

# Physiological responses to water stress and stress memory in *Argania spinosa*

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## ABSTRACT

Drought stress is one of the major abiotic stress factors shifting the physiology and metabolism of the plants. In semiarid areas, drought stress events are often recurrent, and plants have developed strategies to remember a first so-called priming stress to eventually respond more effectively to a second triggering stress.

In this study, we tested several physiological and morphological variables in *Argania spinosa* (L.) Skeels plants, growing in greenhouse conditions under a drought treatment induced by water withholding. Two sequential one-month periods of water stress were imposed to understand the stress memory events in this species. The plants were divided into two groups depending on the treatment applied (irrigated and stressed) which later, after one month of a recuperation phase, were divided again into two more groups (having a total of four at the end of the study). Leaf samples were periodically taken and relative water content, gas exchange, chlorophyll content and other variables were measured, analysed and compared between those groups. Plants, which have suffered the two sequential water stress periods, were more affected according to several variables than plants subjected to only one stress treatment, so we finally conclude that this species does not seem to have any drought stress memory mechanisms for the studied variables, under the conditions of this experiment.

## 1. Introduction

Drought is a very important source of stress for plants, and it can produce several deleterious effects on them. The adverse effects produced by water stress are responsible for reduced yield in the agricultural context and for reduced fitness in the ecological context (wild populations) (Cortés et al., 2013; Sah et al., 2020; Pintó-Marijuan et al., 2017; Seleiman et al., 2021).

To add even more importance to the problem, the ecosystems are currently living in a context of global change that involves, among many other things, a raise of the average temperature because of greenhouse effect and an increase in desertification all around the globe (Burrel et al., 2020; Huang et al., 2020). Moreover, extreme weather events are every time more frequent causing stronger effects on plants and ecosystems (Smith 2011).

In arid and semi-arid regions of the world, where annual rainfall is limited and unequally distributed over the year, plants are constantly exposed to water stress. To cope with these conditions, they have developed many different physiological, biochemical, and morphological responses (as leaf shedding; stomatal control; accumulation of

osmolytes or antioxidant compounds; limiting growth; among many others) (Farooq et al., 2009; Osakabe et al., 2014; Zunzunegui et al., 2011).

Additionally, plants from recurrent arid zones might exhibit other strategies, as the capacity of stress memory. In several species, after an initial stress, called alarm phase, the plant can recover if conditions improve; but if there is a new stress period in the future, this first stress could have marked the plant with an imprint that modify its responses to subsequent stress episodes (Fleta Soriano et al., 2016). Stress memory is then defined as the improved response to a certain stressful event when it is faced for the second time, as a result of physiological, morphological and genetic changes caused by the first exposition to that given stressful event (Fleta Soriano et al., 2016; Walter et al., 2011, 2013; Wojtyła et al., 2020).

*Argania spinosa* (L.) Skeels commonly known as the argan tree, is a spiny, slow growing tree endemic to the Southwest of Morocco. It is a member of the Sapotaceae family, typical of tropical and subtropical zones. It lives in arid and semiarid regions, (annual precipitation ranging from 100 to 400 mm, irregularly distributed over the year). Following several authors, the argan forests are part of a transitional zonation from

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Mediterranean to Saharan type climate (McGregor et al., 2009).

This species plays an essential role for the communities that coexist with it, both for ecological and economical purposes. First, it is the only tree in these arid regions protecting the soil from erosion and slowing down aridification processes, while it shades other crops and maintains soil fertility. On the other hand, it is a very important source for economical income for the people who exploit it, especially for women, mainly for the oil from the kernels of its fruits (the argan oil, world known for cosmetic purposes). Moreover, the wood is used as fuel and the leaves as “hanging forage” to feed goats and other animals. Despite its importance, the argan tree populations are drastically reducing both in density and in surface covered, due to overgrazing of the aerial parts, increasing aridity and the ongoing substitution of argan groves by irrigated crops, which are more profitable in the short term but eventually end up deteriorating the soil (Díaz-Barradas et al., 2010; Chakhchar et al., 2017a).

From the ecological point of view, this species can grow, producing fruits while living in a very arid environment, with very infrequent rain events and a long summer drought. It has been shown, in previous studies, that the argan tree has several morphological and physiological strategies to resist water stress, as stomatal control in response to an increase of VPD or a decrease in leaf water potential, accumulation of antioxidant defense systems, leaf shedding under extreme dry conditions, irregular canopy which can favor stem flow, dimorphic root system related to flexible water uptake pattern, association with arbuscular mycorrhizae (Díaz-Barradas et al., 2010; Chakhchar et al., 2015a, b; Zunzunegui et al., 2018).

In spite of different authors recognizing that stress memory might be an important strategy to face a changing climate with frequent drought events, there is currently little information available about the existence of stress memory in native plants from environments subjected to drought conditions in a cyclical manner (Alves et al., 2020).

It is possible that *A. spinosa* might exhibit any kind of stress memory responses in order to survive under these extreme environmental conditions. Our hypothesis is that plants subjected to two sequential periods of water stress will respond significantly better to the second stress treatment than the ones that were previously irrigated and later subjected to water withholding, as it has been shown in previous studies with other plant species (Pintó-Marijuan et al., 2017; Kulak, 2020; Alves et al., 2020).

To test our hypothesis, we set a particular experimental design intended to assess the existence of stress memory mechanisms in *A. spinosa*. The presence or not of stress memory mechanisms has never been tested in this species.

Our aims were to record different morphological and physiological traits in plants subjected to three water cycles, the first month corresponded to a water withholding period, the second month a recuperation period with full irrigation and the third to a new water withholding period in which previously irrigated and stress plants were exchanged trying to find evidence of drought memory.

## 2. Materials and methods

### 2.1. Study species

*Argania spinosa* (L.) Skeels has been selected as a model species for our experiment of stress-memory because it is the only tree capable of growing in arid and semiarid conditions of its distribution range, thanks to a highly conservative use of water, water-saving and drought avoiding strategies combined with tolerance mechanisms (Díaz-Barradas et al., 2010). It has also showed to be a facultative semi-deciduous tree, being capable of partially or totally shedding its leaves if a severe summer drought occurs (Ain-Lhout et al., 2015).

### 2.2. Experimental design

In June 2017, Dr. Fatima Ain-Lhout has collected seeds of *A. spinosa* from the Souss valley in the surroundings of Agadir (Morocco). She brought the seeds in paper bags to the University of Seville (Spain), seeds were soaked in tap water for 24 h and then placed in cells of germination trays, filled with potting soil, in the greenhouse of the University of Seville. Seeds germinated after 20 days, and they were kept in these trays for two months. Approximately with 70-day-old, the seedlings were transferred to small pots and a few months later, with 8-month-old, to larger ones (5,8 L volume, 29 cm height). The plants grew with Blumenerde universal substrate (a commercial substrate which includes peat, plant compost, coconut fiber, perlite, NPK + Mg fertilizer) and were not fertilized at any moment. In October 2021 we selected 36 *Argania spinosa* seedlings, with 4-year-old and 40–50 cm height.

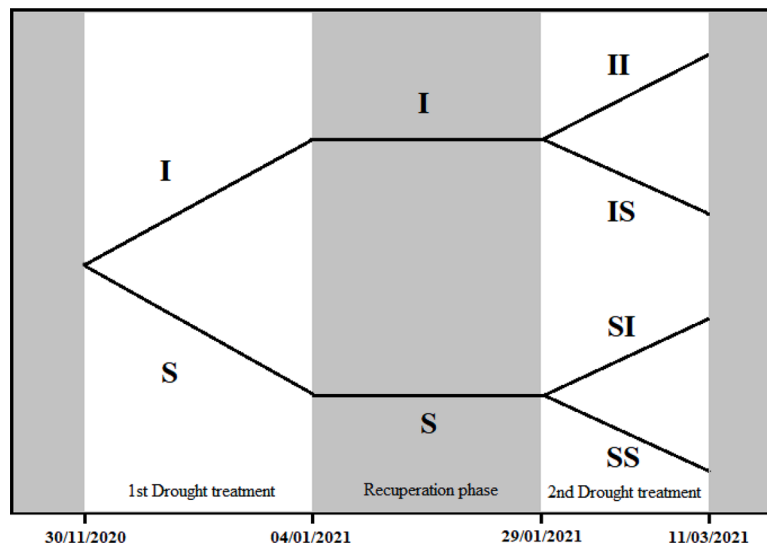
Inside the greenhouse, the temperature ranged from 16 to 24 °C, relative humidity between 40 and 60% and natural light was supplemented with artificial light to maintain the photoperiod of 14 h. At the beginning of the experiment, all the plants were pruned to have approximately the same height. Besides that, weeds that eventually invaded the pots (mainly *Oxalis* sp.) were eliminated every sampling day and the substrate was refilled to the initial level, when necessary, due to losses produced by irrigation.

The experiment was designed in three monthly periods, following the method by Muhittin Kulak (Kulak, 2020) with slight modifications (Fig. 1). Initially, we divided the 36 seedlings into two different groups of 18 plants: water stressed group (named S for Stressed), in which plants were irrigated weekly with 10 mL (approximately 2.5% of the field capacity), except for the first week, at the beginning of the experiment, when plants were irrigated with 25 mL and control group (named I for Irrigated), in which plants were irrigated until field capacity (400 mL of water per plant and week). Then, the plants were subjected to a recuperation period, in which all of them were irrigated to field capacity for approximately one month. After that, each group was divided into two more groups, so that the plants that were irrigated the first time were divided into an irrigated and a stressed group with 9 plants each, and the same was applied to the first stressed group. This means that at this third monthly step of the experiment we had 4 different groups: Irrigated-Irrigated (II), Irrigated-Stressed (IS), Stressed-Irrigated (SI), and Stressed-Stressed (SS). IS and SS plants were irrigated with 10 mL every week and II and SI at full field capacity (Fig. 1 and Table 1).

### 2.3. Greenhouse measurements

Every sampling day, all the 36 plants with their pots were weighted, using a GRAM electronic balance (model SBZ-10 K) just before the irrigation and, the relative weight loss on subsequent days was calculated dividing each pot weight by the original weight recorded the first day which was considered as 100%. Pot weight was measured on 9 different days, the first 5 days belonging to the first drought treatment (the 3rd, 10th, 16th, 23th, 28th days of the experiment) and the last 4 days belonging to the second drought treatment (the 64th, 70th, 79th and the 94th days of the experiment). No measures were recorded during the recuperation phase due to technical problems.

Four leaves per plant from 20 different plants (10 plants × 2 treatments × 4 leaves=80 leaves in the first drought and recuperation periods and 5 plants × 4 treatments × 4 leaves = 80 leaves in the second) were collected for analysis at the laboratory. The leaf area was measured using the program Midebmp (R. Ordiales, CSIC, Spain, 2000), from scanned images of the leaves and they were weighted using a Nahita Blue electronic precision balance (serial number: 5173). Three leaves per plant were used to calculate relative water content (RWC), leaf mass-area index (LMA) and leaf dry matter content (LDMC) and the fourth leaf for stomatal quantification. For the first purpose, fresh weight was measured immediately after collecting the leaves, turgid weight after applying them water and keeping them for 24 h in the fridge, and dry



**Fig. 1.** Experimental scheme of the study, each step of the experiment last for 1 month approximately and the total experiment lasted for 101 days. II: Irrigated-Irrigated, IS: Irrigated-Stressed, SI: Stressed-Irrigated, SS: Stressed-Stressed. (Modified from Kulak 2020).

**Table 1**  
Plants groups per treatment, with their abbreviations.

Treatment	Group	Abbreviation	Plants
First drought treatment	Irrigated	I	C1-C18
	Stressed	S	S1-S18
Second drought treatment	Irrigated-Irrigated	II	C1-C9
	Irrigated-Stressed	IS	C10-C18
	Stressed-Irrigated	SI	S1-S9
	Stressed-Stressed	SS	S10-S18

weight after keeping the leaves at 80 °C for 24 h.

The fourth leaf per plant was used to calculate stomatal density. For that purpose, clear nail varnish was applied to the underside of the leaves. When the nail varnish dried out, it was removed using transparent adhesive tape and then stuck to a slide. Then, stoma-number were counted using a microscope with 400x augmentation and 0.1046 mm<sup>2</sup> diameter of field of view. Leaf traits were measured on the 10th, 21st, 35th, 58th, 70th, 84th days of the experiment.

Gas exchange measurements were performed in the same 20 plants (10 plants per treatment in the first drought and recuperation periods and 5 per treatment at the second and, always we measured 2 leaves per plant), using a portable photosynthesis system (model LI-6400, from Li-Cor Inc, Nebraska, USA). Measurements were made in situ from 9 h am (solar time) to 12.00. The measured gas exchange variables were net CO<sub>2</sub> assimilation rate (A), stomatal conductance to H<sub>2</sub>O (g<sub>s</sub>), Intercellular CO<sub>2</sub> concentration (C<sub>i</sub>) and transpiration rate (Tr). Water use efficiency (WUE=A/g<sub>s</sub>) and Carboxylation efficiency (CE= A/C<sub>i</sub>) were calculated using these data. The leaf-chamber was set with the following conditions, 1400 μmol m<sup>-2</sup> s<sup>-1</sup> PPFD, 1.0–1.5 kPa VPD and 400 μmol mol<sup>-1</sup> CO<sub>2</sub>.

Several leaves (10–15) from the 16 remaining plants (divided equally between treatments) were collected for photosynthetic pigment analysis. The leaves were weighted using a Nahita Blue electronic precision balance and 0.3 g of leaves per plant were mixed with 10 mL of acetone and crushed using a mortar in order to extract and dissolve the pigments. These pigments were kept in the fridge, inside test tubes covered with Parafilm® to avoid evaporation. The next day the samples were filtered using filter paper and their volumes were recorded. For the analysis, samples were diluted 3 times with acetone, and the absorbance curve of the sample was calculated using a spectrophotometer (double beam UV/VIS spectrophotometer, model DU-8800D (Drawell International Technology Limited, Chongqing, China), set in wavelength scan mode

(350–700 nm). Photosynthetic pigments samples were taken on the same days as the gas exchange measurements, which were on the 16th, 58th and 94th days of the experiment. The pigment content was calculated from the absorbance curve obtained by the spectrophotometer, using the Gaussian Peak Spectrum (GPS) method for pigment quantification. The GPS equations were run using a ready-to-use fitting library for SigmaPlot, provided by Küpper et al. (Küpper et al., 2007). The pigments measured were chlorophyll a (Chla), chlorophyll b (Chlb), violaxanthin (V), antheraxanthin (A), zeaxanthin (Z), β-carotene and trans-lutein. Chla/Chlb, total chlorophyll/total carotenoids and (A + Z)/(V + A + Z) ratios were also calculated.

Close to the end of the experiment (which corresponded to the day 94 of the experiment); the plant macroscopic analysis of every individual was visually evaluated and catalogued inside one of these three categories: “green” (all leaves green coloured), “chlorotic” (less than half of the leaves yellow coloured) and “very chlorotic” (more than half of the leaves yellow coloured).

At the end of the experiment (the 101st day), 12 plants (3 of each treatment) were collected for biomass weight. Leaves, stems and roots were separated, dried for 24 h at 80 °C and weight, with a Nahita Blue electronic precision balance for leaves, while stems and roots were weighted using a LaboLan.

#### 2.4. Data analysis

All the data was statistically analysed using SPSS 26.0 statistical package (SPSS, Inc., Chicago, IL, USA). The Kolmogorov-Smirnov test was used to test for normality, aside from Levene’s test to assess variance homogeneity. When normality and variance homogeneity were confirmed, one-way ANOVA tests were performed in order to compare the means of the different variables depending on the different treatments. In the second drought-stress treatment in which we had 4 different groups and significant differences were found, a post-hoc test (Duncan’s test) was performed in order to determine individual differences between groups. The significance was always set to  $p = 0.05$ , except indicated otherwise. In the few cases in which the data did not adjust to a normal distribution, the Kruskal-Wallis test followed by Mann Whitney test were used to compare means between treatments (significance was corrected using Bonferroni’s correction). For the last time-point we have used a two-way ANOVA using drought treatment (yes or no) and intensity (single or double) as factors.

In the case of plant macroscopic analysis, a chi-squared test ( $\chi^2$  test)

was done to compare the observed frequencies with the expected frequencies if the variables were randomly distributed and independent from each other.

### 3. Results

#### 3.1. Pot weight

Due to the different irrigation treatments, there were significant differences between irrigated and stressed plants through all the first stress treatment (Fig. 2).

During the second stress treatment the differences were more complex; the two irrigated groups (II and SI) always present a significant higher water content, while in the stressed group, IS plants exhibited a higher water content than SS plants until the last day in which both groups present a similar and very low water content, close to 40% of the initial weight (Fig. 2).

#### 3.2. Leaf traits (RWC, LMA, LMDC and stomatal density)

Leaf relative water content (RWC) did not show significant differences between irrigated and stress plants during the first stress treatment. During the second drought cycle, although there is a high variability, only SS plants presented significant differences in relation to the other groups (Fig. 3).

Stomatal density was significantly different between control and stressed groups (less density in stressed plants) only on day 35 (January 4, 2021), just before the recuperation phase started ( $p = 0.03$ ). The last measured day, there were no significant differences regarding stomatal density, but a tendency can be observed ( $p = 0.09$ ) where SI plants have the highest stomatal density and IS plants have the lowest value. The two remaining groups present intermediate values (Fig. 4).

No significant differences were found for LMA and LMDC for any group on any of the days.

Some of the measured leaf traits presented significant differences in the two-way ANOVA at the last timepoint. For RWC there were significant differences between treatments, intensity, and the interaction between them, for stomatal density there were almost significant differences in the interaction between the factors and for LMA and LMDC there were not any significant differences for any of the factors (Table 2).

#### 3.3. Gas exchange

Although gas exchange was measured at the first drought treatment, at the recuperation phase and at the second drought treatment, significant differences were only observed on the last one (day 94 since start of experiment). Net CO<sub>2</sub> assimilation rate and stomatal conductance to H<sub>2</sub>O where both significantly different on this day ( $p = 0.021$  and  $p = 0.003$ , respectively). During this day plants from the double-stress treatment (SS) exhibited the lowest assimilation rate and stomatal conductance, while the II plants the highest values (Fig. 5).

Transpiration rate was also significantly different between treatments on the last day ( $p = 0.02$ ), plants from the double-stress treatment (SS) exhibited the lowest transpiration rate, the II plants the highest values, while the other two plant groups SI and IS intermediate values (Fig. 6).

Carboxylation efficiency (CE) differences were almost significant ( $p = 0.056$ ) at the last timepoint, and we can observe a tendency where the SS plants have the lowest CE (but also with a large of dispersion). Surprisingly, out of the other three groups, IS is the one with the highest average CE, though it also has a high dispersion (Fig. 6).

The two-way ANOVA at the last timepoint shows that for net CO<sub>2</sub> assimilation rate there were significant differences for intensity and in the interaction between both factors, stomatal conductance and transpiration presented significant differences for treatment and interaction was also significant for transpiration, for carboxylation efficiency significant differences only appear for intensity and there were not any significant differences for WUE (Table 3).

#### 3.4. Photosynthetic pigments

Our results evidenced that there were not any significant differences in pigment quantitative composition for any of the different treatments in any sampling day (Fig. 7). The only variable that was at least close to be significantly different was the total content of carotenoids on day 16 (during the first drought treatment). Stressed plants seem to have a higher content of total carotenoids than irrigated plants at the end of the first water withholding period, even though differences were not significant ( $p = 0.063$ ). Interestingly, none of the plants showed significant levels of the carotenoid  $\beta$ -carotene. The two-way ANOVA (for treatment and intensity) didn't show any significant difference for any measured photosynthetic pigment.

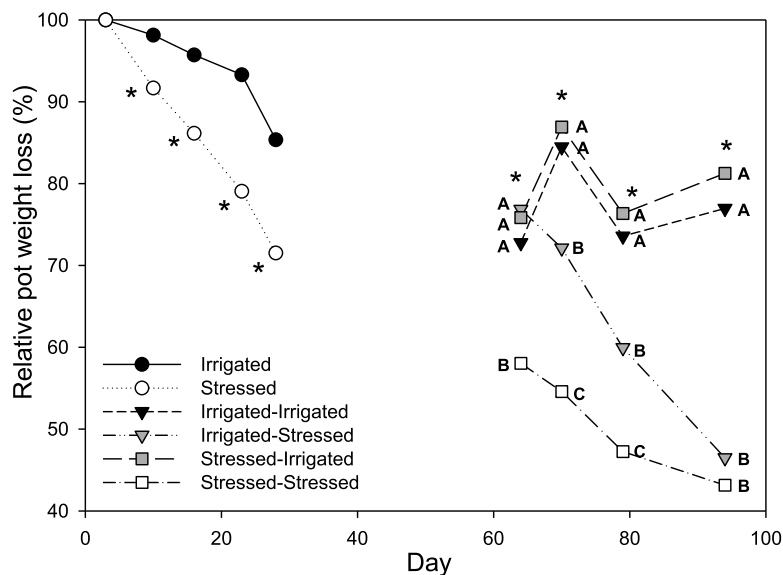
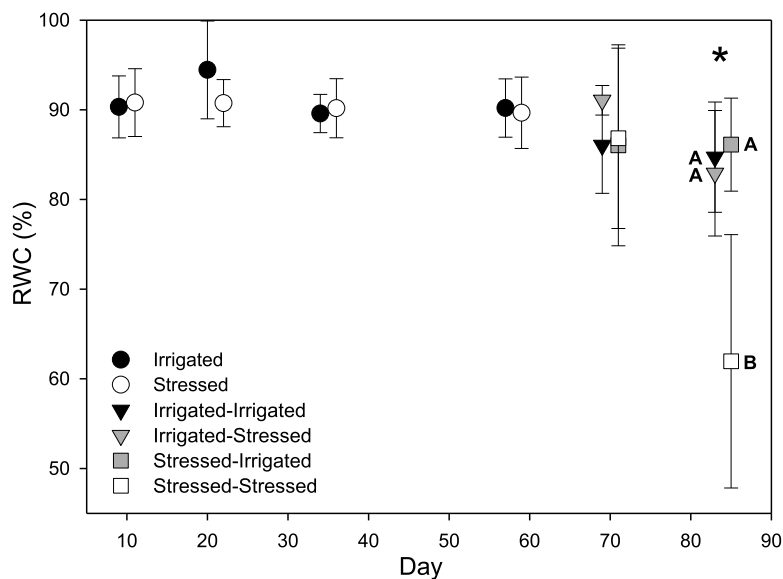
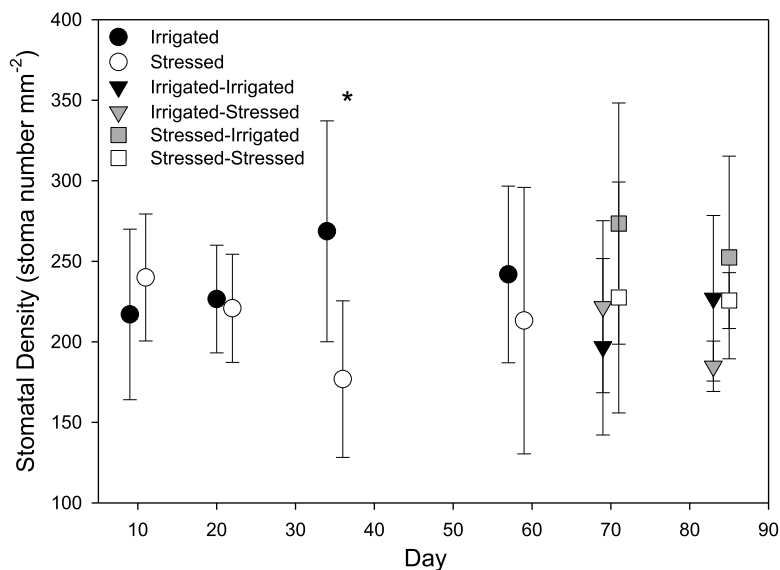


Fig. 2. Relative weight loss of the different experimental pots over time, compared to day 1 of the experiment Asterisks indicate significant differences between treatments ( $p < 0.05$ ), while capital letters indicate different groups created by the subsequent post hoc test.



**Fig 3.** Relative water content (RWC) over time. Asterisks indicate significant differences between treatments ( $p < 0.05$ ), while capital letters indicate different groups created by the subsequent post hoc test. Vertical lines indicate sample standard deviation (Bessel's correction was applied).



**Fig. 4.** Stomatal density over time. Asterisks indicate significant differences between treatments. Vertical lines indicate sample standard deviation (Bessel's correction was applied).

**Table 2**

Results of the two-way ANOVA using drought treatment and intensity as factors for the last timepoint. Only leaf traits which presented significant differences are shown in this table.

	dF	F	p
<b>RWC</b>			
Drought	1	10.76	<b>0.005</b>
Intensity	1	7.99	<b>0.012</b>
D × I	1	6.12	<b>0.025</b>
<b>Stomatal Density</b>			
Drought	1	3.39	0.084
Intensity	1	0.14	0.718
D × I	1	4.21	0.055

### 3.5. Biomass

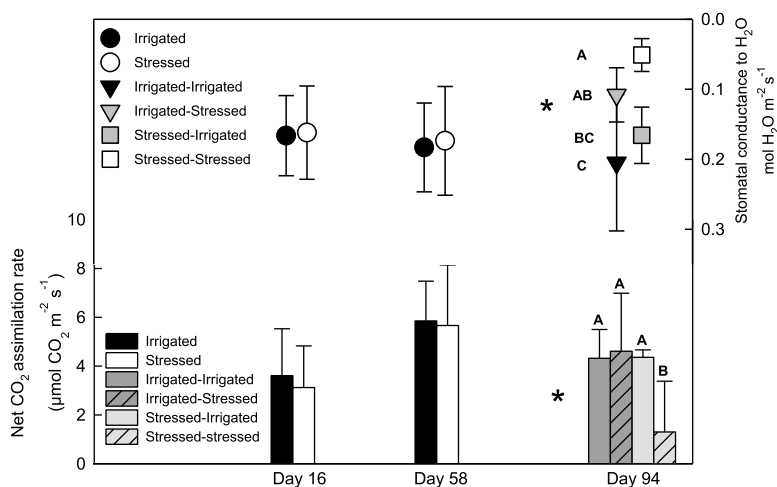
Independent of the treatments, root biomass was always the larger amount (about 60%) of total plant biomass. There were not significant differences between treatments in terms of absolute or relative biomass (Fig. 8). Despite that, observing the data, we can see a tendency towards more leaf relative biomass in the groups that were irrigated close to the end of the experiment (II and SI), although differences were not significant ( $p = 0.166$ ) due to the high dispersion of the data.

The results of the two-way ANOVA (for treatment and intensity) showed that only leaf relative leaf biomass presented significant differences in relation to treatment ( $p = 0.034$ ).

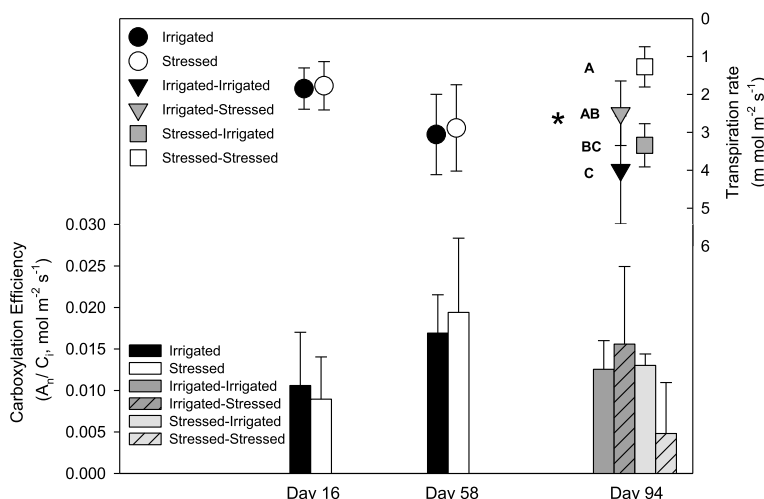
### 3.6. Plant macroscopic analysis

In the case of plant macroscopic analysis at the end of the second drought stress period, plants subjected to the double stress showed a





**Fig. 5.** Net CO<sub>2</sub> assimilation rate and stomatal conductance to H<sub>2</sub>O over time. Asterisks indicate significant differences between treatments ( $p < 0.05$ ), while capital letters indicate significant differences among treatments created by the subsequent post hoc test. Vertical lines indicate sample standard deviation (Bessel’s correction was applied).



**Fig. 6.** Carboxylation efficiency and transpiration over time. Asterisks indicate significant differences between treatments ( $p < 0.05$ ), while capital letters indicate different groups created by the subsequent post hoc test. Vertical lines indicate sample standard deviation (Bessel’s correction was applied).

**Table 3**

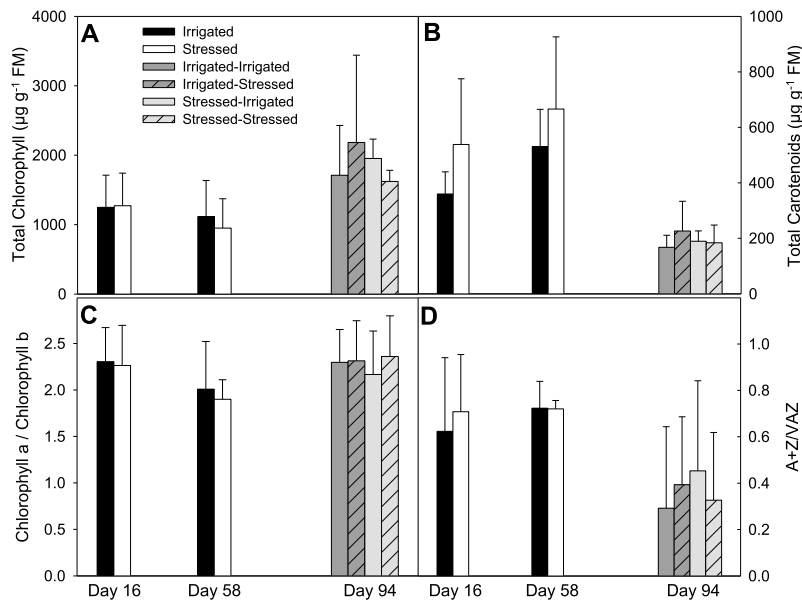
Results of the two-way ANOVA using drought treatment and intensity as factors for the last timepoint. Only gas exchange variables which presented significant differences are shown in this table.

	dF	F	P
<b>CO2 Assimilation</b>			
Drought	1	3.34	0.086
Intensity	1	4.88	<b>0.042</b>
D × I	1	4.67	<b>0.046</b>
<b>g<sub>s</sub></b>			
Drought	1	16.92	<b>0.001</b>
Intensity	1	0.12	0.734
D × I	1	3.50	0.080
<b>Transpiration</b>			
Drought	1	18.92	<b>&lt;0.001</b>
Intensity	1	0.49	0.494
D × I	1	5.21	<b>0.036</b>
<b>Carboxylation efficiency</b>			
Drought	1	0.96	0.342
Intensity	1	4.55	<b>0.049</b>
D × I	1	3.82	0.068

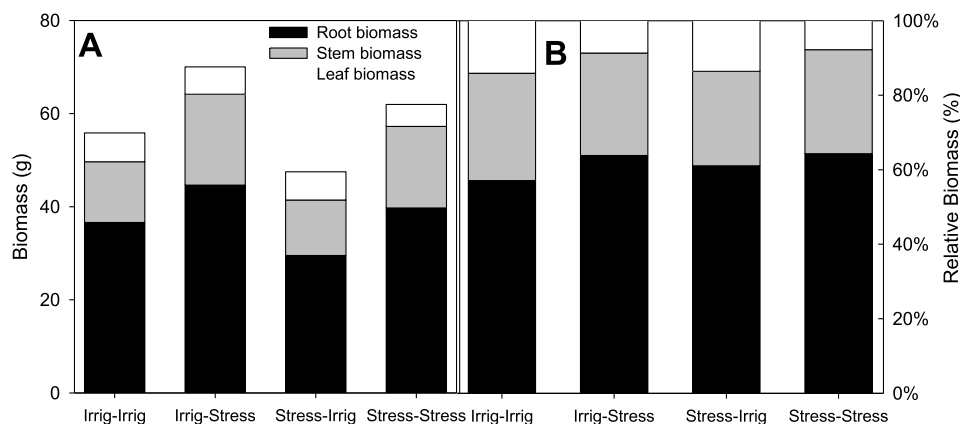
significant higher frequency of chlorotic individuals than the other groups (Fig. 9 and Table 4). The chi-squared test performed resulted in the reject of the null hypothesis that all the variables were independent and randomly distributed, with a confidence interval of 95% ( $p < 0.05$ ).

**4. Discussion**

This study intended to evaluate the capacity of ecological stress memory in *Argania spinosa* as a mechanism to resist water stress in its natural habitat. Our experimental design included a temporal drought, followed by a recovery period and a new drought event, trying to simulate the natural situation of this species under its natural habitat in which rain events might occur occasionally (Ain-Lhout et al., 2015; Chakhchar et al., 2020). Although this species is well adapted to extreme low precipitation regimes, with variability in traits and performance among populations (Diaz Barradas et al. 2010), the results of this experiment suggest that *A. spinosa* does not present signs of water stress memory, at least for the measured variables. Stressed-Stressed was always the most affected group of plants, while Irrigated-Irrigated was the least compromised for all the measured variables.



**Fig. 7.** Total chlorophyll content (A), total carotenoids content (B), Chlorophyll a/chlorophyll b ratio (C) and (antheraxanthin+zeaxanthin)/(violaxanthin+antheraxanthin+zeaxanthin) ratio (D). Vertical lines indicate sample standard deviation (Bessel’s correction was applied). FM: Fresh Mass. The color and pattern of the legend is the same for the four graphics.



**Fig. 8.** Mean biomass (A) and relative biomass (B) of leaves, stems and roots of each group at the end of the experiment. Irrig-Irrig: Irrigated-Irrigated, Irrig-Stress: Irrigated-Stressed, Stress-Irrig: Stressed-Irrigated, Stress-Stress: Stressed-Stressed.

However, regarding gas exchange variables we could find several intermediate responses in the groups that were irrigated only half of the experiment (IS and SI). These differences will be separately discussed for each variable in the following sections.

During the first water withholding period there were no significant differences in RWC, nevertheless these differences only appear at the last day (day 84 of the experiment). Our results emphasize the great capacity of this species to maintain turgid their leaves for an extended period of time with low water input. These results agree with Meslem et al. for seedlings of this same species, where differences in RWC did not start to be significant until 4 weeks of drought, although in that case significant differences showed up in half the time (Meslem et al., 2015).

On the last day, the SS group had a lower RWC value than the IS group. This shows that doubly stressed plants were more affected in terms of water content in leaves, but the results of the two-way ANOVA

for the last time point, exhibited significant differences for treatment, intensity and the interaction between them, which emphasizes that the effect of one or two water withholding periods are also important in the water content of the leaves. These results match with the ones obtained by Pintó-Marijuan et al. (2017) in a stress memory experiment with the invasive CAM species *Aptenia cordifolia*.

Although the two groups of stressed plants showed lower values than irrigated plants in relation to stomatal density differences were not significant. However, the results of the two-way ANOVA were almost significant for the interaction treatment  $\times$  intensity. The high variability in the data in those plants might also indicate an extreme stress situation, as it has been shown by other authors (Zunzunegui et al., 2011). A bigger sample would be needed to confirm if there could be stress memory mechanisms related to stomatal density in this species.

The structural traits LMA and LMDC did not show any significant



Fig. 9. Examples of “green”, “chlorotic” and “very chlorotic” plants, from left to right.

Table 4

Percentage of plant qualitative appearance (%) recorded for each experimental group. II: Irrigated-Irrigated, IS: Irrigated-Stressed, SI: Stressed-Irrigated, SS: Stressed-Stressed.

Group of plants	Green	Chlorotic	Very Chlorotic
II	89	11	0
IS	56	33	11
SI	89	11	0
SS	11	56	33

differences between treatments, suggesting that the leaves are very resistant to water stress. The strategy of this species consist of shedding their leaves instead of reducing their mass, as a part of its facultative semi-deciduous strategy (Díaz-Barradas et al., 2010).

Net CO<sub>2</sub> assimilation data supported that CO<sub>2</sub> assimilation capacity was not compromised by only one water scarcity event, when compared to the fully irrigated plants and, significant differences only appear at the end of the second drought period, in which SS presented significant lower values than the other groups. The results of the two-way ANOVA also emphasize that photosynthetic assimilation is sensible to the effects of the drought stress and its distribution. Lower gas exchange values in the drought stressed plants were also found in previous studies with this species (Díaz-Barradas et al., 2010; Chakhchar et al., 2016).

In the case of stomatal conductance and transpiration rate, Irrigated-Irrigated and Stressed-Stressed plants also had the highest and lowest values, respectively, but here IS and SI constituted two intermediate groups, furthermore in the two-way ANOVA there were significant differences in the interaction treatment and intensity. These results suggest that these variables might be more sensible to water stress. In natural conditions, *A. spinosa* exhibits a very efficient drought-dependent stomatal control, under a decrease in water potential or an increase in VPD (Díaz-Barradas et al., 2010).

It can be noted that for all the gas exchange variables, the values were higher in day 58 than in the first measurements on day 16. This situation could be explained through the meteorological conditions, since day 16 was a moderate cloudy day, while day 58 was a sunny one.

Pigment compositions results, with no significant differences among treatments, contrast with the results from Pintó-Marijuan et al. for *Aptenia cordifolia*, who found differences and stress memory for this type of pigments (Pintó-Marijuan et al., 2017). It also contrasts with Meslem et al., who found significant differences in chlorophyll a, chlorophyll b

and total chlorophyll between control and 6-week water stressed *A. spinosa* seedlings (less chlorophyll in stressed plants) (Meslem et al., 2015).

Probably, the pigments (chlorophyll, xanthophylls and β-carotene) don't actually play a relevant role in water stress response in *A. spinosa*, while other compounds that were not quantified in this experiment may participate in water stress response and could be used as better biochemical stress markers for this species, like thiol compounds, superoxide radicals, hydrogen peroxide, free proline, poly-phenol content, epicuticular wax loads and several stress-related enzymes like peroxidases (Chakhchar et al., 2015a, 2015b; Chakhchar et al., 2016, 2017b). Free proline, for example, was higher in more stressed populations of this species as seen in previous studies. (Díaz-Barradas et al., 2010; Meslem et al., 2015)

Although, differences in absolute biomass were not recorded, it was possible to observe a tendency towards reduced leaf biomass in the stressed groups and there were significant differences in relative biomass in response to the treatment in the two-way ANOVA. This makes sense according to the fact that *Argania spinosa* is described as a facultative deciduous species (Díaz-Barradas et al., 2010), also called semi-deciduous or drought deciduous. This type of plants that are typical of semi-arid and savannah-like climates, shed their leaves temporarily during the dry season, when long term drought occurs, in order to save water trough reduction of transpiration (Marchin et al., 2010; Dahlin et al., 2017).

Even though root biomass is the main deposit of plant biomass, a typical trait of plants from arid climates (Chachchar et al., 2020), we didn't find any difference in root biomass or root/to/shoot ratio in any type of stress treatment, in comparison with irrigated plants. This result agrees with Chakhchar et al., 2018, as they have found that argan seedlings subjected to water stress for 40 days didn't change significantly root length, diameter or root-to-shoot ratio in comparison with control plants. But, in the same study, the authors have found a considerable reduction of root-hydraulic conductivity of seedlings subjected to a severe drought stress. This strategy may reduce water loss through plant vessels and protect leaves against dehydration (Chachchar et al., 2020).

At the end of the experiment, it was evident (and statistically significant) that stressed plants had more chlorotic leaves and they were in the process of shedding them. No previous papers discussing leaf chlorosis or shedding in *A. spinosa* were found.

This agrees with the conclusion reached in biomass and with the



facultative deciduous strategy of this species. Additionally, we can see that the plants which have suffered a double stress (SS) have more chlorotic leaves, which again contradicts the hypothesis that this species has water-stress memory mechanisms, which would lead to an improved performance in comparison to plants without a persisting stress memory (Walter et al., 2013).

We can conclude that, in the conditions of this experiment, *Argania spinosa* does not seem to have any form of stress memory mechanism, at least in what respects to drought stress and for the variables measured in this experiment. Our results suggest that the first water withholding treatment causes any type of negative imprint, to face other stress events.

In natural conditions the different populations of *Argania spinosa* are well acclimated to the conditions of their habitat (Díaz Barradas et al. 2010), but stress memory is not acclimation per se and enables the plant to respond quicker and better to a new stress event (Water et al., 2013). In plants, stress memory seems to be associated to epigenetic marks on genomes, but the complete mechanistic background is still unclear (Bhar et al., 2022).

This knowledge will be essential for a more efficient management of the argan tree, promoting its conservation in its natural habitat and practices that improve agricultural production in a context of increasing temperature and aridity due to global change.

#### Author contributions

JL de la Fuente and MC Díaz-Barradas have participated in the experimental work and in the preparation and correction of the manuscript. M Zunzunegui has participated in the preparation and correction of the manuscript.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data Availability

Data will be made available on request.

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