

Article

Effects of Water Stress on Gas Exchange, Water Relations and Leaf Structure in Two Ornamental Shrubs in the Mediterranean Area

Alessandro Tribulato ¹, Stefania Toscano ^{1,*} , Virginia Di Lorenzo ¹ and Daniela Romano ^{1,2} 

¹ Department of Agriculture, Food and Environment, Università degli Studi di Catania, Via Valdisavioia 5, 95123 Catania, Italy

² CNR—Trees and Timber Institute (IVALSA), via P. Gaifami 18, 95126 Catania, Italy

* Correspondence: stefania.toscano@unict.it; Tel.: +39-0954-7833-03

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Abstract: One of the main problems in the Mediterranean area is the long dry season, and hence there is a need to individuate plants that are tolerant to low water availability. The mechanisms adopted by different plant species to overcome drought stress conditions and reduce water loss could allow the identification of tolerant species to drought stress, thereby increasing the sustainability of ornamental plant utilization in green areas. In this regard, the aim of this study was to investigate the morphological, physiological, and anatomical responses of *Polygala myrtifolia* L. and *Viburnum tinus* L. ‘Lucidum’ irrigated under different irrigation deficits. In pot plants, four water regimes were adopted (10%, 20%, 30% and 40% of water container capacity (WC)). Drought stress significantly reduced the biomass accumulation in both shrubs. In *Viburnum*, total dry biomass reduction was observed only in 10% WC with a reduction by 33%, while in *Polygala*, this was observed both in 20% WC and 10% WC (~48%). The higher deficit irrigation conditions improve the root-to-shoot ratio, which was increased in *Polygala* 20% WC (by 20%) but not in *Viburnum* ones. The latter species shows higher drought tolerance, as demonstrated by the gas exchange values, chlorophyll fluorescence, leaf structure, and water relationship.

Keywords: *Polygala myrtifolia* L.; *Viburnum tinus* L. ‘Lucidum’; leaf anatomy; chlorophyll a fluorescence; leaf water content; water use efficiency

1. Introduction

The use of ornamental plants to develop green areas can be a response to some problems linked to urbanization (i.e., pollution, heat island, etc.), but their use traditionally needs a large amount of water for irrigation. To realize an environmentally friendly landscape, it is necessary to save water, partly by making suitable plant choices.

In the Mediterranean regions, water deficit is one of the main problems for ornamental plant use, and global changes will predictably amplify the present issues, especially in urban areas [1]. In this context, identifying plants that are tolerant to water shortages has attracted considerable interest.

The lack of water is a great problem in the Mediterranean region, characterized by warm summers with little rainfall and, as a consequence, long periods of water unavailability [2], which leads to water stress in plant species. Over time, drought stress, which is frequent in Mediterranean areas, selects species which are particularly suitable to overcoming water deficit [3]; the selective pressure of the Mediterranean climate has modified the plant traits of different regions leading to vegetation communities dominated by sclerophyllous, evergreen shrubs [4].

Traditionally, for ornamental purposes, exotic species have been adopted in Mediterranean gardening, while over recent years, research has been focused on the study of the response of

Mediterranean native shrub species to water stress [5], also allowing the identification of new ornamentals suitable for sustainable landscaping. The ability of Mediterranean shrubs to tolerate severe drought conditions, one of the main factors affecting plant survival and species distribution, increases their use in the revegetation of semiarid areas [6].

Different species growing in Mediterranean areas could provide good candidates for ornamental plants regarding their tolerance to water shortages. Drought stress harms aesthetic aspects and consequently affects ornamental plant quality. The latter in fact mainly depends on visual appearance, identified as leaf size and color, flower characteristics, number and longevity [7]. Drought stress can also dramatically reduce plant survival after transplantation with high economic losses and impacts on the aesthetic quality of green areas [8].

The successful establishment of ornamental green areas in Mediterranean regions can be achieved through the adequate selection of plant species characterized by a high tolerance to water stress [9]. Tolerant plants are those plants that have the ability to modify their metabolism to guarantee vital and essential physiological processes with a limited amount of water [10]; most of them are Mediterranean-native species.

Drought-tolerant plants are able to modify, during water stress conditions, their morphology and to activate physiological and biochemical processes able to cope with water deficit [11,12]. The morphological modifications linked to drought stress reduce shoot and leaf growth, although the modality and the intensity of these phenomena depend on the genotypes, even within the same species [12]. Previous results showed that in *Lantana camara* L., the differences in total leaf area are linked to the leaf number, while in *Ligustrum lucidum* W.T. Aiton, they are related only to the leaf sizes [13]. With the avoidance mechanisms to limit water losses, plants reduce the canopy area and hence increase their root/shoot ratio. The response to drought stress also involves anatomical changes: in severe drought stress conditions, *Lantana* plants increased their spongy tissue [13] to facilitate CO₂ diffusion, enhancing the competition among cells for CO₂ and light [14].

The reduction of the specific leaf area results in an increasing of the water use efficiency (WUE) [12]; the higher content of chlorophylls and proteins in thicker leaves, the greater photosynthetic capacities per unit leaf area [15].

Water stress, indeed, reduces plant growth and development by decreasing respiration, growth, photosynthesis, assimilate partitioning, moisture, and nutrient relationships [16–18].

Water shortage typically increases stomatal closure in plants, hence reducing gas exchanges and decreasing photosynthetic activity [19]; in some species water unavailability determines smaller stomata formation, able to have faster dynamic characteristics [20]. Under severe drought stress conditions, the decrease of photosynthetic activity often results in plant death [21]. Stomatal closure reduces water loss and also nutrient uptake; therefore, the metabolic pathways are altered and growth is reduced. Some Mediterranean plants, such as *Pistacia lentiscus* L., when subjected to drought stress, are able to limit water losses through an efficient transpiration control [10].

The efficiency of the PSII photosystem and indirectly the physiological state of the plants can be measured through the measurement of chlorophyll *a* fluorescence and in particular of the Fv/Fm ratio [22]. According to different studies, with values between 0.78 and 0.85, the plant is not stressed [23]; values below 0.78 indicate damage to the PSII photosystem [22].

The analysis of the mechanisms adopted by different species to overcome drought stress and reduce water loss could allow the identification of the most tolerant species to be used in arid and semi-arid environments, thus increasing the sustainability of ornamental plant maintenance.

In this context, the purpose of this study was to investigate morphological, physiological, and anatomical responses in *Polygala myrtifolia* L. and *Viburnum tinus* L. ‘Lucidum’ irrigated at different irrigation deficits.

Polygala myrtifolia L., the common name of which is Milkwort, is an erect, much-branched evergreen shrub, of 1–2.5 m in height, with glossy green leaves, native to South Africa [24]. Due to its attractive shape and prolonged blooming, from spring to autumn, the plant is widely used as a pot

plant and in gardens in regions with a mild climate, because it is sensitive to temperatures below 5 °C. The species is considered to be drought tolerant [25].

Viburnum tinus L., the common name of which Laurustinus, is a typical Mediterranean species [26]. Within this species, the cultivar ‘Lucidum’, characterized by wider and bright leaves, is quite spread in ornamental green areas. Although for some authors it is a different species (*Viburnum. lucidum* Mill.) or a botanical variety (*Viburnum. tinus* var. *lucidum* (Mill.) Aiton), according to The Plant List website (<http://www.theplantlist.org/>), it seems that both terms are referred to *Viburnum. tinus* L. ‘Lucidum’. It is a much-branched evergreen shrub that is 3.5 m in height, with a rounded form and a very dense habitat. The leaves are from narrow-ovate to ovate oblong, shiny dark green; flowering occurs during the winter [24]. It is a Mediterranean sclerophyllous plant with a broad distribution range [27]. The species is considered as a low water-use plant [28,29] and is able to grow in regions with mild winters [30].

Both species are widespread in the Mediterranean area of Italy, in gardens and green areas, because of their ornamental value and adaptability to many environmental conditions. Different studies were carried out on *Viburnum* to investigate on salt stress response [28,31], but little information is available in terms of growth parameters, photosynthetic efficiency, leaf functional anatomical traits in drought stress conditions.

These two species were chosen on the basis of our previous studies [32]; *Polygala myrtifolia* L. and *Viburnum tinus* L. ‘Lucidum’ were found, respectively, to be moderately sensitive and sensitive to salinity. Since salinity and drought stresses share important adaptation strategies, this work was carried out to verify if unavailability of water activates the same adaptation strategy observed under salinity stress. Unfortunately, in Mediterranean areas, drought is associated with salinity, especially in the coastal areas. If the strategies adopted by plants are the same or similar, it is possible individuate genotypes able to cope together water and salt stress thus improving the compatibility of green areas to Mediterranean environments.

2. Materials and Methods

2.1. Experimental Conditions and Plant Materials

The experiment was carried out on two ornamental shrubs, *Polygala myrtifolia* L. and *Viburnum tinus* L. ‘Lucidum’, grown in a cold greenhouse in Catania (South Italy, 37°30′ N 15°06′ E; 20 m above sea level (m.a.s.l.)). Three-month-old rooted cuttings of both species were transplanted into 2.7 L pots (one plant per pot), filled with sand, silt and clay (75%, 18% and 7%, respectively), and fertilized with 2 g L⁻¹ of Osmocote Plus (14/13/13, N, P, K + microelements). The substrate contained 120.0 g/kg organic matter, 102.7 mg/kg hydrolyzed nitrogen, 116 mg/kg available P and 359 mg/kg available K. The substrates had a pH of 5.9 and EC of 2.2 dS/m.

At the beginning of the experiment, the dry biomass of the plants was on average 53.7 ± 2.3 g and 109.5 ± 10.2 g for *Polygala. myrtifolia* L. and *Viburnum. tinus* L. ‘Lucidum’, respectively.

Three replicates of 12 plants per treatment (144 plants in total per species) were adopted.

2.2. Substrate Moisture Measurements and Water Supply

To determine the volumetric content of water in the substrate, an automated management system with dielectric sensors EC 5TE (Decagon Devices Inc., Pullman, WA) was used. Two sensors were used for each treatment and data were recorded using a data acquisition system (data logger) CR1000 (Campbell Scientific Ltd., Loughborough, UK).

Sensors were calibrated following the Starr and Paltineanu [33] protocol, with slight modifications, where a series of each measurements is taken in connection with samples of volumetric soil to quantify the relationship between the substrate (measured with the 5TM) and the volumetric water content (WC). The sensors were installed 10 cm below the substrate surface at the center of each sample pot. Measurements were regularly taken during the experiment, every 5 min, from 10 July to 20 October.

The use of the probes was preceded by their calibration to determine the real content of water in the substrate samples, which were placed in a thermo-ventilated oven at 70 °C until the constant weight (W cal.) reached ($R^2 = 0.9674$). The irrigation, scheduled twice a day (at 7.00 am and at 6.00 pm), was activated when the water content dropped below the pre-set threshold values of 10% WC, 20% WC, 30% WC and 40% WC (control) of the volume of the substrate. The same threshold values were adopted to determine irrigation interruption. The electrical conductivity of the water was 0.9 dS m⁻¹. The experiment lasted 100 days. Figure 1 shows the trend of water volumetric content (%) in the different treatments during the trial.

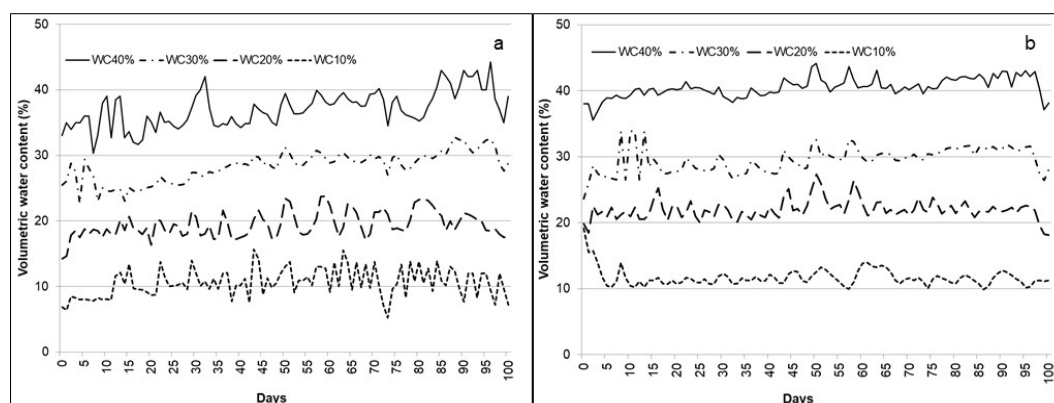


Figure 1. Volumetric water content (%) in Polygala (a) and in Viburnum (b) during the trial.

2.3. Meteorological Data

The mean air temperature, relative humidity and global radiation during the experiment were measured by a data logger CR1000 (Campbell Scientific Ltd., Loughborough, UK). Average temperatures ranged between 20 °C and 30 °C, while the relative humidity (RH) was between 44% and 89% (Figure 2). The mean photosynthetic active radiation (PAR) ranged from 10 MJ m⁻² and 15 MJ m⁻² (data not shown).

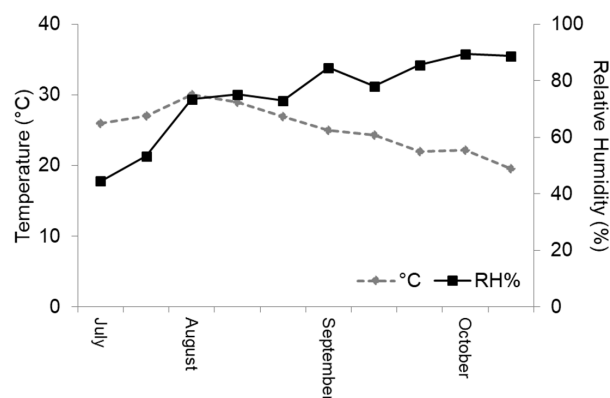


Figure 2. Mean temperature (°C) and relative humidity (%) during the trial.

2.4. Biomass, Leaf Area and SPAD Index

At the end of the experiment, 12 plants per treatment (four per replicate) were separated into stems, leaves and roots. The dry matter (DM) of the different sections was obtained by putting weighed samples in a thermo-ventilated oven at 70 °C until they reached a constant weight. The specific leaf area (SLA) was determined in the same plants used to determine the dry biomass by the ratio between leaf area and leaf dry biomass. Total leaf number was measured and the unit leaf area was determined as the ratio of the total leaf area to total leaf number. The leaf area was determined with an area meter (Delta-T Devices Ltd, Cambridge, UK). SPAD index was measured on twenty fully expanded leaves by means of a portable chlorophyll meter SPAD-502 (Minolta Camera Co., Osaka, Japan).

2.5. Leaf Anatomy

At the end of the experiment, in order to study the anatomy of leaves in relation to the water treatments, 12 mature ones were removed from the plants. Six longitudinal section per treatment were embedded in a cold-polymerizing historesin (Tecnovit 7100, Heraeus Kulzer GmbH & CO. KG, Germany). With the use of a microtome (Leica Microsystems GmbH, Wetzlar, Germany), samples were sliced into four to six μm thick sections and placed on slides for further microscopy analysis using an image analyzer (Leitz mod. ASM 68K, Hexagon Metrology GmbH, Wetzlar, Germany). The abaxial side of leaf sections was observed in order to study the stomata density using the silicon leaf impression method. Both, *Polygala* and *Viburnum* were hypostomatic. With the use of a $\times 400$ microscope both number and size of the stomata was determined on four different fields of vision of separate impressions of the lamina [13].

2.6. Plant Physiological Measurements

2.6.1. Leaf Gas Exchanges and Chlorophyll a Fluorescence

Midday gas exchange measurements were determined during the experimental period, every 25 days, between 10:00 and 14:00. On six leaves from each treatment, the assimilation rate (A_N), the stomatal conductance (g_s) and instantaneous water use efficiency (WUE) were determined with a portable IRGA (LCi, ADC Bioscientific Ltd., Hoddesdon, UK). After measuring photosynthesis, in the same leaves, the quantum yield of PSII was estimated with the modulated chlorophyll fluorimeter OS1-FL (Opti-Sciences Corporation, Tyngsboro, MA, USA). Each leaf was dark-adapted using cuvette clips (Opti-Sciences Corporation, Tyngsboro, MA, USA) for 20 min.

The saturation of the photosystem was performed using a filtered 35 W halogen lamp (350 to 690 nm). The Fv/Fm ratio, used to express the chlorophyll a fluorescence, was calculated according to Schreiber et al. [34].

2.6.2. Leaf Water Content

Every 25 days, the predawn (ψ_{pd}) and midday (ψ_{md}) leaf water potential was determined in six plants per treatment. The method described by Scholander et al. [35] was adopted to estimate the leaf water potential, using a pressure chamber (PMS 600, PMS Instruments co., Corvallis, OR, USA).

2.7. Statistical Analysis

The experiment was conducted as a randomized complete block design with three replicates. The statistical analyses were carried out using CoStat version 6.311 (CoHortSoftware, Monterey, CA, USA), and two-way ANOVA was used. The differences between the means were determined using Tukey's test ($p < 0.05$). The interactions, when significant, are presented separately in the figures. Results of physiological measurements are shown as means \pm SE. Differences among treatments at each date were analyzed by one-way ANOVA at a significance level of $p < 0.05$ according to Tukey's test. Principal component analysis (PCA) was performed over the correlation matrix among 20 selected variables using Minitab 16, LLC. The chosen variables were able to effectively indicate the stress situation. The plot of PC1 (43.6%) vs. PC2 (33.5%) is presented.

3. Results

3.1. Biomass and Leaf Area

Biomass accumulation was reduced in both ornamental shrubs due to the different irrigation treatments (Table 1, Figure 3). Total dry biomass in *Polygala* was reduced in 20% WC and 10% WC (by $\sim 48\%$), whereas in *Viburnum*, the reduction was only in 10% WC by $\sim 33\%$ compared to the other treatments (Figure 3a).

Table 1. Effects of species and water supply treatments on total, shoot and root dry biomass, root/shoot ratio (R/S), number of leaves, total and unit leaf area, specific leaf area (SLA) and SPAD index of potted Polygala and Viburnum plants. Four water supply treatments were considered: control irrigation (40% WC); light deficit irrigation (30% WC); medium deficit irrigation (20% WC) and severe deficit irrigation (10% WC).

Species	Treatments	Total Dry Biomass (g plant ⁻¹)	Shoot Dry Biomass (g plant ⁻¹)	Root Dry Biomass (g plant ⁻¹)	R/S Ratio (g g ⁻¹)	Leaf (n. plant ⁻¹)	Total Leaf Area (cm ² plant ⁻¹)	Unit Leaf Area (cm ² plant ⁻¹)	SLA (cm ² g ⁻¹)	SPAD Index
Polygala		146.7 ± 8.6 ^b	110.8 ± 9.6	35.9 ± 5.1 ^b	0.31 ± 0.0 ^b	4443.6 ± 507.9 ^a	7304.6 ± 779.9 ^a	1.7 ± 0.1 ^b	99.8 ± 3.1 ^a	53.7 ± 0.9 ^b
Viburnum		167.2 ± 9.5 ^a	109.4 ± 6.8	57.9 ± 4.2 ^a	0.54 ± 0.0 ^a	117.8 ± 14.5 ^b	3408.7 ± 393.9 ^b	29.5 ± 0.7 ^a	56.3 ± 2.3 ^b	73.8 ± 1.5 ^a
	40% WC	192.2 ± 5.8 ^a	136.4 ± 2.7 ^a	55.8 ± 6.2 ^a	0.41 ± 0.0	3170.0 ± 578.7 ^a	7488.9 ± 1131.1 ^a	15.3 ± 6.1	88.1 ± 5.7 ^a	60.4 ± 2.4 ^b
	30% WC	193.9 ± 4.9 ^a	135.3 ± 5.3 ^a	58.6 ± 3.0 ^a	0.44 ± 0.0	2949.8 ± 670.9 ^a	6952.7 ± 1214.2 ^a	15.4 ± 6.1	82.0 ± 4.5 ^{ab}	62.7 ± 2.6 ^b
	20% WC	134.0 ± 12.9 ^b	92.4 ± 3.4 ^b	43.6 ± 4.8 ^{ab}	0.46 ± 0.1	1713.9 ± 533.4 ^b	3971.9 ± 680.2 ^b	15.3 ± 6.2	74.8 ± 4.2 ^b	63.3 ± 2.6 ^b
	10% WC	105.6 ± 8.0 ^c	76.2 ± 2.3 ^c	29.5 ± 6.2 ^b	0.32 ± 0.1	1289.0 ± 560.6 ^b	3013.3 ± 527.4 ^c	16.4 ± 6.6	67.0 ± 3.7 ^c	68.6 ± 1.1 ^a
Significance ¹										
Species (S)		**	ns	***	***	***	***	***	***	***
Treatments (T)		***	***	***	ns	***	***	ns	***	**
S × T		**	*	*	**	***	***	ns	ns	ns

¹ The statistical analysis was performed via two-way ANOVA; n.s. not significant; * significant at $p < 0.05$; ** significant at $p < 0.01$; *** significant at $p < 0.001$. Values in the same column followed by the same letter are not significantly different at $p < 0.05$ (Tukey's test).

The reduction of shoot dry biomass in *Polygala* had a similar trend for the total dry biomass (by ~44%), while in *Viburnum*, the shoot dry biomass decreased in 20% WC and 10% WC (by ~33%) compared to the control and 30% WC (Figure 3b). Root dry biomass was only modified in *Polygala* with a reduction of 63% in 20% WC and 10% WC compared to the control and 30% WC; *Viburnum* plants, considering this parameter, were not affected by the drought stress (Figure 3c).

The root-to-shoot ratio increased in *Polygala* plants grown under higher deficit irrigation conditions (10% WC) (Figure 3d).

At the end of the trial, with the intensification of water stress (20% WC and 10% WC), a reduction in the leaf number and area was observed. In particular, a reduction in leaf number by 50% and 54% in the 20% WC treatment was observed in *Polygala* and *Viburnum*, respectively (Figure 3e). The reduction in total leaf area was similar to the reduction of leaf area with a decrease of about 50% in *Polygala* and by 53% in *Viburnum* (Figure 3f). The unit leaf area was significantly affected only by species (Table 1), while the SLA values was influenced by species and water treatments; in both species a significant reduction in 20% WC and 10% WC of 17% was observed (Table 1).

The SPAD index was influenced by species and water treatments; in particular the severe deficit irrigation (10% WC) showing on average an increase of 10% on both species (Table 1).

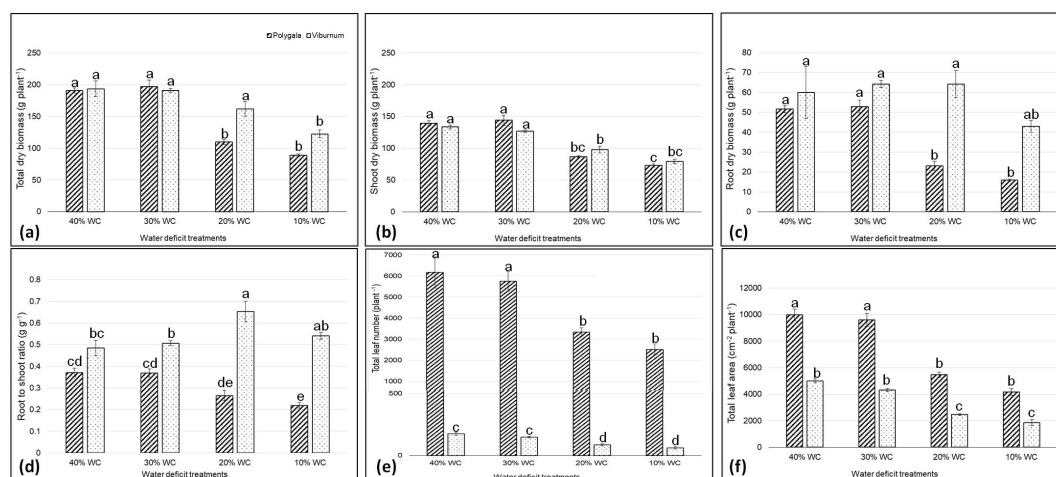


Figure 3. Interactions between species (*Polygala* and *Viburnum*) and water deficit treatments (40% WC, 30% WC, 20% WC and 10% WC) on total and shoot dry biomass (a,b), root dry biomass (c), root to shoot ratio (d), total leaf number (e) and total leaf area (f). Columns with the same letters are not significantly different, as determined by Tukey's test ($p < 0.05$).

3.2. Leaf Anatomy

In both species, water deficit treatments induced a modification of leaf total thickness (Table 2, Figure 4a). In *Polygala*, a reduction by ~25% in 30% WC compared to plants irrigated at 40% WC, 20% WC and 10% WC was observed. Plants of *Viburnum* subjected to drought stress treatment (30% WC, 20% WC and 10% WC) showed an increase of 21%, which significantly differed from the control plants (Figure 4a).

In *Polygala*, no significant modification was observed in palisade parenchyma, while, on the other hand, *Viburnum* showed an increase in 20% WC of 46% (Figure 4b).

In *Polygala*, no significant modifications were observed in spongy parenchyma; in *Viburnum*, 20% WC showed an increase by 27% (Figure 4c).

Stomata density showed significant differences among the irrigation treatments only in *Viburnum*, with the highest density in the control (40% WC) and the lowest in the more severe water stress treatments (Figure 4d). The water deficit treatments affected the stomata size only in *Viburnum* plants. The latter, in fact, showed a modification in the severe water treatments (10% WC) with a significant reduction by 16% (Figure 4e).

Table 2. Effects of species and irrigation treatments on total number, palisade, spongy thickness (μm), stomatal density (n/mm^2) and size (μm) of leaves of Polygala and Viburnum plants. Two-way ANOVA was used and the differences between the means were determined using Tukey's test ($p \leq 0.05$).

Species	Treatments	Thickness (μm)			Stomata	
		Total	Palisade	Spongy	Density (n/mm^2)	Size (μm)
Polygala		284.7 ± 10.0^b	69.9 ± 2.5^b	170.3 ± 6.2^b	175.8 ± 2.5^b	2.9 ± 0.0^b
Viburnum		432.4 ± 14.8^a	146.1 ± 11.3^a	228.7 ± 9.9^a	204.0 ± 5.6^a	2.6 ± 0.1^a
	40% WC	331.6 ± 15.6^b	81.0 ± 8.2^c	203.2 ± 15.7	199.5 ± 9.4^{ab}	2.8 ± 0.1^a
	30% WC	331.5 ± 41.7^b	103.4 ± 21.0^b	184.2 ± 20.1	172.5 ± 4.8^c	2.9 ± 0.1^a
	20% WC	384.5 ± 45.7^a	117.8 ± 20.7^{ab}	211.1 ± 22.1	185.3 ± 3.0^{bc}	2.9 ± 0.0^a
	10% WC	386.7 ± 33.3^a	129.8 ± 23.6^a	199.4 ± 8.2	202.3 ± 7.0^a	2.5 ± 0.1^b
Significance [†]						
Species (S)		***	***	ns	***	***
Treatments (T)		***	***	***	***	***
S \times T [†]		***	***	*	***	***

[†] Values are means for the main effects of species (S) and irrigation treatment (T). ns—not significant; * significant at $p \leq 0.05$; ** significant at $p \leq 0.01$; *** significant at $p \leq 0.001$. The values in the same column followed by the same letter are not significantly different at $p \leq 0.05$ (Tukey's test).

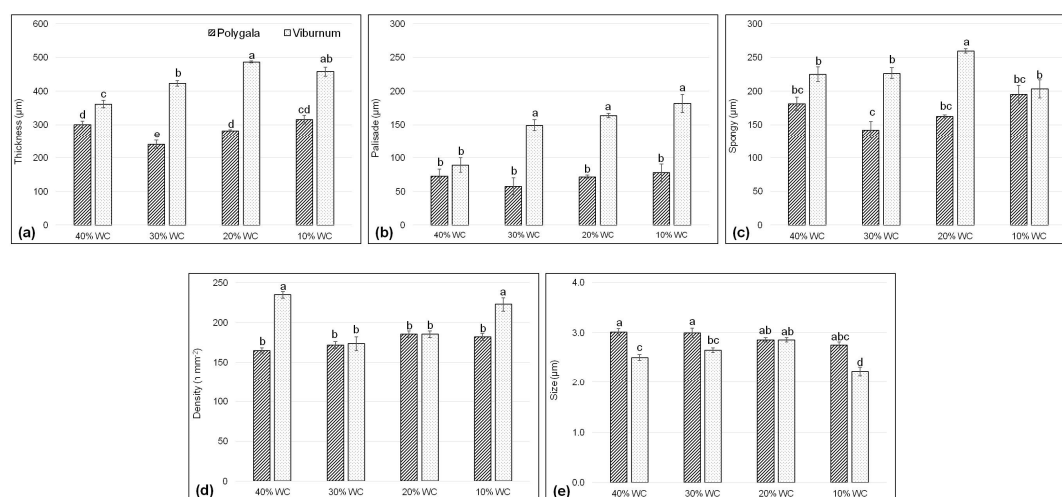


Figure 4. Interactions between species (Polygala and Viburnum) and water deficit treatments (40% WC, 30% WC, 20% WC and 10% WC) on thickness (a), palisade (b), spongy (c), stomata density (d) and size (e). Columns with the same letters are not significantly different, as determined by Tukey's test ($p < 0.05$).

3.3. Plant Physiological Parameters

Significant effects of water stress treatments in both species for net photosynthesis and leaf conductance were observed (Figure 5).

Since the first measurement, Polygala plants grown under severe water deficit (10% WC) showed a reduction of 80% in A_N compared to the control; a similar decrease was detected at the end of the trial in 20% WC plants. However, differences compared to the control occurred only after 50 days after the start of the experiment. The 30% WC plants did not show any significant differences as compared to control plants for the entire experimental time.

In Viburnum plants, after 25 days of water deficit, the decrease of net photosynthesis was significant for the more stressed thesis (20% WC and 10% WC) and remained thereafter significant for the entire experimental period. Indeed, at the end of the trial, compared to the control, a reduction by 66% and 86% in 20% WC and 10% WC, respectively, was observed. In 30% WC Viburnum plants, a similar behavior was observed to that of control plants (Figure 5).

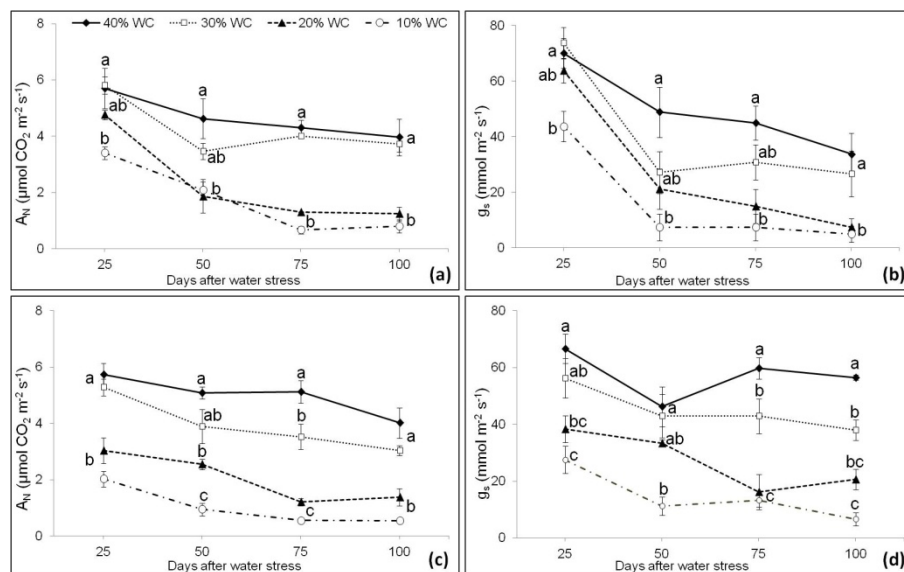


Figure 5. Net photosynthesis (A_N) and leaf conductance (g_s) in Polygala (a,b) and Viburnum (c,d) during the experiment. Plants were irrigated to container capacity (40% WC) or subjected to water stress treatments (30% WC, 20% WC and 10% WC). Mean values \pm S.E. ($n = 6$). An absence of bars denotes a size smaller than the symbol. Different letters indicate significant differences between treatments at each sampling time as determined by Tukey's test ($p < 0.05$).

Similar results were detected for both species regarding leaf conductance (Figure 5).

In the two shrubs, a reduction in photosynthesis was linked to stomatal closure. A positive correlation was found between the photosynthetic rate and stomatal conductance of the two species during the experimental period with R^2 values of 0.8849 in Polygala and 0.9415 in Viburnum (Figure 6).

In Figure S1, trends of the transpiration rate and sub-stomatal CO_2 are shown. Since the first measurement, Polygala plants grown under more stressed treatments (20% WC and 10% WC) showed a reduction by 47% in E compared to the control and 30% WC; a similar decrease was detected on the 75th day, while at the end of the trial, the treatments did not show significant differences (Figure S1a). In Viburnum plants, the decrease of E was significant for the more stressed thesis (20% WC and 10% WC) and remained thereafter significant for the entire experimental period. (Figure S1c). In Polygala, water use efficiency (WUE) did not show any significant differences among the control and the other treatments; only 20% WC, 75 days after starting the experiment, showed lower values (Figure S1b). Viburnum 10% WC showed an increase by 40% after 75 days.

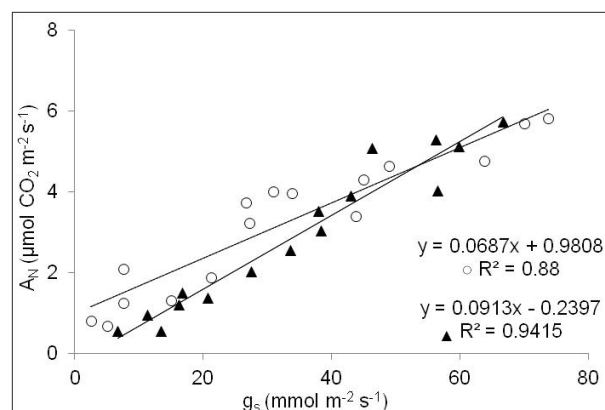


Figure 6. Correlation between net photosynthesis (A) and leaf conductance (g_s) in Polygala (\blacktriangle) and Viburnum (\circ) during the experiment.

In both species, water use efficiency (WUE) did not show any significant differences among the control and the other treatments; only 10% WC, 75 days after starting the experiment, showed lower values (Figure 7) both in *Polygala* and *Viburnum*.

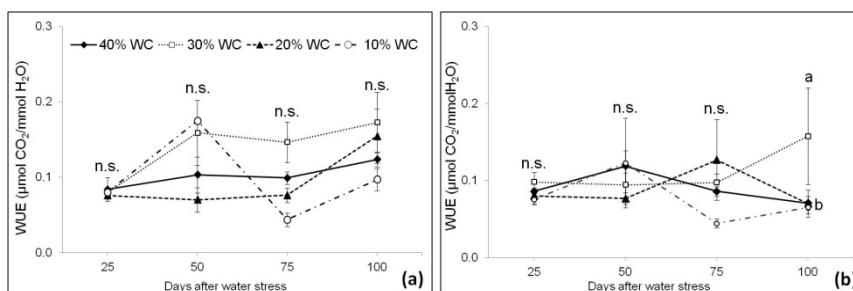


Figure 7. Water use efficiency (WUE) in *Polygala* (a) and *Viburnum* (b) during the experiment. Plants were irrigated to container capacity (40% WC) or subjected to different levels of water stress treatments (30% WC, 20% WC and 10% WC). Mean values \pm S.E. ($n = 6$). An absence of bars denotes a size smaller than the symbol. Different letters indicate significant differences between treatments at each sampling time as determined by Tukey's test ($p < 0.05$).

The Fv/Fm ratio in *Polygala* showed significant differences in 20% WC and 10% WC treatments during the trial, with the lowest values at 75 and 100 days (0.75 and 0.73, respectively). No significant differences for Fv/Fm were observed between 30% WC and the control (Figure 8a). In *Viburnum*, a reduction in the maximum quantum efficiency was observed only in severe deficit irrigated plants (10% WC) starting from the 50th day until the end of the experiment with a value of 0.77 (Figure 8b).

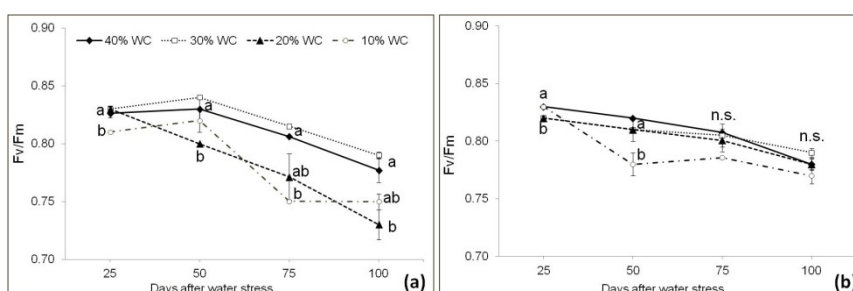


Figure 8. Maximum quantum efficiency of the PSII (Fv/Fm) in *Polygala* (a) and *Viburnum* (b) during trial. Plants were irrigated to container capacity (40% WC) or subjected to water stress treatments (30% WC, 20% WC and 10% WC). Mean values \pm S.E. ($n = 6$). An absence of bars denotes a size smaller than the symbol. Different letters indicate significant differences between treatments at each sampling time as determined by Tukey's test ($p < 0.05$).

Leaf water potential at pre-dawn (Ψ_{pd}) in *Polygala* showed a significant reduction in 10% WC for irrigated plants only. Compared to the mean value of the other three irrigation treatments, a decrease of 75%, 82%, 66% and 74% was detected after 25, 50, 75 and 100 days. A similar trend was also observed in plants treated with a severe water deficit, such as for *Viburnum* (10% WC) with a reduction of 70%, 122%, 117% and 72% of leaf water potential over the average of the other treatments (Figure 9a,b).

In *Polygala*, the leaf water potential at midday (Ψ_{md}) significantly decreased in all stress treatments compared to the control, reaching values of -1.49 , -1.62 and -2.04 MPa, respectively at the end of the experiment, in 30% WC, 20% WC and 10% WC treated plants (Figure 9c). In *Viburnum*, a significant reduction was observed starting from the 75th from the experiment onset until the end of the trial, reaching values of -0.82 , -0.93 , -1.22 and -1.48 MPa, respectively, in 30% WC, 20% WC and 10% WC treatments (Figure 9d).

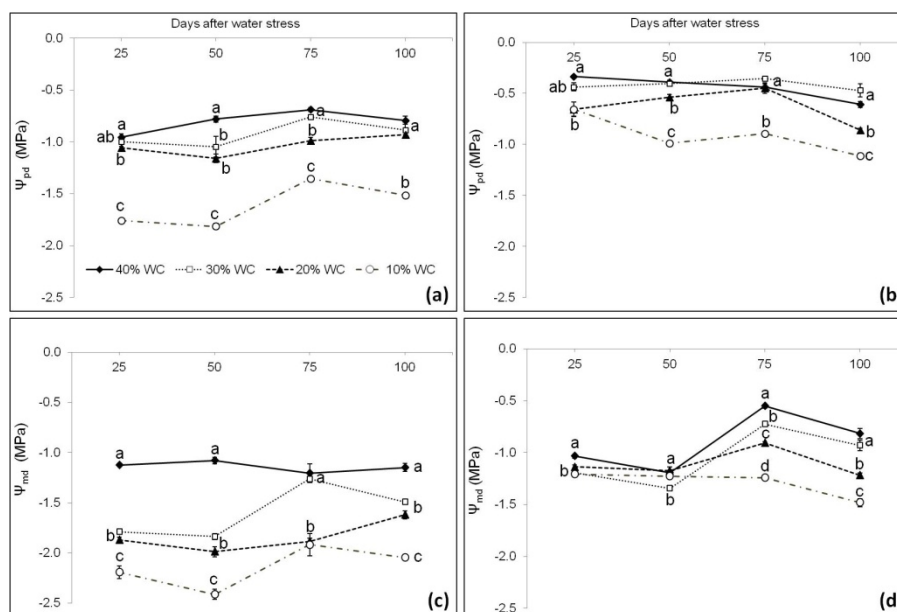


Figure 9. Leaf water potential (Ψ , MPa) at pre-dawn (a,b) and at midday (c,d) in *Polygala* (on the left) and in *Viburnum* (on the right) during the course of the trial. Plants were irrigated to container capacity (40% WC) or subjected to water stress treatments (30% WC, 20% WC and 10% WC). The vertical bars represent the S.E. of means ($n = 6$). The absence of bars indicates that the size was less than the symbol. Different letters indicate significant differences between treatments at each sampling time as determined by Tukey's test ($p < 0.05$).

3.4. Principal Component Analysis (PCA)

Processed data on the principal component analysis (PCA) resulted, for both species, in a cumulative variance of 43 and 77% for PC1 and PC2 respectively, associated to Eigen values of 8.7 and 6.7. From the present analysis appeared quite clear how the two species, subjected to different drought stress treatments, clearly resulted separated in the distribution plot (Figure 10) showing proportional values of 43.6 and 33.5 for PC1 and PC2 respectively.

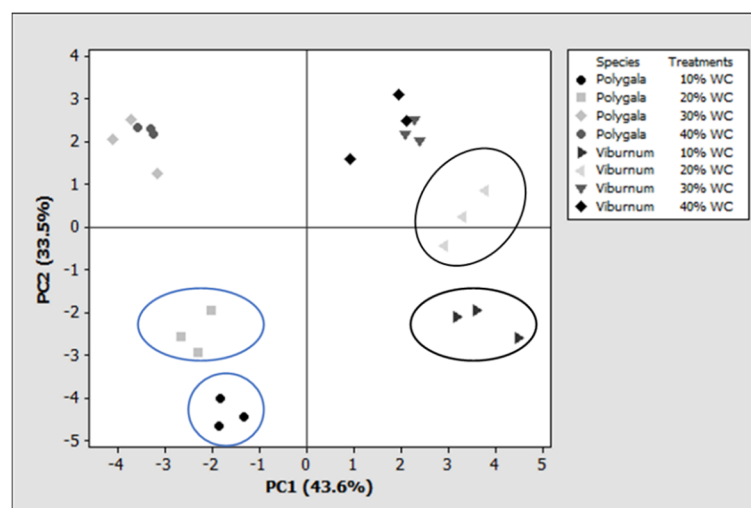


Figure 10. Principal component analysis (PCA) was performed over the correlation matrix among 20 selected variables. The plot of PC1 (43.6%) vs. PC2 (33.5%) is presented.

Furthermore, also water shortage treatments at 10% WC and 20% WC resulted clearly separated, indicating a specific and stress-intensity response in both species, whereas 30% WC and 40% WC didn't

show any clear spatial distribution, indicating that the stress intensity did not affect any physiological and morphological plant response.

In the PCA, PC1 vs. PC2 a negative correlation was detected in SLA, leaf number, stomata size, total leaf area, shoot dry biomass, A, gs, WUE and total dry biomass, while a positive correlation was detected in Ψ_{pd} , Fv/Fm, Ψ_{md} , root dry biomass, R/S ratio, unit leaf area, spongy palisade thickness stomata density and SPAD (Figure 11), describing the tendency of the plants to allocate the synthesized organic matter in the roots instead of the shoots in order to cope with water stress conditions. The main changes in the above ground part of the plants were detected on a general reduction of leaves number and, as a consequence, on the total leaf area. At the same time, the plants modify the unit leaf area (Figure 12).

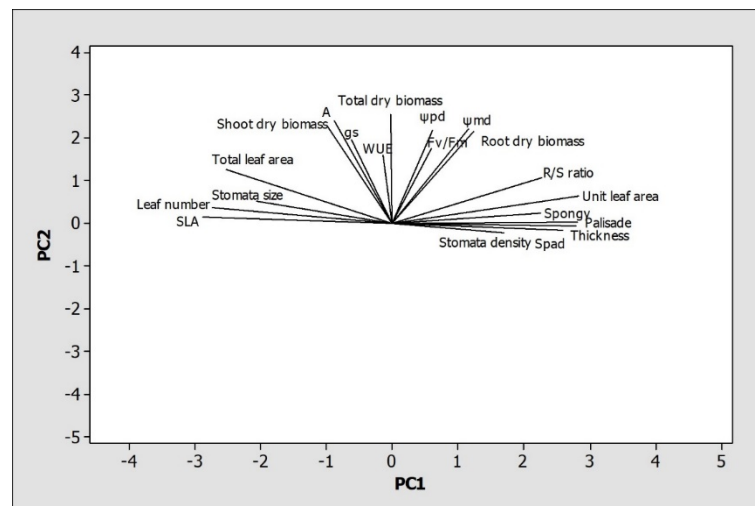


Figure 11. Principal component analysis (PC1 vs PC2) of *Polygala* and *Viburnum* species subjected to drought stress treatments: loading plot and distribution of samples in the consensus space.

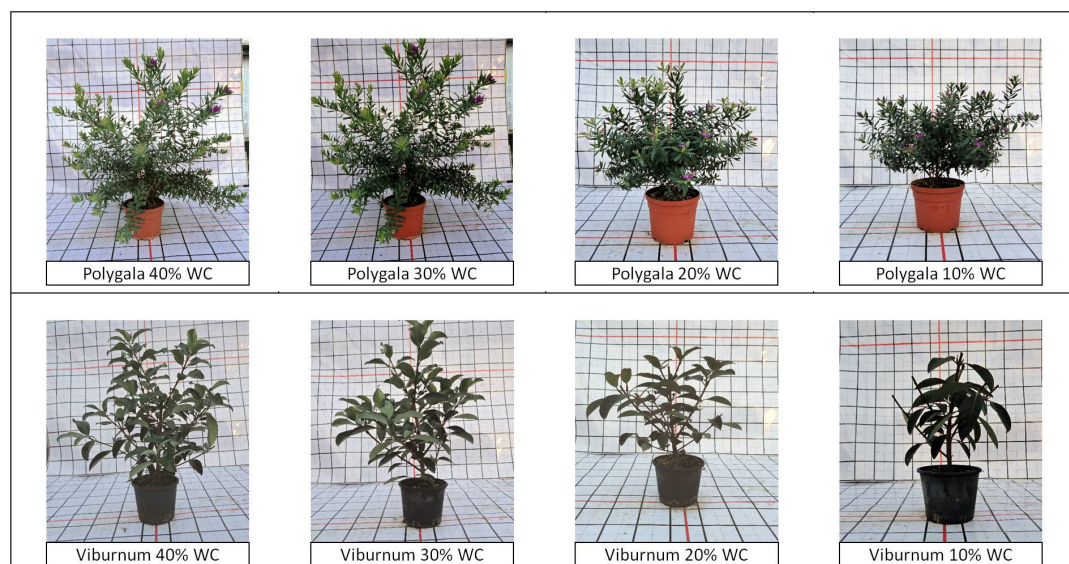


Figure 12. *Polygala* and *Viburnum* plants irrigated to container capacity (40% WC) or subjected to water stress treatments (30% WC, 20% WC and 10% WC) at the end of the trial.

4. Discussion

The selection of highly tolerant drought stress species plays an important role in planning private and public ornamental green areas in urban and peri-urban environments. In many urban areas, the irrigation of green areas represents a high amount of urban water consumption (between 40%

and 70%) [36]. However, in environments with limited water availability, the selection of ornamental plants with a high WUE is almost compulsory. The sustainability of green areas greatly depends on the performance of ornamental plants under a deficient water supply. Nevertheless, the plant chosen should have a drought tolerance threshold compatible with the water fraction available without showing any ornamental quality losses such as leaf yellowing or abscission.

Regarding the suitable selection of drought-tolerant plants, it is important to understand the physiological and/or biochemical responses of plant species to water stress [13,37]. Our results showed that there are differences in the drought responses of two shrubs, despite both of them being able to overcome this environmental limitation. The drought stress, in fact, had different effects on the biomass repartition according to the species.

Viburnum and *Polygala*, in fact, showed different morphological adaptations to overcome the imposed drought stress, reducing leaf number and area, and dry biomass. These adaptations are similar to those found in roses [38], *Callistemon* [8,39], *Spirea nipponica* ‘Snowmound’, *Pittosporum eugenioides* ‘Variegatum’, *Viburnum nandum* [40], and some Mediterranean shrubs [41].

The growth reduction of plants, and hence the lower plant biomass, represents the final expression of a number of processes analyzed to determine salt and water stress tolerance [28,32,42]. In our study, in both species, the severe drought stress (20% WC and 10% WC) led in particular to a decrease of the shoot dry biomass. The tolerance level to drought stress should be considered an important factor to save water. Even if shrubs reduced biomass, they managed to maintain their ornamental value. Deficit irrigation reduces plant growth in several ornamental species [12,43–47], although the reaction to drought stress widely varies among species, being a typical species-specific response. The lower shoot growth is also common in salt stress, caused by reduction of new leaf emission and leaf growth [48]. The leaf area reduction allow the plants to reduce water loss by transpiration. This is a typical stress-avoidance mechanism adopted by plants to overcome water stress condition, as reported for different shrub species in drought environments [12,13,39,41]. In the face of a marked reduction of shoot biomass, the root biomass is increased to allow the better exploration of the soil to find available water. In fact, the plants increase the root to shoot ratio to overcome the drought stress, which reduces water consumption and increases water absorption [49,50]. This response was observed in *Viburnum* plants (20% WC and 10% WC), where there has been a redistribution of the dry biomass to benefit the roots at the expense of shoots (higher root/shoot ratio). The increase in R/S ratio observed in our study was the result of a greater reduction in shoot biomass rather than an increase in root biomass. A higher root/shoot ratio could allow us to obtain “hardy” plants which are able to rapidly overcome the transplant crisis [51] and better adapt to subsequent water shortages [52]. A lower deficit irrigation (30% WC) in the nursery phase is able to reduce the excessive shoot growth without compromising the ornamental value and avoiding chemical growth retardants [53]. Unlike, in fact, what occurs for salt stress or from marine aerosol, browning on the leaf blade rarely takes place.

A drastic water shortage reduced the number of leaves and total leaf area in both studied species. Different papers have also reported the decrement of these parameters in relation to low soil water availability [54–59]. *Polygala* drought-stressed plants showed a more evident reduction in leaf number; this is correlated with the high number of leaves that characterized this species. One of the main plant responses to drought is the reduction of leaf area, because reducing the surface area limits water loss via transpiration [60]. The leaf area represents the leaf adaptation to environmental changes, and it is connected to plant growth, light interception, gas exchange [61], and also ornamental plant value. The plant capacity to reduce leaf area is typically a drought-tolerant response [61]. This strategy is similar to plant response to salt stress; in this condition, however, the degree of tolerance is strictly related to the aesthetic value, which can be compromised by browning on the leaf blade [62].

High water deficit can also modify the concentrations of chlorophyll and carotenoid [63]. A reduced chlorophyll content was found in *Catharanthus roseus* [64], *Helianthus annuus* [65] and *Vaccinium myrtillus* [66] grown in severe water stress conditions; an increase of this parameter was observed in moderate and severe water stress conditions, similar to that shown in our results [67]. In our study,

an increase of SPAD index was found in severe drought stress (10% WC). Furthermore, the increase in chlorophyll content due to moderate and intense water stress could be the effect of the growth decline in cells related to chlorophyll synthesis [68]. At any rate, the influence of chlorophyll content on the photosynthetic rate in *Thypha latifolia* is slight [69]. The chlorophylls and carotenoids are important pigments which contribute to the visual appearance and ornamental quality of the plants. Therefore, ornamental drought-tolerant plants should not show significant variations under the water regimes available.

During drought stress conditions, changes occur in the leaf anatomy. Some studies indicated that slight variations in the leaf thickness determined significant modifications in gas exchange in some plant species, while others demonstrated large plasticity in terms of leaf thickness with little variation in photosynthetic capacity [70]. Studies on leaf anatomical characteristics of *Passiflora* species showed that *P. setacea* was the one, among the five species of the same genera investigated, with the highest survivability under water limitation due to its capacity to tolerate severe drought stress [71]. In our study, *Viburnum* plants subjected to drought conditions showed an increase in palisade parenchyma thickness; this is in accordance to Guerfel et al. [72], who observed in two Tunisian olive cultivars subjected to water stress an increase in palisade parenchyma thickness during water stress in drought-tolerant plants.

The palisade parenchyma is directly linked to photosynthesis, because the higher development of this tissue could enable a greater fixation of CO₂ with faster stomata opening [73]. Under low water availability, plants modify the size and density of their stomata as an adaptation strategy to water stress. In our study, both shrubs significantly reduce the size of their stomata. Only *Viburnum*, however, reduced its stomata density. The reduction of the stomata number and size on the leaf surface has an evident influence on water loss.

Smaller stomata allow a better control of drought stress and regulation of water use efficiency [20]. The modification of the size and the density of stomata represents a typical avoidance strategy during drought stress conditions.

With the reduction of transpiration, the water use efficiency generally increases [74]. Water use efficiency (WUE, A/E) is adopted to indicate the water amount used by plants for growing. Some papers reported that stressed plants are more able to utilize the energy obtained by photosynthesis, thanks to higher A/E [15,75]. In our study, drought stress did not affect this process, indicating that the two shrubs could efficiently use water resources under moderate and severe water stress conditions.

The leaf water potential was highly different between the control and severe water stress conditions in both species. In different species, subjected to drought stress conditions stomata closure is a consequence to water potential adjustment [20]. The water potential measured predawn appeared to be a very effective parameter for highlighting the different degree of drought stress to which *Polygala* and *Viburnum* plants, in relation to the different water stress treatment, encountered during the trial. For both species, in fact, the values of the water potential were very low at 10% WC, although the differences, compared to the control, were clearly lower for *Viburnum* than for *Polygala*, confirming that the latter has a poor tolerance to water deficit. The reduction of leaf water potential is a frequent response to water stress, also in drought-tolerant plants [76]. The capacity to maintain a high leaf water potential at 40% WC showed that the plants did not experience water limitation.

Net photosynthesis showed different values in relation to water stress conditions during the experimental period. This parameter, in both species, decreased with the lowering of the water content. The differences among the treatments were particularly evident at the end of the trial, especially in *Polygala* plants.

The closure of stomata was a major factor affecting the reduction of g_s and A_N under drought stress conditions in both species. The variations in the photosynthetic activity are largely attributable to changes in stomatal conductance, as can be seen from the high degree of positive correlation between these two parameters. The result confirms what was found by other authors in different shrub species [77]. The decrease in g_s represents a mechanism to overcome drought stress and limit water

loss. This is according to Hessini et al. [78], where in plants of *Spartina alterniflora* subjected to drought at the end of the trial, the stressed plants showed significantly lower stomatal conductance.

Drought stress influences the sensitivity of the photosynthetic apparatus to photoinhibition [79–81]. Beyond affecting stomatal closure, drought stress reduced gas exchange in the plants by reducing transpiration and photosynthetic rates [41].

Under drought stress, photoinhibition can increase because the plant's capacity to use the available light can be reduced [80,82]. Our results showed that water stress did not influence photoinhibition, because the values of F_v/F_m were within the typical ranges of healthy plants [0.80; 22]. This demonstrates that the damage of the foliar tissues is not irreversible, that PSII was not permanently damaged [32], and that the chloroplasts of Mediterranean species are able to adopt different mechanisms during drought stress conditions to avoid photo-inhibitory processes, such as the mechanism to dissipate the reducing power produced by the PSII.

This parameter also did not show wide variation in the previous experiment conducted by Farieri et al. [32], indicating that the plants, even in conditions of saline aerosol, were able to preserve leaf functionality.

The PCA analysis demonstrated that the treatments with higher water ability did not differ between both species, in turn indicating that the level of stress did not affect the plant morphology and physiology. Moreover, the data demonstrated that the two species have different drought responses, since there was no overlap among species and treatments.

The plants try to implement a series of modifications to compensate the reduction of leaf area, leading to a series of structural modifications of the leaf.

5. Conclusions

The results of this study indicate that the ornamental shrubs developed avoidance mechanisms in response to drought stress. As for saline aerosol, even for drought stress, net assimilation is the most complete parameter of leaf functionality associated with chlorophyll a fluorescence. The ornamental shrubs have modified gas exchange, leaf number and area, and root-to-shoot ratio. These morpho-physiological adjustments could to some extent mitigate the effect of drought stress under field conditions.

Overall, our shrubs were tolerant to drought treatment, particularly the *Viburnum* plants. These findings supported the use of this species in Mediterranean areas, where the spring and the summer are very warm and dry and hence are characterized by drought stress. The species' adaptability to water stress was similar to that observed with salt aerosol stress, as well as the degree of adaptation. The similar plant strategies to overcome these two frequent stresses in the Mediterranean coastal areas, linked to the same strategies used by plants, allow us to assume that the possibility of identifying plants that can simultaneously counter environmental stresses is concrete. The environmental sustainability of the green areas in urban and peri-urban sites is extremely important in locations with lower water availability. The innovative and original results arising from our work are represented by the strategies for the identification of water regimes that can be compatible with low water availability and the retaining of ornamental quality. In particular, the water deficit of 30% for the two species and 20% for *Viburnum* can be satisfactorily used from a broad sustainability point of view.

Supplementary Materials: The following is available online at <http://www.mdpi.com/2073-4395/9/7/381/s1>, Figure S1: Transpiration rate (E) and Sub-stomatal CO₂ (C_i) in *Polygala* (a,b) and *Viburnum* (c,d) during the experiment.

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