Physiological and morphological traits associated with germinative and reproductive stage of garden orache (A. hortensis L. var. rubra) under water stress

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Abstract

Background: Drought is a major problem limiting the growth and development of plants in the world and especially in Tunisia. Halophytes constitute a renewable wealth and they offer great flexibility with regard to abiotic stresses, and they are evaluated for their ecological and potential food use.

Results: The proposed work identifies the response of Atriplex hortensis var. rubra to the germinal stage and the reproductive stage under a deficient water regime to measure the drought resistance of this plant that has very interesting forage production abilities. The morphological and water parameters are used to characterize the physiological response of this species to the effects of water deficit. For the germination test, four levels of osmotic potential caused by PEG-6000 solutions at different levels of water potential (−0.1, −0.5, −1.0, −1.5 MPa) were adopted in seed of A. hortensis germination media. The methodology adopted in the second experiment is based on the cultivation of potted plants stored in a semi-controlled greenhouse at flowering stage. The water deficit was imposed on the plants by watering stop for a week, and the control plants are subjected to a water regime maintained irrigated at 100% of the capacity in the field. Drought tolerance was scored 30 days after the drought stress commenced based on the number of branches and leaf, dry biomass, relative water content, leaf water potential, and nitrogen content. No significant difference was observed in germination rates for all PEG concentrations throughout the experiment which are still close to 60%. The results obtained for the second experiment show a high tolerance of A. hortensis under water stress. Drought induced decreases in two physiological parameters, the number of branches and leafs, and the relative water content of annual Atriplex. Heatmap and PCA data revealed that physiological parameters are more sensitive than morphological parameters in distinguishing the control and drought treatments.

Conclusions: Indeed, the orache is distinguished by a great ability to retain water potential after a month of stress. Thus, height, number of branches, leaf and shoot dry weight, and percentage of nitrogen were significantly similar for controls and stressed for A. hortensis. On the other hand, measured root length and basic and midday water potential show significant variability between controls and stressors. In addition, these results highlight the importance of the resistance of Atriplex halophyte forage to drought.

Keywords: Water deficit, Atriplex hortensis L., Germination, PEG, Morphophysiological parameters, Water potential, Nitrogen, PCA

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Background
The environmental stress such as salinity (soil or water) and drought are serious obstacles for horticulture and field crops in further areas of the world, especially arid and semiarid regions. Stresses caused by abiotic and biotic factors have long-threatened sustainable development of agricultural production. Water-deficit stress is a major problem in agriculture and most crop plants show high sensitivity to this stress than others abiotic constraint conditions. Caused by reduced precipitation and increased temperature [1], drought has been the most important limiting factor for crop productivity and, ultimately, for food security worldwide [2]. Drought is considered as a major threat, limiting growth and yield of plants [3, 4], water stress is caused by insufficient rainfall that results in soil drying. High temperature, low humidity in atmosphere, and water deficiency are the main causes of drought [5, 6]. Drought stress affects germination rate and early seedling growth of the plant [7, 8]. Under water-deficit conditions, a significant reduction in germination, hypocotyl length, root and shoot fresh, and dry weight were observed, whereas the root length is increased [9]. It also affects the carbon assimilation and phenology of the plant [10]. Germination of each seed is considered as one of the first and most fundamental life stages of a plant, so that the success in growth and yield production is depending on this stage. Among the stages of the plant life cycle, seed germination, seedling emergence, and establishment are the key processes in the survival and growth of plants [11]. Germination is regulated by duration of wetting and the amount of moisture in the growth medium [12, 13]. Water stress acts by decreasing the percentage and rate of germination and seedling growth [14]. Water stress not only affects seed germination, but also increases mean germination time in crop plants [15].

The germination rate, germination potential, and germination index of the seeds mirror the germination speed, uniformity and the strength potential of seedlings, all of which declined dramatically with the increasing of drought stress intensity [16, 17]. Polyethylene glycol is the best solute that we are aware of for imposing a low water stress that is reflective of the type of stress imposed by a drying soil [18–20]. Water stress due to drought is probably the most significant abiotic factor limiting plant and also crop growth and development.

Over 100 plant species belong to Atriplex genus. The common cultivated crop is garden orache (Atriplex hortensis L.), named also as mountain spinach, sea purslane, or saltbush [21]. This species, same as spinach (Spinacia oleracea), belongs to Chenopodiaceae family. *Atriplex hortensis*, a glycine betaine natural accumulator also called mountain spinach, can tolerate harsh conditions such as cold, drought, and high salinity [22–24]). Garden orache herb (Herba Atriplicis hortensis) is characterized by a high content of flavonoids, vitamin C [25], mineral components [26], and amino acids [27]. Garden orache belongs to a group of plants of leaves being a rich source of protein [28]. Leaves of orache can be consumed either fresh or boiled, separately or together with other vegetables [29].

*Atriplex hortensis* has been recognized for its medicinal properties which were shown to improve digestion, increase circulation, and boost the immune system [30]. Additionally, *A. hortensis* has been used in land rehabilitation projects because of its ability to establish well, grow rapidly, reduce soil erosion, and compete with native plants [31, 32]. As a result, *A. hortensis* is important for both domestic and wild browsing animals where other forage crops are lacking. Despite its affinity for low-to-moderate saline areas where it has little competition from non-halophytes, *A. hortensis* can also grow where total soluble salts are low, making it well suited to a multitude of different environments [33]; in addition, *A. hortensis* as well, a promising potential cash crop halophyte for revegetation or fodder production in arid environments [34, 35]. Pathways required for flowering have response systems that mediate survival under various stresses. In response to stress, such as drought, flowering pathways are accelerated to produce flowers and seeds more rapidly [36, 37].
The aim of the presented experiment was to estimate physiological and morphological traits associated with germinative and reproductive stage of annual Atriplex (*A. hortensis* L. var. *rubra*) under water stress. In this work, pot experiment with randomized block design was conducted for garden orache for assaying the physiological response to drought tolerance during flowering stage.

**Materials and methods**

**Germination analysis**

Six replicates of 20 seeds were set in Petri dishes containing three germitest papers. Papers were moistened with 7 ml of different polyethylene glycol (PEG-6000) solutions to simulate drought stress at different water potential levels (−0.1, −0.5, −1.0, and −1.5 MPa). Distilled water was used as the control (0.0 MPa). The amounts of PEG used in the experiment were calculated following Villela et al. (1991) [38]. Samples were stored in germination chambers at 25 °C, 12-h photoperiod. Seeds were transferred to new petri dishes every 3 days to maintain osmotic levels.

The variables analyzed to verify seed water stress tolerance were germination percentage and vigor index (VI) as described by Maguire [39]. The experiment was conducted for 15 days. Seeds were considered as germinated when radicle protrusion reached 2 mm.

The final germination percentage (GP) was calculated using the following equation:

\[
GP = \left(\frac{\text{Total number of germinated seeds}}{\text{total seed}}\right) \times 100.
\]

The vigor index was calculated according to the following formula:[40]

\[
\text{Vigor index (VI)} = \text{[seedling length (cm)]} \times \text{germination percentage].}
\]

**Experimental design and data processing**

A second experiment was conducted under reproductive stage drought stress condition with the objective to determine the effect of water-deficit stress on morphophysiological parameters of *Atriplex hortensis*. Growth conditions and stress treatments of garden orache (*A. hortensis* L.) was conducted in a greenhouse at the Experimental Station of INRAT in Tunisia (35°87 N, 9°96 E). When plants reached flowering, drought was imposed to stress pots with similar weight by withholding water for a week, while non-stressed plots continued receiving irrigation. The experiment design was split-plot with six replications.

**Leaf water potential analysis**

Measurements of leaf RWC and Ψleaf were made on the same leaf. Ψleaf was measured with a pressure chamber following the precautions recommended by Turner [41] between 6:30 and 7:30 a.m., each treatment included six replications.

**Leaf relative water content analysis**

The methods used to determine leaf RWC as follows: first, leafs (4–5 leaves from top 2 or 3 branches) were sampled and weighed immediately to obtain the fresh weight (FW), and then, the leafs were placed in tubes with freshly distilled water for 8 h, surface dried with filter paper and weighed to obtain the saturated weight (SW), and then dried it at 80 °C in a forced-draught oven for 24 h to obtain the dry weight (DW). The leaf RWC was calculated as:

\[
RWC = \left[\frac{\text{FW} - \text{DW}}{\text{SW} - \text{DW}}\right] \times 100.
\]

**Growth analysis**

At harvest, plant height, the number of branches and leafs and root length on the main stem were recorded. Stems, leaves, and roots were separated from each plant, and total dry weight per plant was calculated by adding the dry weight of different plant components after oven drying at 80 °C for 5 days.

**Estimation of total proteins**

N content was estimated by Kjeldahl method and N percentage was calculated by the following equation [42]:

\[
N\% = 0.56 \times t \times a - b \times VW \times 100DM.
\]
Fig. 1  The effect of increasing water stress (0, −0.1, 0.5, 1.0, and −1.5 MPa) on cumulative percent germination and vigor index of *Atriplex hortensis* after 15 d. The data are the average SD of eight independent replicates. The mean values represented by the different letters were significantly different in Tukey’s test $P < 0.05$. 
\( t = \text{the concentration of acid used for titration (mol kg}^{-1}), \)
\( a = \text{the amount of acid used as a sample (ml)}, \)
\( b = \text{the amount of acid used as control (ml)}, \)
\( V = \text{the volume of extract obtained from digestion (ml)}, \)
\( W = \text{the weight of plant sample for digestion (g)}, \)
\( \text{DM} = \text{dry matter percentage}. \)

**Data processing**

All data were processed with Satistica. The two-way analysis of variance and the least significant difference (LSD) were used to compare the differences between different data sets. All results are given as means ± SE. The significance of the correlations between different parameters was determined by bivariate correlations based on Pearson’s correlation (two-tailed). Origin statistical software (PCA analysis) was used to determine the correlations between physiological and morphological traits, and to perform principal component analysis of the traits.

**Results**

**Germination under drought stress**

Seeds need a suitable condition to have a good germination, with polyethylene glycol (PEG-6000) used to simulate drought stress, and the percentage of Atriplex seeds were affected by levels of drought stress. Drought stress simulated by polyethylene glycol PEG-6000 significantly reduced seed germination percentage (Fig. 1). Under the action of severe drought stress (−1.5 MPa), the seed germination rate was extremely high (60%), and the difference between the drought treatments reached a no significant level (\( P < 0.05 \)), showing that it is feasible to study the drought resistance of Atriplex with PEG solution of different osmotic potential gradients to simulate drought stress.

Drought significantly (\( p < 0.05 \)) affected vigor index (Fig. 1), and it was considerably reduced under stress condition as compared to control. The data presented in Fig. 1 revealed that the average seedling vigor index of different concentrations of PEG was ranging

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Fig. 2 The effect of water deficit on plant height of Atriplex hortensis harvested 30 days after withholding irrigation. The data are the average SD of five independent replicates. The mean values represented by the different letters were significantly different in Tukey’s test \( P < 0.05 \).
between 260 and 450. Seedling vigor index of *A. hortensis* is considerably decreased with increasing PEG concentrations. The highest Vigor Index was achieved with control associated with their more shoot lengths as compared to other treatments.

**Evaluation of growth performance**

The effect of drought stress on plant performance was evaluated by analysis of the changes in the DW and RWC after 30 days of withholding irrigation. Essential traits, root length, and shoot length are also important indicators in response to water stress. Drought stress imposed during the reproductive stages of Atriplex plants also had a transferable effect on shoot growth and developmental traits (Figs. 2 and 3). Parameters measured at 30 days after water stress, decreased in response to increasing soil moisture stress for plant height and biomass components. The plant height (Fig. 2) and the shoot and leaf dry weights (Fig. 4) of the Atriplex plants grown under both control (100% FC) and after withholding irrigation were significantly similar from control treatment (100% FC) and stressed. Nearly, a significant variation was measured on root length and number of branches and leaves among the plants under normal growth conditions and drought-affected plants (Figs. 3 and 5).

**Leaf osmotic potential analysis**

Similar to shoot and root traits, physiological and gas-exchange traits of the Atriplex plants were also affected by water deficit. Drought stress treatment significantly reduced the midday and pre-dawn leaf water potential of *A. hortensis* relative to the control (Fig. 6).
To evaluate the degree of damage caused by water stress on Atriplex plant, relative water contents (Fig. 7) of *A. hortensis* leaves under different treatments were determined. Compared to the control, the relative water content of the leaves treated with drought stress decreased compared with the control; however, the change of water content was significant with increasing water deficit.

**Nitrogen content and correlation analysis**

Subsequently, we studied the percentage of nitrogen of *A. hortensis* under control and drought stress (30 days after withholding irrigation) conditions in differently treated samples showed no significant difference (Fig. 8).

Significant and positive correlations were observed between plant dry biomass and nitrogen content ($R^2 = 0.69; p < 0.001$) under drought stress condition (Fig. 9). However, changes of nitrogen percentage were positively correlated with dry biomass, suggesting that tolerant plants of Atriplex contain less nitrogen in their shoots but keep it better under drought stress conditions. The current study indicated that the physiological and biochemical traits have direct or indirect effect on yield performance of Atriplex plant under water stressed environment at reproductive stage.
Physiological and morphological evaluation of the drought responses of Atriplex plants by PCA methods and heatmap

Furthermore, complete data sets, one showing the relative physiological changes under drought stress, were subjected to principal component analysis (PCA). Principle component analysis was performed using the relative values of all physiological traits to comprehensively evaluate the differences in plant physiological responses and the final total score was calculated to represent physiological responses. Pearson correlations were calculated to determine the relationship among the physiological parameters of physiological responses. To evaluate the contributions of each parameter in the control and drought-treated Atriplex plants, we performed PCA using four physiological parameters [(leaf water potential midday (LWPmid), LWPpre leaf water potential pre-dawn, nitrogen content, and relative water content (RWC)] and seven morphological traits [dry weight shoot (DWsh), dry weight leaf (DWlf), plant height, root length, number of branches (NubBran), number of leaves (Nubleaf), and dry weight leaf/shoot (DWleaf/shoot)]. The physiological parameters contributed more than the morphological parameters to the separation of the control and drought-treated groups (Fig. 10). The seven morphological measurements, which reflect relative long-term response to abiotic stress, were clustered together (top of Fig. 10, circled). The principal

Fig. 5 Number of branches and leaves of A. hortensis under control and drought stress (30 days after withholding irrigation) conditions. The data are the average SD of five independent replicates. The mean values represented by the different letters were significantly different in Tukey’s test $P < 0.05$
components reflected different aspects of physiological responses in *A. hortensis* under drought stress.

We evaluated the Atriplex plants for their responses to drought treatment. Drought responses in both well-watered and drought-stressed plants were measured using both physiological (LWPmid, LWPpre, Nitrogen content, and RWC) and morphological (DWsh, DWlf, plant height, root length, NubBran, Nuleaf, and DWleaf/shoot) parameters collected from plants after 30 days of drought treatment. To identify the key parameters for assessing drought tolerance in Atriplex, both physiological and morphological measurements were used to plot a heatmap. As shown in Fig. 11, the morphological and physiological measurements of *A. hortensis*, grown under either drought treatment or well-watered conditions (control), were used for hierarchical (row) clustering. This clear clustering demonstrates that in comparison to control conditions, drought stress treatment alters both the physiological and morphological characteristics for Atriplex plants. The heat map clearly reveals considerable variation among Atriplex plants in their physiological responses to drought stress and well watered (Fig. 11).

**Fig. 6** Midday and Pre-dawn leaf water potential of *A. hortensis* under control and drought stress (30 days after withholding irrigation) conditions. The data are the average SD of five independent replicates. The mean values represented by the different letters were significantly different in Tukey's test *P* < 0.05.
Discussion

Drought stress is a serious threat which decreases crop production. Seeds are an important stage in the life history of a plant, and an important time for the study of drought resistance of the plant [43]. The present study was taken up to study the effect of drought stress at germination and reproductive stages on potential physiological and biochemical responses in garden orache. Seed germination data are important to explain the total viability of seed and lead to estimate the number of seed that will grow into successful seedlings in the field. Under the action of drought stress, the seed germination rate of A. hortensis was extremely great (more than 60%), the seedling vigor index is considerably decreased, implying that even if the seeds were germinated, the growth of the seedlings was significantly inhibited. Drought stress delayed or inhibited seed germination and seedling growth by creating low osmotic potential preventing water uptake [44, 45].

It is known that the reproductive stage of plants is more susceptible to drought. Therefore, an
understanding on the responsive mechanisms during this stage of *A. hortensis* will not only be important for basic plant physiology, but the knowledge can also be used for crop improvement via either genetic engineering or molecular breeding. Root length, shoot height, and leaf area are considered as major determinants to evaluate drought response during reproductive stage. A positive relationship exists between root traits and resistance to drought [46, 47]. At reproductive stage, drought stress affects relative growth, leaf water potential, and relative water content of leaves (RWC %). Nosalewicz et al. [48] reported that exposing barley (*Hordeum vulgare* (L.)) to drought stress during reproductive stages decreased the shoot:root ratio and the number of thick roots. Moreover, exposing *Astragalus nitidiflorus* to drought stress increased seed dormancy [49]. In this study, the shoot growth and developmental parameters of *A. hortensis* also decreased in response to water stress. In addition, relative water content and the number of branches and leaves were also lower in soil water stressed compared to the optimum irrigation. Root growth of *A. hortensis* increases relatively to shoot
growth to acquire more water under drought stress conditions. Roots are the first organs to sense drought stress and have been proposed as an important avenue of research to improve crop adaptation for their regulation of water availability to drought stress. Screening root traits at early stages of plant development could be a proxy trait at mature stages under drought stress [50]. Having a longer tap root system could be a drought-adaptive mechanism to increase water and nutrient uptake under stressed conditions [51, 52].

Significant and positive correlations were observed between plant dry biomass and nitrogen content under drought stress condition of Atriplex plant at reproductive stage. A significant linear correlation was observed between plant N accumulation and dry biomass, indicating that plant N accumulation was intimately associated with dry biomass accumulation [53, 54]. Drought induces decreases in the soil water content and increases in plant water deficit, which cause subsequent decreases in the leaf carbon assimilation rate and soil available nutrients, leading to plant N limitation [55, 56].
It is still challenging to reliably analyze and interpret large physiological datasets collected from plants grown under drought and well-watered conditions. Various methods and statistical models have been proposed for such analyses. Correlation analysis, PCA, and clustering are considered to be good methods for evaluating the relationships between the parameters and their principal components for drought tolerance [57, 58]. In this study, PCA and correlation analysis showed that the differences in drought tolerance among Atriplex plants were largely due to variations in physiological parameters. A heatmap is a visual method that can be used to explore complex associations between multiple parameters collected from various treatments. It is often useful to combine heatmap with hierarchical clustering, which is a way of arranging items in a hierarchy based on the distance or similarity between treatments [59]. After drought stress, most of the measured traits in Atriplex plant approached to control levels, but there was still considerable variation in physiological parameters (Fig. 11).

**Fig. 10** Principal component analysis (PCA) for physiological responses in Atriplex plant. PC1–PC2 variables loading plots during drought stress. PC1–PC2: the first and second principal component. DWsh dry weight shoot, DWlf dry weight leaf, LWPmid leaf water potential midday, LWPpre leaf water potential pre-dawn, Nitrogen content, DWleaf/shoot dry weight leaf/shoot, RWC relative water content, Height, Root length, NubBran number of branches, NubLeaf number of leafs.
Conclusion

Drought has become major abiotic limitation factor on forage production under warming climate. The improved performance of drought tolerant Atriplex was associated with more efficient physiological and biochemical factors under conditions of stress where drought is frequent, particularly at reproductive stage.

To combat water stress, there is need to explore the resilient genetic resources and their utilization in breeding program. These results provide a foundation for future research directed at understanding the molecular mechanisms underlying annual Atriplex tolerance to drought.
Abbreviations
GP: Germination percentage; PEG: Polyethylene glycol; VI: Vigor index; RWC: Relative water content; SW: Saturated weight; DW: Dry weight; PCA: Principal component analysis; ANOVA: Analysis of variance; SD: Standard deviation.

Acknowledgements
The authors acknowledge the National Academies of Sciences, Engineering, and Medicine (USA) and the United States Agency for International Development (USAID) for the financial support to publication the present work. The assistance provided by lab staff of Animal and Forage Production of INRAT is also gratefully acknowledged.

Authors’ contributions
SS and SB conceived and designed the experimental strategies and manuscript. SS performed all experiments and data analysis. AZ helped in statistical analysis of raw data. SA and AE provided valuable understandings to improve experimental strategy. All authors read and approved the final manuscript.

Funding
The authors acknowledge the National Academies of Sciences, Engineering, and Medicine (USA) and the United States Agency for International Development (USAID) for the financial support. Partnerships for Enhanced Engagement in Research (PEER) and the PEER program cooperative agreement number: AID-OAA-A-11–00012.

Availability of data and materials
The data sets supporting the conclusions of this article are included within the article.

Declarations

Ethics approval and consent to participate
This manuscript is an original research, and has not been published or submitted in other journals.

Consent for publication
All the authors agreed to publish in the journal.

Competing interests
The authors declare that they have no competing interest.

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Received: 18 August 2020 Accepted: 13 March 2021 Published online: 14 May 2021

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