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Growth, Physiological, Biochemical, and Transcriptional Responses to Drought Stress in Seedlings of *Medicago sativa* L., *Medicago arborea* L. and Their Hybrid (Alborea)

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Abstract: *Medicago sativa* L. is a tetraploid perennial forage legume of great agronomical interest. The increasing need for its use under water-deficit conditions as well as low-input systems demands further improvement of its drought tolerance. On the other hand, *Medicagoarborea* L. is a perennial leguminous shrub, which is knownas a drought-tolerant species. In the present study, drought stress responses of the aforementioned medicago species, along with their hybrid, named Alborea, were comparatively assayed at the morphological, physiological, biochemical, and transcriptional levels. In particular, transcript abundance of representative genes that: (a) control ion transport, *intracellular* Na^+/H^+ *antiporters*(*NHX1*) and *rare cold inducible2A* (*RCI2A*); (b) have an osmotic function *Δ1-pyrroline-5-carboxylate synthetase 1* (*P5CS1*); and (c) participate in signaling pathways and control cell growth and leaf function *stress-induced mitogen-activated protein kinases kinases* (*SIMKK*), *Zinc Finger* (*ZFN*), *apetala2/ethylene-responsive element binding* (*AP2/EREB*), *basic leucine zipper* (*bzip*) and *Medicago sativa Helicase 1*(*MH1*) were evaluated. Under well-watered conditions, the studied population of Alborea showed the highest stem elongation rate and photosynthetic rate that were dramatically reduced under drought conditions compared to *M. sativa* and *M. arborea*. Under drought conditions, the studied population of *M. arborea* showed less reduction of relative water content, all gas-exchange parameters, less lipid peroxidation, and more antioxidant capacity. Moreover, transcriptional analysis demonstrated that the population of *M. arborea* exhibited significantly higher transcript levels of drought-responsive genes in both leaves and roots under drought stress conditions. *M. sativa* has better antioxidant capacity than Alborea and had a higher induction of stress-related genes, thus it performs better than Alborea under drought conditions. Among the studied genes, it seems that *AP2/EREB* play a critical role in the response of the studied population to drought stress.

Keywords: Medicago species; water deficit; gene expression; antioxidant capacity; leaf gas-exchange parameters

1. Introduction

Drought is one of the most important environmental stresses limiting crop yields and productivity all over the world [1]. As the world's population is increasing rapidly, the amount of available agricultural land is shrinking mainly due to habitat use and climate change. Therefore, it is of great importance to exploit drought-affected land to meet the increasing world food demand and energy needs [2].

Plants react to the water deficit by the regulation of different physiological and biochemical processes such as water relation, gas exchange, photosynthesis, and the metabolism of organic compounds [3] as well as by adjustments of the membrane system [4]. Water deficit severely affects the photosynthesis process in all its phases mainly due to decrease of CO_2 diffusion to the chloroplast and to metabolic constraints [5]. In addition, plants accumulate compatible solutes such as proline that can stabilize proteins, to facilitate water absorption by decreasing the cytoplasmic osmotic potential, and to remove excess levels of Reactive Oxygen Species (ROS) maintaining cellular redox balance [1,6]. Plants possess an efficient antioxidant (enzymatic and non-enzymatic) defense system to cope with ROS-induced oxidative stress [7–9]. Antioxidant enzymes such as superoxide dismutase (SOD), and peroxidase (POD) could suppress the oxidative damage. The contribution of antioxidants to drought tolerance has been reported in several cases [10–12]. Plants respond to drought stress at the molecular level mainly by altered gene expression. Several different pathways are activated that interact with each other, forming a complex network which finally leads to modification of target proteins responsible for cellular responses at the physiological, biochemical, and molecular levels [13,14].

Alfalfa or *Medicago sativa* L. (*M. sativa*) is a tetraploid perennial forage legume of great agronomical interest, due to its low production cost and high nutritive value. Moreover, its deep noduled-root system improves soil properties and it is a source of biologically fixed nitrogen that also prevents water loss in semi-dry lands [15]. Recently, there is an increasing interest for its use as a bioenergy feedstock [16]. Furthermore, the tendency to shift from high-input farming systems based on inorganic-N fertilizers to low-input systems, increases the need for alfalfa. Previous agronomical, physiological, and biochemical studies suggest that alfalfa is a plant species relatively tolerant to drought. However, its use under water-deficit conditions as well as low-input systems, demands further improvement of its drought tolerance [17]. Compared to other crop species, restricted information is available regarding mechanisms by which genetic and physiological factors contribute to drought tolerance in alfalfa. This is mainly due to its large, tetraploid genome and out-crossing nature both of which hinder the genomic and genetic research. Nevertheless, the use of –omic technologies shed some light on the mechanisms underlying alfalfa drought tolerance [18–20]. However, comparative studies on the expression of drought-induced genes in different medicago species are limited.

Medicago arborea L. (*M. arborea*) is a perennial leguminous shrub that is well adapted to the Mediterranean area and is used for forage production during summer and winter. It is also considered to be the oldest species of the genus and is known as a drought-tolerant species [21]. Researchers have attempted the introgression of favorable traits from *M. arborea* genome to *M. sativa* such as drought tolerance. Hybrids between *M. sativa* and *M. arborea* named Alborea have been produced in the USA and Australia [22,23].

In our previous work [24], transcriptional responses of several salt induced genes have been studied in the parent species and their hybrid. The aforementioned genes had been categorized into three groups: a. genes encoding ion transporters, *intracellular Na^+/H^+ antiporters* (*NHX1*) and *rare cold inducible2A* (*RCI2A*), b. osmolytes, *Δ1-pyrroline-5-carboxylate synthetase 1* (*P5CS1*) and c. transcription factors and signal transduction proteins, *apetala2/ethylene-responsive element binding* (*AP2/EREB*), *stress-induced mitogen-activated protein kinases kinases*(*SIMKK*), *Zinc Finger* (*ZFN*)). The initial responses of plants to salt and drought stress are similar as they must cope with water deficit in both stresses [25]. In this regard, the aim of the present work was to study the transcriptional responses of the same

set of genes in the parental species and Alborea under drought stress. Additionally, in the present study, we have investigated the expression of two more gene, a DEAD-box *helicase* gene previously name *Medicago sativa Helicase 1* (*MH1*), possibly involved in ROS scavenging [26] and *a basic leucine zipper* (*bzip*) transcription factor previously reported to play a role in both drought and salt tolerance in *M. sativa* [27]. Our main interest was to explore the mechanisms for drought tolerance of the parental species and their hybrid giving special attention to leaf gas-exchange parameters, antioxidant capacity and membrane stability in drought conditions.

2. Materials and Methods

2.1. Plant Material

The seeds of *M. arborea*, *M. sativa* and Alborea used in the present study was obtained from E. Bingham from the Agronomy Department, University of -Madison- Wisconsin,,Wisconsin, United States. The development of Alborea was described in detail by [22,24].

2.2. Seeds Pretreatment

Seeds of parental species along with their hybrid (hereafter entries) were scarified as it was described by [24] Then the seeds were placed on agar plates, turned upside down and remained at 4 °C overnight. Finally, they were incubated to 20 °C for 3–4 days in the dark to ensure uniform germination.

2.3. Growth Conditions and Drought Treatment

Thirty germinated seedlings of the entries were transferred into 10 cm pots containing a commercial growing medium (Kronos N 50–300 mg/L, P_2O_5 80–300 mg/L, K_2O 80–300 mg/L, pH 5–6.5, salinity <1.75 g/L) and were placed in a growth chamber under a 16/8 h day/night regime, 23 °C, 55–65% relative humidity in a completely randomized block design. All the pots were normally watered in the growth chamber for four weeks before the implementation of the drought stress. Twenty-four plants per each entry were selected according to their uniformity of growth. Half of the plants were put under drought stress imposition by withholding water (drought treatment) for almost three weeks while another half were maintained in optimal water availability conditions (control) until harvesting or used for physiological measurement. Each treatment had 4 replicates consisting of three plants. The controls were irrigated with Hoagland solution [28] twice during the experiment. All pots were randomized within each treatment biweekly. The drought treatment was initiated on the 20th of November and was completed on the 7th of December. Shoots and roots were individually harvestedand immediately placed into liquid nitrogen. All plants during harvest were in the vegetative stage, and their roots did not show nodules.

2.4. Growth and Physiological Parameters

The stem length was measured every three days during the experimental period. Stem elongation rate (SER) was calculated as follow: (SER) = (T2 − T1)/t, where T1 and T2 are the stem length at the beginning and at the end of a time (T) respectively.

Leaf relative water content (RWC) was determined weekly according to the following equation RWC = (FW − DW)/(SW − DW) * 100 [29] where FW is leaf Fresh Weight, DW is leaf Dry Weight after being dried at 85 °C for 48 h and SW is turgid weight of leaves after soaking in water for 24 h at room temperature (20 °C).

Leaf gas-exchange parameters (net photosynthetic rate (Pn), stomatal conductance (gs), and transpiration rate (E)), were measured twice, after the 1st and 2nd week of drought stress, using the upper fully developed leaves. The measurements were carried out with a portable phytosynthesis system Li-6400 (LiCor, Lincoln, NE, USA) under constant conditions, air flow rate 500 μmol s^{-1}, PAR500 μmol $m^{-2}s^{-1}$, 400 μmol CO_2 mol^{-1}, while leaf temperature ranged between 19 °C and 23 °C. Water use efficiency (WUE) was calculated as the ratio between Pn and E.

2.5. Sample Preparation for Antioxidant Assays

For enzyme antioxidant assays, plant extracts were prepared, from frozen (–80 °C) leaf and root tissues. The tissues were powdered with liquid nitrogen and were extracted (solid tissue/buffer ratio was 1:10) with ice-cold 50 mM Potassium phosphate buffer (pH 6) containing 0.1 mM EDTA and 1% (*w*/*v*) polyvinylpyrrolidone (PVP). Samples were centrifuged at 13,000 rpm for 5 min. The supernatants were collected and used for antioxidant and enzyme activity assays. All assays were performed in duplicate.

2.6. Total Antioxidant Capacity Assays

Ferric Reducing Antioxidant Power (FRAP) assays were performed according to [30]. The FRAP reagent was freshly prepared by adding 10 mM of 2,4,6-Tris (2-pyridyl)-1,3,5-triazine (TPTZ) (dissolved in 40 mM of HCl), 20 mM of $FeCl_3$ and 300 mM of acetate buffer (pH 3.6) at a ratio of 1:1:10. Total antioxidant capacity (TAC) based on FRAP, was expressed as µg ascorbic acid equivalents/mg of FW. 2,2′-Azino-bis 3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assays were accomplished according to published methods [31,32]. The results were expressed as % radical scavenging activities/mg of FW.

2.7. MDA

Malondialdehyde (MDA) was measured by the colorimetric method described by [33]. Trichloroacetic acid 0.1% (*w*/*v*) was added in the extracts at a ration 4:1. After centrifugation (10 min, 13,000 rpm, 4 °C), the supernatant was collected (500 µL) and mixed with 1 mL volume of 20% (*w*/*v*) trichloroacetic acid solution and 0.5% thiobarbituric acid (TBA) and the sample incubated at 95 °C for 25 min. The reaction was stopped by incubating on ice. The samples then centrifuged at 13,000 rpm for 5 min. The absorption of supernatant was recorded at 532 nm as described by [34]. The amount of MDA was calculated using as extinction coefficient 155 $mM^{-1}cm^{-1}$. The results were expressed as nmol MDA/mg of fresh tissue.

2.8. Antioxidant Enzyme Assays

SOD activity was determined according to [35] by measuring the inhibition of cytochrome c reduction in a coupled enzyme system with xanthine oxidase at pH 7.8 and 25 °C. In a 1 mL reaction mix, containing 50 mM potassium phosphate, 0.1 mM EDTA, 0.01 mMcytochrome c, 0.05 mM xanthine, 0.005-unit xanthine oxidase and the appropriate volume of plant extract. SOD activity was determined at the absorption of 500 nm. One unit of enzyme activity was taken as the quantity of enzyme, which reduced the absorbance reading of uninhibited rate to 50%.

POD activity was determined at 436 nm according to the method of [36]. The method is based on the conversion of guaiacol to tetraguaiacol (ε = 26.6 mM^{-1} cm^{-1}). The reaction mixture contained 100 mM Potassium phosphate buffer (pH 7.0), guaiacol 20 mM, H_2O_2 10 mM and plant extract. The increase in absorbance was recorded at 436 nm for 3 min. The results were expressed as U/mg of protein. One unit of enzyme activity is defined as the amount of enzyme that catalyses the turnover of 1µmol of substrate per min.

2.9. Protein Determination

The protein content of samples was determined according to the Bradford method, using bovine serum albumin as a standard [37]. Results were expressed as mg of protein/mg fresh tissue.

2.10. Primer Design

Primer sequences were designed based on expressed sequences tags (EST) sequences that were retrieved after homology search on GenBank and on National Center for Biotechnology Information (NCBI). Primers (Table 1) for quantitative real time PCR (qPCR) experiments were designed using the NCBI Primer designing tool.

Table 1. The set of primers used for qPCR experiments (*Msactin2*, *MsAP2/EREB*, *MsNHX1*, *MsP5CS1*, *MsZFN*, *MsSIMKK*, *MsRCI2A*, *MsHelicase* and *MsbZIP*)

Sequence Name	Genbank Number	Primer Sequence	Amplicon Size
Ms-actin2-F	JQ028730.1	TTCTCACCACACTTCTCGCC	173bp
Ms-actin2-R		CCAGCCTTCACCATTCCAGT	
Ms-AP2/EREB-F	Not deposited	AATGGGTGGGGAAACGGAAC	95bp
Ms- AP2/EREB-R		TTTGGTGGTGGAGTGTGGTT	
Ms-NHX1-F	AY513732.1	GCCATGAAATTCACCGACCG	118bp
Ms-NHX1-R		CTGCCACCAAAAACAGGACG	
Ms- P5CS1-F	X98421.1	TTTGCGGTCGGAAGGTGTTA	119bp
Ms- P5CS1-R		CGATTTCCAAGGTGCAAGCC	
Ms-ZFN-F	JX131368.1	CCCAAGCTGCAAGTTTGACC	154bp
Ms-ZFN-R		TGAGCCCGACTCAACAAGTC	
Ms-SIMKK-F	AJ293274.1	ACCAGAAGCTCCAACGACTG	94bp
Ms-SIMKK-R		CCTCGAAGCAGTCCATCTCC	
Ms-RCI2-F	JQ665271.1	GTTGTCAGGGGCGTCATTCT	169bp
Ms-RCI2-R		TCCAAGCAGGACAAAACGGA	
Ms-Helicase-F	EF011022.1	CCGGATCTTCAGGTTTGCCT	131bp
Ms-Helicase-R		TGCTTGATGCCCTCCAATGT	
Ms-bZIP-F	HQ911778.1	GGTGACAGTGGTTCAGAGGG	109bp
Ms-bZIP-R		CGTTGGCTCCATCAACAAGC	

Ms: *Medicago sativa*; AP2/EREB:apetala2/ethylene-responsive element binding; NHX1: intracellular Na^+/H^+ antiporters; P5CS1: Δ1-pyrroline-5-carboxylate synthetase 1; ZFN: Zinc Finger; SIMKK:stress-induced mitogen-activated protein kinases kinases; RCI2: rare cold inducible2A; bZIP: basic leucine zipper

2.11. cDNA Synthesis and Gene Relative Expression Ratios

Four-week-old plants were used for expression analysis. Total RNA was isolated with the Trizol isolation reagent (Invitrogen, Carlsbad, CA, USA) for reverse transcription. Both quality and quantity of nucleic acid were evaluated by agarose gel electrophoresis and NanoDrop™ UV, spectrophotometer respectively. The total RNA of roots and shoots was reverse-transcribed using the Superscript II enzyme (Invitrogen, Carlsbad, CA, USA), and the synthesized cDNA was used as a template in the qPCR. qPCR experiments were employed on a Step One Plus qPCR system (Applied Biosystems, Foster City, CA, USA). Samples were prepared using 10 µL of Sybr Select Master Mix (Applied Biosystems, Foster City, CA, USA), 1.5 ng/µL cDNA as sample, 200 nM of each primer and Molecular Biology Reagent water (Sigma) up to a final volume of 20 µL. For all target genes assayed the qPCR amplification thermal profile was programmed to run for 40 cycles, during which the temperature was set to be at 95 °C for 15 s, and at 60 °C for 30 s, followed by a melting curve analysis of the transcripts. Melting curves were programmed as follows: 15 seconds at 95 °C, 15 seconds at 60 °C, 20 minutes' slow ramp, and 15 seconds at 95 °C. The data for the melting curve were captured during this slow ramp, and the melting curve was visualized using the Step One Plus software. Relative expression ratios were calculated according to the $2^{-\Delta\Delta Ct}$ method [38]. More specifically to calculate ΔΔ*C*t value, the control sample of each entry (*M. arborea*, *M. sativa* and Alborea) was used. MS-Actin-2 was used as endogenous gene for normalization.

2.12. Statistical Analysis

The effect of treatment and entries on seedlings height and SER was detected by using the Repeated-Measures Analysis of Variance (ANOVA) with General Linear Model. The within-subject factors were the dates and the treatments and the between-subject factor the entries. The antioxidant capacity was estimated as the ratio of the enzymatic activity of plants under drought stress in relation to the plants under well-watered conditions. A 2-way ANOVA was used for detecting the effect of entries and organs on the ratio of the plant's enzymatic activity. Moreover, the effect of treatments, entries and organs on gene expression was detected by using a 3-way ANOVA. The Tukey test at

0.05 probability level was chosen for detection of differences among means. The IBM SPSS Statistics 23 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis.

3. Results

3.1. The Effect of Drought Stress on Seedling Growth

The morphological parameters (height and Stem Elongation Rate) were significantly reduced under drought stress (Table 2) compared to the control. Significant interaction for both seedling height and SER was detected between entries (*M. sativa*, *M. arborea*, Alborea) and dates of drought stress indicating a different response of the entries during the drought treatment (Figure 1a,b). Particularly, the seedlings height of Alborea's population was significantly reduced at the end of the drought treatment by about 20% (Figure 1a), while for the studied population of *M. sativa* and *M. arborea* was reduced by only 13% and 5% respectively (Figure 1a,b). However, it is noteworthy that Alborea was taller than the parental species even under drought stress (Figure 1a). Additionally, overall (across dates) the height of the parental species did not significantly differ under both control and drought stress. Similarly, the SER of Alborea's population was rapidly declined by about 98% after three weeks of water deficit, whereas the SER of population of *M. sativa* and *M. arborea* was gradually reduced by about 90% and 75% respectively (Figure 1b).

Table 2. The height of seedlings, Stem Elongation Rate (SER), Relative Water Content (RWC), Photosynthetic rate (Pn), Stomatal conductance, transpiration rate (E), Water Use Efficiency (WUE) of the seedlings (mean ± SE) under the control and the drought treatment.

		Height (cm)	SER	RWC	Pn (μmol CO_2/m^2s)	Gs (mol CO_2/m^2s)	E (mmol H_2O/m^2s)	WUE (μmol (CO_2) mmol (H_2O)$^{-1}$
Drought	Control	23 ± 0.75a *	0.8 ± 0.06a	0.9 ± 0.09a	13.2 ± 0.5a	0.14 ± 0.01a	1.7 ± 0.12a	8.2 ± 0.3b
	Drought	21 ± 0.52b	0.4 ± 0.04b	0.8 ± 0.01b	9.3 ± 0.4b	0.08 ± 0.006b	1.0 ± 0.08b	10.2 ± 0.5a
Entries	*M. arborea*	16 ± 0.21c	0.3 ± 0.03b	0.87 ± 0.01a	9.7 ± 0.5b	0.07 ± 0.004b	0.9 ± 0.07c	11.1 ± 0.50a
	M. sativa	19 ± 0.41b	0.4 ± 0.04b	0.85 ± 0.01a	10.6 ± 0.5b	0.11 ± 0.009a	1.3 ± 0.08b	8.3 ± 0.34b
	Alborea	31 ± 0.77a	1.0 ± 0.09a	0.8 ± 0.02b	13.4 ± 0.9a	0.14 ± 0.015a	1.8 ± 0.20a	8.1 ± 0.60b
Dates	1 (20/11/15)	17 ± 0.59e						
	2 (23/11/15)	20 ± 0.78d	0.94 ± 0.10a					
	3 (26/11/15)	22 ± 1.04c	0.84 ± 0.10a	0.87 ± 0.01a	11.8 ± 0.5a	0.11 ± 0.008	1.4 ± 0.11	9.1 ± 0.4
	4 (30/11/15)	24 ± 1.13bc	0.51 ± 0.05b					
	5 (3/12/15)	25 ± 1.25ab	0.46 ± 0.07b	0.85 ± 0.01b	10.7 ± 0.7b	0.10 ± 0.010	1.3 ± 0.14	9.2 ± 0.5
	6 (7/12/15)	26 ± 1.33a	0.26 ± 0.04b	0.8 ± 0.02c				
Source of Variation	Drought (A)	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$
	Entries (B)	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$
	Dates (C)	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	ns	ns	ns
	AXB	ns **	$p < 0.05$	ns	$p < 0.05$	$p < 0.05$	$p < 0.05$	ns
	AXC	ns	ns	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	ns
	BXC	$p < 0.05$	$p < 0.05$	$p < 0.05$	ns	ns	ns	ns
	AXBXC	ns	ns	ns	ns	ns	ns	ns

* Means followed by the same letter in the column for the same parameter did not significantly differ ($p > 0.05$) according to Tukey test. ** ns: not significant at 0.05 level.

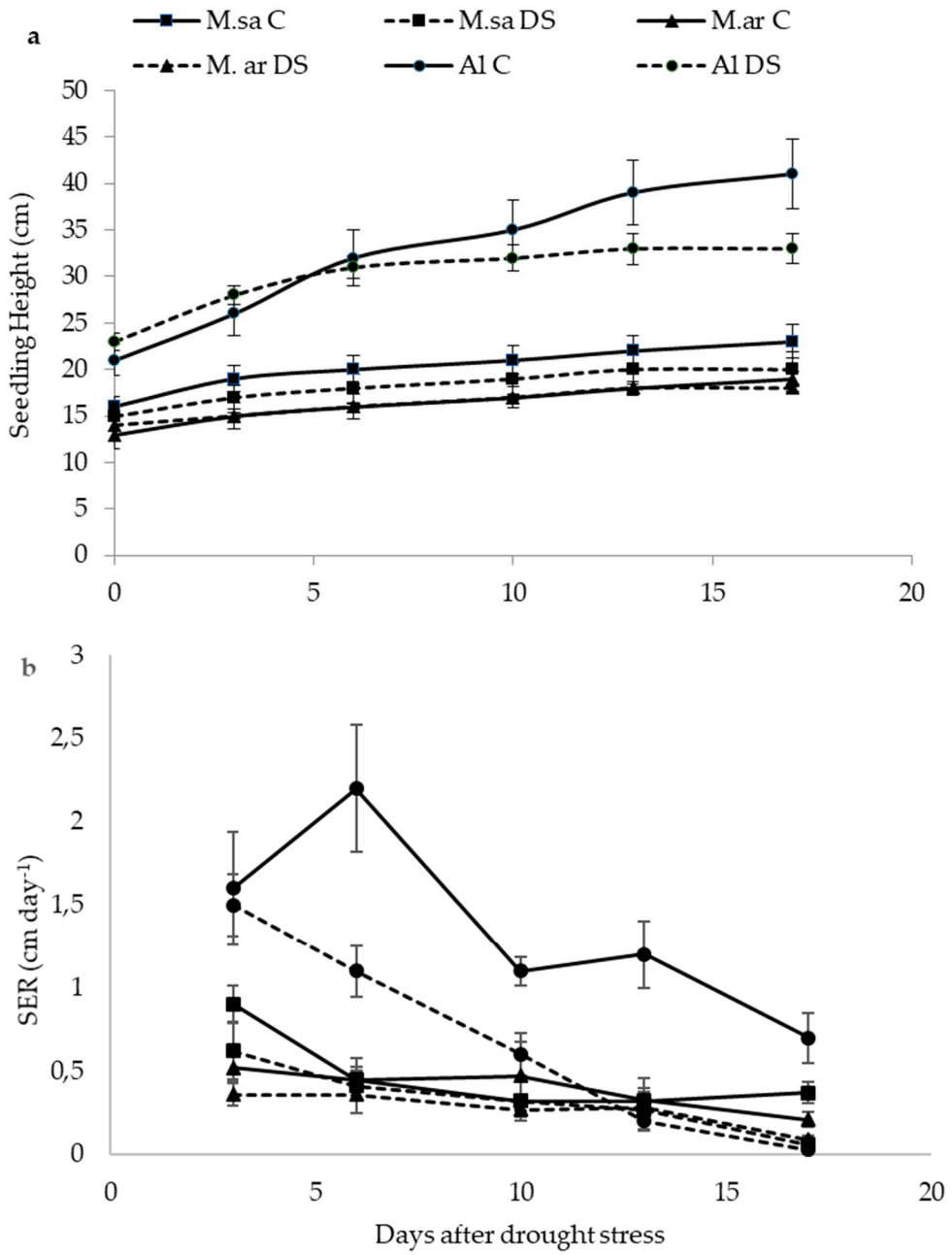

Figure 1. The seedlings height (**a**), and the seedlings Stem Elongation Rate (SER) (**b**) of *M. arborea* (M.ar), *M. sativa* (M.sa) and Alborea (Al) under control (C) and drought stress (DS) during the experimental period.Error bars indicate the mean SE.

3.2. The Effect of Drought Stress on Physiological Parameters

RWC and gas-exchange parameters of net photosynthetic rate (Pn), stomatal conductance (gs) and transpiration rate (E) were significantly reduced under drought stress conditions, while the WUE increased (Table 2). Among the entries, RWC, an indicator of leaf water status was lower in the studied population of Alborea compared to population of *M. sativa* and *M. arborea* (Table 2). The RWC of Alborea's leaves was about 29% lower compared to the control after three weeks of drought stress, while the corresponding reduction of *M. sativa* and *M. arborea* was about 19% and 15% respectively (data not shown). The population of Alborea had the highest Pn under well-watered conditions, while after two weeks of drought stress the Pn of the entries did not significantly differ (Figure 2a). The Pn of Alborea was reduced after 1st week of water deficit but it was still higher than Pn of *M. sativa* and

M. arborea. However, the Pn of *M. sativa* and *M. arborea* was significantly reduced after the 2nd week of drought stress (Figure 2a). On the other hand, the population of *M. arborea* had the lowest gs and E under both well-watered and water-deficit conditions, but the highest WUE (Figure 2b–d). For all the entries, the gs and E was gradually decreased after two weeks of drought stress, while the WUE increased (Figure 2b–d).

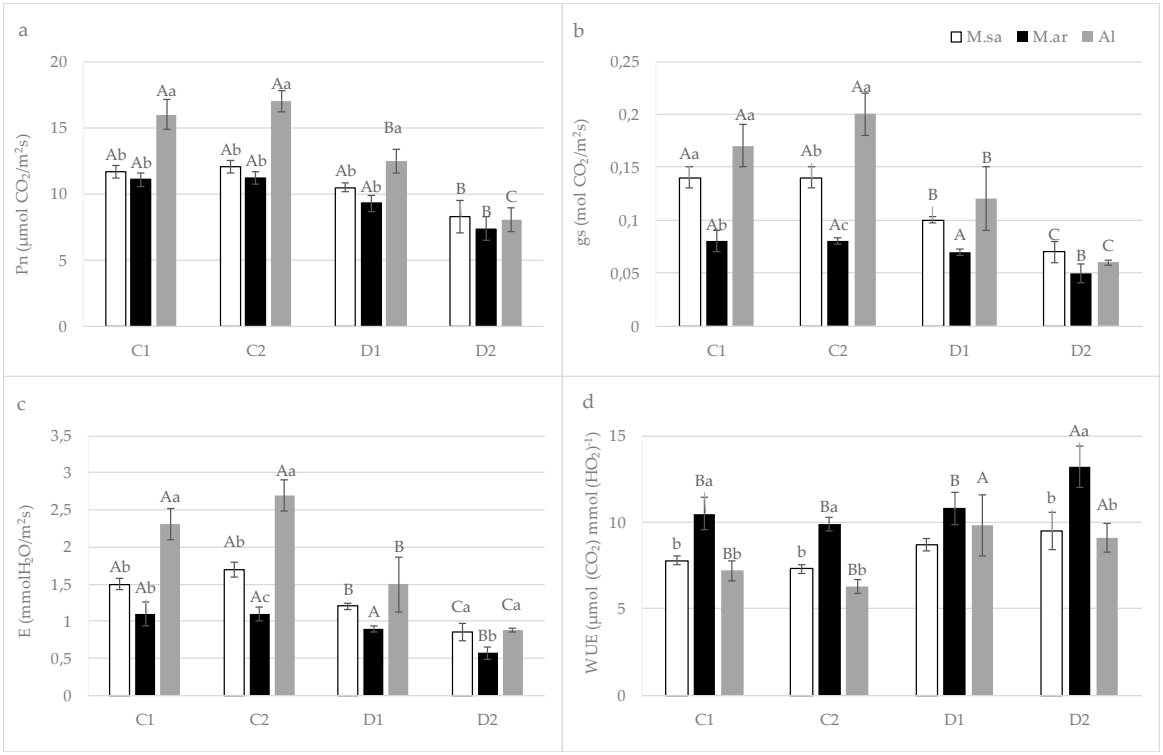

Figure 2. The net photosynthetic rate (Pn) (**a**), stomatal conductance (gs) (**b**), transpiration rate (E) (**c**) and Water Use Efficiency (WUE) (**d**) as the rate Pn/E of *M. arborea* (M.ar), *M. sativa* (M.sa) and Alborea (Al) under control (C) and drought stress (D) after the 1st (C1 and D1) and 2nd (C2 and D2) week of drought. Error bars indicate the mean ± SE of four independent samples. The different capital letters refer to the significant differences between the same entries among the treatments (C & D) at $p < 0.05$ (Tukey test). The different small letters refer to the significant differences between the entries in the same treatment at $p < 0.05$ (Tukey test).

3.3. The Effect of Drought Stress on the Antioxidant Capacity

Antioxidant capacity reflects the ability of plant to reduce harmful intracellular ROS concentration. ROS scavenging capacity determined by ABTS+ (2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) increased 2.1-fold in stressed plants of *M. sativa's* population ($p < 0.05$), with significant correlation between the entries and organs. On the other hand, FRAP assays did not indicate any significant correlation between drought, entries, and organs (Table 3).

SOD activity increased in leaves and roots compared to the control, but with no statistically significant correlation. Peroxidase (POD) activity and protein content increased significantly for all the entries under drought treatment (Table 3) compared to the control, with the population of Alborea exhibiting the highest ratio in POD activity (1.4 fold) and of *M. arborea* the highest protein content (2.3 fold). Significant correlation between organs and entries was obtained in both POD activity and protein content.

Malondialdehyde (MDA) assay revealed that all the entries were prone to lipid peroxidation ($p < 0.05$) under drought stress, with leaves being more susceptible ($p < 0.05$) in comparison with roots.

MDA content ratio shows that the population of *M. arborea* was the least susceptible under stress (Table 3).

Table 3. The ratio of the enzymatic activity of plants under drought stress in relation to the plants under well-watered conditions (mean ± SE).

	ABTS	FRAP	SOD	POD	MDA	Total Proteins
Entries						
M. arborea	1.7 ± 0.33ab *	2.4 ± 0.4	1.1 ± 0.2	0.7 ± 0.20b	1.3 ± 0.31b	2.3 ± 0.2a
M. sativa	2.1 ± 0.18a	2.1 ± 0.2	0.8 ± 0.1	0.5 ± 0.04b	1.6 ± 0.13ab	2.0 ± 0.6a
Alborea	1.2 ± 0.22b	1.9 ± 0.4	1.5 ± 0.1	1.4 ± 0.10a	1.8 ± 0.36a	0.8 ± 0.1b
Organs						
Leaves	1.9 ± 0.23a	2.0 ± 0.3	1.3 ± 0.1	0.8 ± 0.2	2.2 ± 0.2a	1.9 ± 0.4
Roots	1.4 ± 0.21b	2.3 ± 0.3	1.0 ± 0.2	1.2 ± 0.1	0.9 ± 0.1b	1.4 ± 0.3
Source of variation						
Entries (A)	$p < 0.05$	ns	ns	$p < 0.05$	$p < 0.05$	$p < 0.05$
Tissues (B)	***p < 0.05***	***ns***	***ns***	***ns***	***p < 0.05***	***ns***
AXB (Interaction)	ns **	ns	ns	$p < 0.05$	ns	$p < 0.05$

* Means followed by the same letter in the column for the same parameter did not significantly differ ($p > 0.05$) according to Tukey test. ** ns: not significant at 0.05 level. ABTS+ (2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)); (FRAP) Ferric Reducing Antioxidant Power; (SOD) Superoxide Dismutase; (POD) Peroxidase; (MDA) Malondialdehyde.

3.4. The Effect of Drought Stress on Transcriptional Responses

The results of the ANOVA regarding the expression levels of the studied genes are presented in Table 4. Down-regulation of *ZFN* under drought stress was recorded for all the entries in both leaves and roots (Figure 3a,b). Other down-regulated genes under drought stress were *NHX1* (leaves and roots) and bzip (roots) for *M. sativa* and *NHX1* (roots), P5CS1 (leaves and roots) and *MH1* (roots) for Alborea (Figure 3a,b). Generally, the population of *M. arborea* showed the highest accumulation of gene transcripts, followed by that of *M. sativa*, while the population of Alborea showed the lowest. On average, all genes examined were preferentially up-regulated in leaves compared to roots, except for SIMKK followed by *P5CS1* (Figure 3a,b). In the population of *M. arborea*, all genes except for *ZFN* were highly expressed in leaves and high levels of *SIMKK*, *P5CS1*, and *NHX1* transcripts were also detected in roots (Figure 3a,b). Regarding the population of *M. sativa*, it had significantly lower induction levels for all the studied genes in both leaves and roots compared to that of *M. arborea* except the *MH1* in roots (Figure 3a,b). However, the genes that showed profound differences in their induction levels in leaves compared to control were *AP2/EREB*, and *P5CS1*, *bZIP* and *MH1*, whereas *SIMKK* and *MH1* were highly induced in roots. Finally, in Alborea, minor differences in expression levels compared to control were recorded in leaves for the studied genes and only *AP2/EREB* transcripts was highly abundant in roots (Figure 3a,b).

The heat map was based on gene expression levels of the examined genes. All gene expression levels were colored black, dark gray or gray to represent high, moderate, and low expression ratio respectively (Figure 4). As highlighted in the figure, Alborea exhibited the lowest induction of all genes. Furthermore, the most pronounced differences in gene expression between control and drought stressed plants were recorded for *AP2/EREB* gene.

Table 4. The effect of drought stress, species, and tissue on genes expression (mean ± SE)

	AREB	*bZIP*	*P5CS1*	*ZFG*	*NHX*	*MH1*	*SIMKK*	*RCI2A*
Source of variation								
Drought (A)	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$
Entries (B)	$p < 0.05$	$p < 0.05$	$p < 0.05$	ns	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$
Organs (C)	$p < 0.05$	$p < 0.05$	ns *	$p < 0.05$	$p < 0.05$	ns	ns	$p < 0.05$
AXB (Interaction)	$p < 0.05$	$p < 0.05$	$p < 0.05$	ns	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$
AXC (Interaction)	$p < 0.05$	$p < 0.05$	ns	$p < 0.05$	ns	ns	ns	$p < 0.05$
BXC (Interaction)	$p < 0.05$	$p < 0.05$	ns	ns	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$
AXBXC(Interaction)	$p < 0.05$	$p < 0.05$	ns	ns	ns	$p < 0.05$	$p < 0.05$	$p < 0.05$

AP2/EREB:apetala2/ethylene-responsive element binding; NHX1:intracellular Na^+/H^+ antiporters; P5CS1:Δ1-pyrroline-5-carboxylate synthetase 1; ZFN:Zinc Finger; SIMKK:stress-induced mitogen-activated protein kinases kinases; RCI2:rare cold inducible2A; bZIP:basic leucine zipper; **MH1**:Medicago sativa Helicase 1. * n.s not significant.

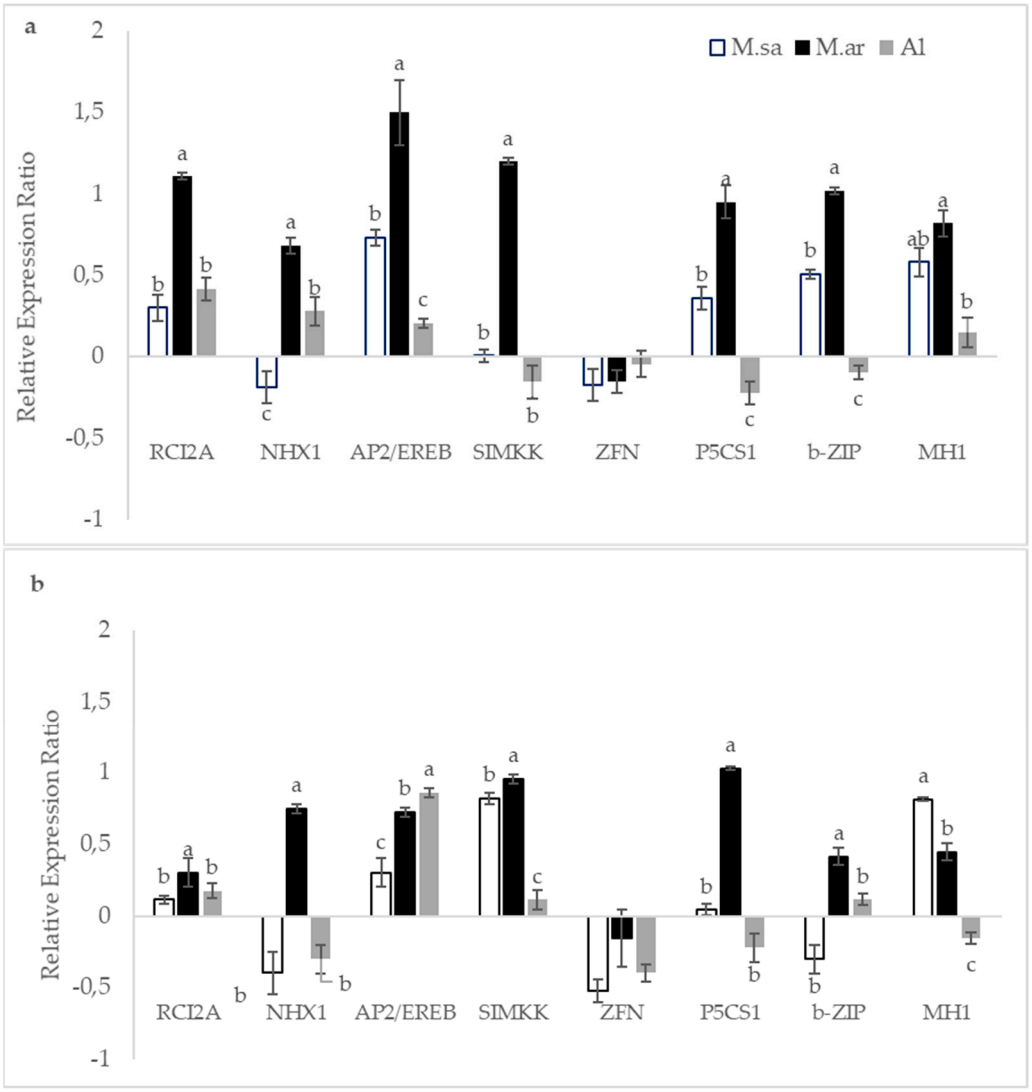

Figure 3. *RCI2A*, *NHX1*, *AP2/EREB*, *SIMKK*, *ZFN*, *P5CS1*, *bzip*, and *MH1* relative expression level for *M. arborea* (M.ar), *M. sativa* (M.sa) and Alborea (Al) under drought stress for leaves (**a**) and roots (**b**). Error bars indicate the mean ± SE. The different small letters refer to the significant differences between the entries in the same treatment at $p < 0.05$ (Tukey test). Relative expression ratios were calculated according to the $2^{-\Delta\Delta Ct}$ method. More specifically to calculate ΔΔCt value, the control sample of each entry (*M. arborea*, *M. sativa* and Alborea) was used. MS-Actin-2 was the endogenous gene used for normalization.

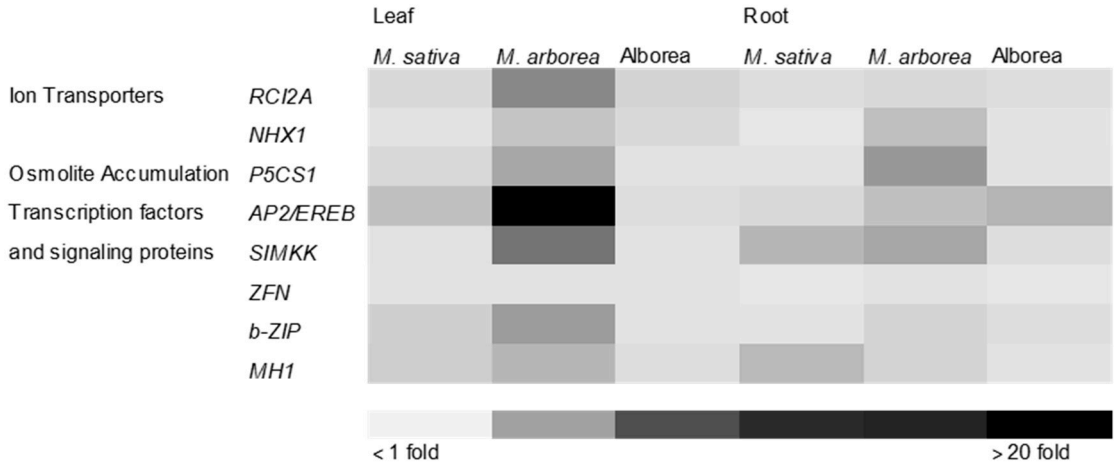

Figure 4. General expression level of *RCI2A*, *NHX1*, *AP2/EREB*, *SIMKK*, *ZFN*, *bZIP*, *MH1* and P5CS1 under drought treatments for *M. arborea*, *M. sativa* and Alborea. Expression levels are black and gray-coded to depict the fold change as follows: black (high expression level >20-fold) to gray (low <1-fold).

4. Discussion

4.1. Seedling Growth and Physiological Responses under Drought Stress

Drought is the major threat for the productivity of fodder crops in the Mediterranean area. The water deficit results in the reduction of seedling growth, which is a critical factor for plant productivity [39]. According to the results of the present study, the seedlings' height and the stem elongation rate of *M. sativa*, *M. arborea* and their hybrid was reduced under drought conditions which is consistent with many other studies [40], including medicagos [41–43]. The inhibition of stem elongation decreases the demand for metabolites and allows the plants to synthesize protective compounds for osmotic adjustment [44].

The hybrid, which had remarkably higher stem elongation rates compared to the parent species under well-watered conditions, exhibited the most dramatic reduction under drought stress. Similar results have been reported by Clifton-Brown and Lewandowski [45] for ***Miscanthus x giganteus***, a sterile hybrid of *M. sacchariflorus* and *M. sinensis* parentage [40], which is highly productive but less drought-tolerant compared to its parent species.

The leaf RWC is considered an essential indicator of water status in plants, signifying the balance between water supply and transpiration rate in leaf tissue [46] and is a meaningful determinant of plants' drought tolerance. In this regard, the studied population of *M. arborea* maintains a better water status than that of *M. sativa* and Alborea, which is critical for its physiological functioning and survival under drought. The decrease in RWC in plants with low tolerance against drought was observed in many species and has been accompanied with plant vigor reduction [47].

Stomatal closure is considered to be an early plant response to drought that allows RWC maintenance [48,49] and consequently reduces photosynthesis [50,51]. In the present study, the most profound decline of gs was observed in the population of Alborea followed by that of *M. sativa* in drought conditions that was simultaneously accompanied with a reduction in Pn. Nevertheless, Alborea had higher Pn than the parental species the first week of drought stress. On the other hand, the population of *M. arborea* that was characterized by a lower Pn and gs under well-watered conditions, preserved similar rates under water-deficit conditions. The lowest reduction of Pn, E and gs found in population of *M. arborea* between control and stressed plants may play a critical role in its better adaptive responses to decreased water supply.

It is well documented that higher WUE species can be better adapted to drought. The increase in WUE under water deficit is generally attributed to the following: a. the water use is reduced more than

biomass production [52] or b. transpiration rate decreased more than net CO_2 assimilation rate [53]. According to our results the higher WUE was recorded for the population of *M. arborea* under drought stress and this could be a consequence of lower decrease of Pn rate compared to the decrease of E rate.

On the other hand, the strategy of Alborea's and *M. sativa's* population under drought was to sharply drop Pn. This can be advantageous under short periods of severe drought stress but could have a negative impact when drought stress is imposed for long periods. Under the latter conditions, the strategy of *M. arborea's* population (gradual reduction in Pn) could be more efficient.

It must be mentioned that the population of Alborea had the highest Pn under well-water conditions and that of *M. arborea* the lowest. In general, high Pn contribute to high growth rates. Nevertheless, the growth rate also depends on nutrient availability and on the genetic potential [54]. Our results confirm the hypothesis that slow growing species such as *M. arborea* that permit conservation of resources (conservative strategy) are better adapted to environmental stresses compared to species (Alborea) that allow rapid acquisition of resources (acquisitive strategy) [55,56].

4.2. Drought Stress and Antioxidant Capacity

One of the plant's first response to environmental stressors (abiotic and/or biotic), is the production of ROS, that functions as a signal to molecules in the complex network of a plant's stress response mechanism [10,57–59]. However, continuous stress can cause an accumulation of ROS at the plasma membrane, and subsequent cell damage. Thus, the generation of ROS requires the up-regulation of antioxidant/detoxifying systems such as SOD and POD [60]. In the present study, no significant increase of SOD activity was observed while a substantial increase was measured for POD for all the entries.

As previously mentioned, accumulation of ROS can cause increase of membrane lipid peroxidation leading to high levels of MDA, which is used as an indicator of oxidative damage [18,61,62]. Moreover, ROS generation inhibits protein synthesis or provokes protein denaturation [63].

Compared to the population of Alborea, the population of *M. arborea* and *M. sativa* exhibited lower levels of lipid peroxidation (MDA content), higher protein content as well as higher non-enzymatic antioxidant capacity. These observations suggest that these species acquire a more efficient protective mechanism against oxidative damage than their hybrid (Alborea). Several reports have demonstrated that tolerant genotypes have a high ability to circumvent drought stress due to lower lipid peroxidation, better antioxidant capacity [64,65] and high protein content [63].

4.3. Drought Stress and Transcriptional Responses

In the present study, we examined the transcriptional profile of eight stress -related genes under drought conditions (*AP2/EREB*, *ZFN*, *bZIP*, *NHX1*, *RCI2A*, *MH1*, *SIMKK* and *P5CS1*). Special attention was given to the role of the aforementioned genes to enhanced antioxidant capacity, protein protection and membrane stability.

The ion transporters *NHX1* and *RCI2A* genes are induced by several environmental factors that decrease the availability of water such as chilling, salt, pathogen attack and obviously water stress [66,67]. They function as membrane stabilizers by controlling the uptake of cations and/or the efflux of anions. They also affect the cellular pH and subsequently, the cellular ROS levels, cell membrane homeostasis and enhanced ROS scavenging [68]. Expression of *RCI2A* in transgenic tomato enhanced cold tolerance by upregulating the activity of peroxidase and ascorbate peroxidase (APX) by conferring better antioxidant capacity, reduced lipid peroxidation, and ultimately reduced membrane damage [68]. Our results show elevated transcript levels of *NHX1* in both leaves and roots and profound up-regulation of *RCI2A* gene in leaves of the studied population of *M. arborea*, which possibly causes enhanced protection against cell damage and preserved cell membrane integrity possibly by better antioxidant capacity.

The *P5CS1* gene encodes a key component in proline biosynthesis and accumulation. Many studies have highlighted the positive correlation of proline accumulation with adaptability of plants in

response to several stresses such as drought and salt stress [69–71]. Greatly higher expression of *P5CS1*, especially in roots, has been detected in the population of *M. arborea* compared to that of *M. sativa* and Alborea. Moreover, a down-regulation of *P5CS1* was monitored in roots of Alboreasubjected to drought stress compared to the control. As several studies have highlighted the role of proline as an osmoprotectant and free-radicals scavenger, it can be assumed that the population of *M. arborea* and to a lesser extent the population of *M. sativa* have a better antioxidant capacity than that of Alborea in order to protect protein integrity and to gain plant tolerance to drought stress [72,73].

Since *bzip* transcription factors are directly activated by the ABA-dependent pathway, in many cases they improve plant tolerance to both salt and drought stresses [74,75]. The same studies reported that usually *bzip* transcription factors form complexes with other transcription factors (such as *Myb* or *AP2/EREB*), acting together to activate ABA-responsive genes or to bind to promoters of other defense related genes (i.e., *NHX1*). Additionally, both *AP2/EREB* transcription factors and ZFNs are involved in many aspects of abiotic and biotic stress responses [76–78]. The population of *M. arborea* exhibited the highest induction of *bzip* transcription factor in both leaves and roots indicating that it is involved in its drought tolerance. On the other hand, the high induction of an *AP2/EREB* transcription factor belonging to the AINTEGUMENTA subgroup [24] highlights its importance regarding the response of the studied populations to drought stress. It is noteworthy that *AINTEGUMENTA* gene was the only gene that was highly expressed in roots of Alborea's population. Interestingly, the transcript levels of *ZFN* were less abundant in the stressed plants in comparison to controls. These findings agree with only one report that correlated the down-regulation of a ZFN protein (namely DST- Drought and Salt Tolerance) with the better drought and salt tolerance thought regulation of H_2O_2 homeostasis [79].

The involvement of mitogen-activated protein kinases (MAPK) in plants adaptation to salt and drought has been highlighted for a plethora of plant species including rice, *Arabidopsis* and *M. sativa* [80–82]. In many instances they play an important role in abiotic oxidative stress responses and ROS metabolism [83]. Consistent with previous results, drought stress led to higher expression levels of *SIMKK* gene (*stress-induced MAPK*) in both leaves and roots of the studied population of *M. arborea* in both leaves and roots and of *M. sativa* in roots, indicating that it might regulate the antioxidant defense system of both species in response to stress signaling.

The adaptive strategy of plants stress tolerance has implicated the involvement of helicases in many cases [26]. Shivakumara et al. [84] demonstrated that even a single *helicase* gene can impart tolerance to multiple stresses in chili plants (*Capsicum annuum*) via reduction of photo-oxidative damage and reduced production of ROS and by enhancing or stabilizing protein synthesis. In the present study, MH1 was the only gene that its induction under drought stress was higher in roots of *M. sativa's* population compared to that of *M. arborea*, while it was similar in leaves of the two species. This further supports the *MH1* involvement in drought tolerance possible by rendering a better protection against ROS production.

5. Conclusions

As mentioned by several researchers, hybrid vigor greatly depends on its increased photosynthetic efficiency [85–87]. Moreover, the increased growth of hybrids and their tolerance to stressful environments act in an antagonistic manner. This is the case with the hybrid in the present study as well. When drought occurs, the hybrid suffers high water losses that cannot be compensated even by early stomatal closure. After stomatal closure, Alborea seems to be unable to synthesize energetically rich compounds that are essential for biosynthetic processes. On the other hand, *M. arborea*, which has lower Pn in well-watered and stressful conditions, does not have to limit its metabolic processes, thus it can increase the efficiency of protective systems. Several other studies have indicated that hybrids with increased drought tolerance also exhibited lesser changes of Pn and gs in water-deficit conditions compared to drought-sensitive hybrids and up-regulated ROS scavengers [65,88].

Moreover, genes that could possibly lead to higher antioxidant capacity, membrane integrity and protein stability such as *RCI2A*, *MH1*, *NHX1*, *P5CS1*, *SIMKK*, were also up-regulated in *M. arborea*

and to a lesser extent in *M. sativa*. Both parents showed better antioxidant capacity than Alborea thus they perform better than Alborea under drought conditions. Among the studied genes an *AP2/EREB* transcription factor belonging to the AINTEGUMENTA subgroup seems to be key player for the response of the studied populations to drought stress. Similar results were obtained from the study of the same population of the species under salt shock and stress [24]. Further research on the contribution of *AINTEGUMENTA* gene to abiotic tolerance is needed as there are only a few studies regarding its role to both salt and drought tolerance.

According to the results, it seems that the parental species and especially *M. arborea* are more tolerant that their hybrid to drought. The fact that Alborea is a high yielding hybrid, with high photosynthetic rates and high biomass, probably affects its drought sensitivity. Consequently, the ability to offer some yield protection in the high yielding hybrid Alborea in higher stress environments yet not sacrifice yield potential in low stress/high yield potential environments should be further explored by improving traits such as stability of leaf gas-exchange parameters and antioxidant capacity.

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