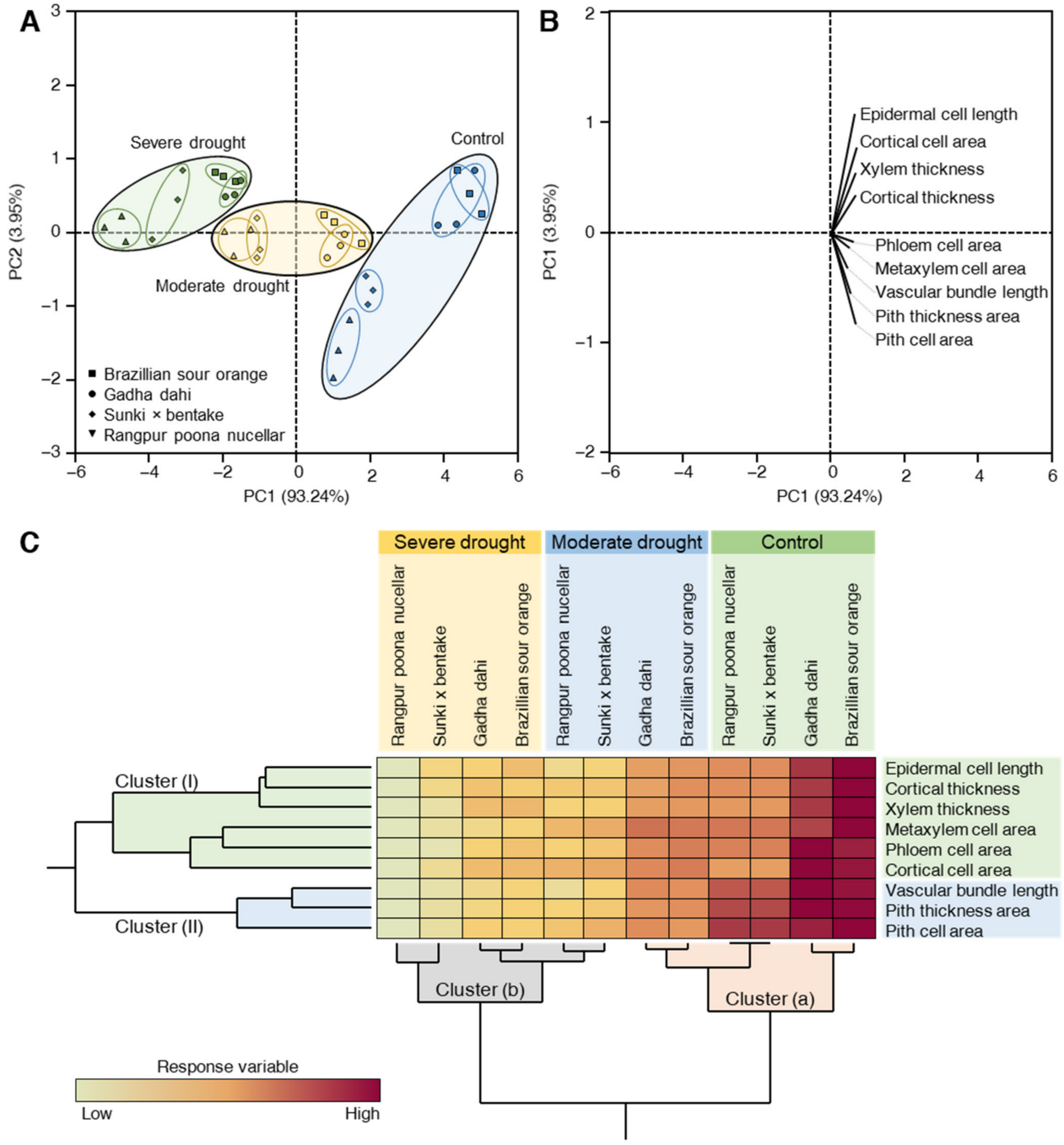

Figure 8. Principal component analysis (PCA) and two-way hierarchical cluster analysis (HCA) of individual leaf anatomical features of two highly tolerant (Brazilian sour orange and Gadha dahi) and two highly sensitive genotypes (Rangpur Poona nucellar and Sunki × bentake) under three water regimes. (**A**) PCA-associated scatter plots, (**B**) PCA-associated loading plots, and (**C**) two-way HCA. Variations in the dependent variables among studied treatments are visualized as a heat map. Rows correspond to dependent variables, whereas columns correspond to different treatments. Low numerical values are light-yellow-colored, while high numerical values are colored dark red (see the scale at the right bottom corner of the heat map).

4. Discussion

Water deficit conditions are a major environmental factor, which frequently limits the growth and productivity of important crop species [17]. Restriction of water supply can severely limit plant growth, development, and production [18,19]. Choice of rootstock is among the most important decisions a grower makes, and implications for yield and

quality are enormous. Rootstock in citrus trees influences the morphological, biochemical, physiological, and genetic characteristics of grafted scion cultivars through the rootstock scion interaction pathway [51]. Citrus rootstocks with better drought tolerance ability can greatly reduce production losses [52]. In this study, plant material consisted of 10 genetically diverse citrus rootstocks belonging to different citrus categories, i.e., oranges, pummelo, lemon, lime, their hybrids, and originating from diverse localities. The leaf shape and size of these rootstocks also varied. These rootstocks are reported to have different tolerance towards some biotic and abiotic stresses. The drought tolerance of these rootstocks was studied in this investigation. Leaf water potential in plants is directly related to water availability [53]. Leaf water potential indicates the whole plant water status, and maintenance of high leaf water potential is found to be associated with dehydration avoidance mechanisms. Our results demonstrated the decrease in leaf water potential as drought conditions become severe compared with control. The maintenance of water potential in leaves is a direct indicator of a plant dehydration avoidance mechanism, as genotypes with better water potential at stress conditions are regarded as drought tolerant [23,54,55]. In our studies, Brazilian sour orange showed drought tolerance by performing best at severe drought and Rangpur Poona nucellar at moderate drought. For insensitive genotypes, the decrease in leaf water potential indicated the mechanical injury of leaf chloroplasts caused by stress conditions which result in reduced transpiration rate and oxidative stress [16].

Citrus rootstocks with high chlorophyll *a* and *b*, and carotenoid contents against the stresses, especially the water stress, are regarded as tolerant rootstocks [56]. The normal functioning of photosynthetic machinery is affected by drought stress, the degradation, and photo-oxidation of chlorophyll caused by transpirational imbalance at water stress hamper the plant's ability to harvest light reducing total photosynthetic output [21,57]. Results showed that photosynthetic pigments chlorophyll *a*, chlorophyll *b*, and carotenoids reduced significantly at elevated stress conditions and overall genotype Brazilian sour orange and Gada dahi had the highest chlorophyll contents at drought conditions highlighting their ability to tolerate drought stress. Plants with dark green leaves (chlorophyll) under drought stress are considered tolerant. Visual assessments indicated the Brazilian sour orange as a tolerant rootstock without changes in leaf green color and leaf necrosis; while, Savage citrange emerged as the most sensitive, with maximum plant death and leaf shedding during stress treatments. The compromised photosynthetic machinery reduces carbohydrate transport and as a result plant growth is also reduced. The ability of plants to maintain growth under limited water supply reveals their tolerance ability [43,58]. Results showed that among citrus rootstocks, Brazilian sour orange and Gada dahi at water stress conditions maintained steady root and shoot growth. The root and shoot moisture content of these rootstocks were also high at stress conditions showing their tolerant nature. While, Rangpur Poona nucellar had the lowest shoot and root growth and moisture content emerged as the most sensitive.

Metabolic imbalances triggered by drought stress cause oxidative stress and as a result ROS are produced and accumulated [59]. The increased oxidation greatly reduces metabolic activities and the normal functioning of cell organelles. To combat oxidative stress, plants also have a built-in antioxidant defense mechanism. Proline is an osmoprotectant that is trigged as a result of ROS production in the cell, its production and accumulation work as ROS scavenging, redox balance, and reduce cell damage which normalizes the functionality of plant cells [60]. In drought-tolerant genotypes, the ROS production is reduced, and proline concentration is increased with increasing severity of stress. The results show that Brazilian sour orange has minimal ROS production and the highest proline concentration at severe drought stress.

In citrus under drought stress, leaves are observed to be shorter with thick epidermal cells which facilitates reducing the transpirational rate and oxidative stress [61,62]. Modifications in vascular anatomy are important for plant acclimation potential. Vascular bundles present in mid rid of leaves serve as a source to distribute nutrients and water. At

stress conditions with reduced leaf size, the reduction in the vascular bundle is an indicator of plants' abilities to modify their anatomy under stress. In the vascular bundle, the xylem acts as a source of water transport. The plants with greater xylem vessel diameter are unable to survive harsh environmental conditions [63–65]. At the onset of drought, stress transpiration, water uptake from roots, and stem hydraulic capacitance begin to decline. That reduces growth, the vascular bundles in the stem are observed to be reduced along with pith cell area and cortical thickness [66,67]. In this study, the results showed that with increasing drought intensity the anatomy of both sensitive and tolerant genotypes was modified, interestingly the two tolerant genotypes Brazilian sour orange and Gada dahi had greater values for all the leaf and stem anatomy parameters at severe drought stress than sensitive genotypes. This could be because of the continuous adaptability of tolerant genotypes which enabled them to maintain growth and function as the amount of water became limited, in response to sensitive genotypes in which the response could have been triggered at very later stages which abruptly affect their growth. These modifications in tolerant genotypes enabled them to maintain steady nutrient transport while reducing the risk of embolisms, increasing water-flow resistance, and constant transport of nutrients across [64].

5. Conclusions

Drought stress adversely affected plant water status, photosynthetic machinery, biochemical balance, and anatomical structure of all the citrus rootstocks studied. The intensifying drought reduced leaf water potential, and compromised the photosynthetic apparatus by damaging photosynthetic pigments (chlorophyll "*a*", "*b*", and carotenoid) apparent from lighter green color. Oxidative stress caused by ROS production which triggered production of proline. Alteration in anatomical structures of leaf and stem were observed. Citrus rootstocks Brazilian sour orange and Gada dahi performed best under drought stress, mitigated damage at molecular biochemical and anatomical levels, while rootstocks Sunki × bentake and Rangpur Poona nucellar were the most sensitive rootstocks.

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