Morpho-Physiological and Biochemical Changes Induced by Drought Stress in Forage Pea (*P.Sativum* Subsp. *Arvense*)

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Abstract

In recent years, drought has been a major problem worldwide. Mediterranean regions have been particularly hard hit, adversely affecting the productivity of plants such as forage species. Using morpho-physiological and biochemical approaches, we have characterized the behavior of a forage pea variety with respect to in vivo water stress, with a view to developing this species for agro-ecological purposes. The study, carried out in pots under semi-controlled greenhouse conditions, was conducted under regular watering for control plants (Control) and with watering stopped for stressed plants (Stress). After 60 days of cultivation, our results revealed highly significant differences for all the parameters studied. Drought stress negatively affected growth, water status and membrane integrity, and an increase in soluble sugars and proline content in the leaves was recorded. At the end of this study, this variety of *P.sativum* subsp. *arvense* can be considered among the cultivars adapted to drought stress since in spite of the reduction in the relative water content, it always keeps a water status of 52% compared to the cultivars of other forage species, a study on open fields is necessary in order to confirm our results.

Keywords: Drought, forage pea, water status, soluble sugars, proline .

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Introduction:

The Mediterranean region suffers from low rainfall (1,2). With global temperatures predicted to reach higher norms in the 21st century, this area is considered a global warming Hot Spot (3). Plant production is low and irregular due to the frequent effects of abiotic constraints. Indeed, drought, salinity and extreme temperatures are serious threats to agriculture (4,5). What's more, in the field these different constraints often occur simultaneously (6). Drought is widely recognized as the primary factor limiting agricultural production worldwide, particularly in the Mediterranean basin (7,2) Yield losses attributed to water stress exceed those caused by the other abiotic factors put together (8). Moreover, due to global warming, drought will become more severe and will have to exert more pressure on agricultural sustainability than in the past (9).

A reduction and/or limitation of water decreases the productivity of many crops (10,11), in particular, if it occurs at the sowing and growth stages (12). This leads to a reduction in growth rate, leaf expansion, stem elongation (12), stomatal conductance and grain filling (13) various morpho-physiological and biochemical processes are inhibited (14). Stomata are closed (15), turgor is diminished (16) photosynthesis is inhibited (17). Husen et al. (14) showed that leaf cell elongation and leaf surface expansion were reduced.

Faced with this stress, plants stop growing, implement a series of measures to maintain survival and/or redirect resources towards flowering and seed production (18,19). These actions include regulation of stomatal conductance (20), osmotic adjustment, maintenance of cell turgor (21) and protection of cell membranes, enzymes and macromolecules from oxidative damage (22,21). However, a plant's ability to survive long-term water deficit depends on the type, duration and severity of the stress (23), as well as on the species and stage of development (25).

In Algeria, the production of forage legumes constitutes, along with cereals, the major agricultural challenges (24). However, due to random rainfall, unpredictable and severe droughts, and the use of cultivars not adapted to our conditions, Algeria suffers from an enormous forage deficit (25). Animals are subjected to frequent periods of food shortages (26). The use of water-tolerant species/cultivars adapted to our environmental conditions would be a judicious solution to this problem (27).

According to Reddy et al. (27), forage legumes can reveal specific mechanisms involved in resistance/tolerance to abiotic stresses. Forage pea is interesting to study because of its agroecological importance thanks to its symbiosis with rhizobial bacteria that promote and enhance seed germination, seedling vigor and emergence, root and shoot growth, plant biomass and seed weight (28-29). It tolerates dry growing conditions and limited rainfall, and is more drought tolerant than vetch and alfalfa (30-1). It is a livestock feed par excellence as it contains high levels of carbohydrates and total digestible nutrients (86-87%), (31-24), various vitamins (B, K and E), minerals, antioxidants, salts, proteins (23-25%) and fiber (5%) (32). For all these reasons, it is considered a potential alternative to soy in Europe (33).

The aim of the present study was to characterize the behavior of the **Sefrou** cultivar (*P.sativum* subsp. *arvense*) in pots, in the face of water stress caused by the cessation of watering. We measured morpho-physiological and biochemical parameters. Knowing how this plant responds to drought via these studied criteria will enable us to know whether it can adapt to our particular climates.

Materials and methods:

Vegetal material:

The seeds of the *P. sativum* subsp. *arvense* varieties used in this study were supplied by the technical institute for field crops (ITGC) (Algeria) in 2022. We chose to test the 'Sefrou' variety. This is an introduced genotype that has long been grown in Algeria (34,24), with the characteristics listed in Table 1.

Table 1. Geographical and climatic data for seed collection station

Collection site	Longitude	Latitude	Altitude	Bioclimatic stage	Pluviometry	Temperatures (°C)			Origin country (Reference)
						m	t	M	
Sidi-Bel-Abbès	0°38'29"O	35°11'38"N	483m	Semi-aride	337,4 mm	10,8	16,3	22,9	Morocco (Ouafi *et al.*, 2016)

Soil sampling:

The soil used in this study was taken from the Specialized Technological Institute for Agricultural Training ITSFA (ex ITMAS) located at Boukhalfa in the wilaya of Tizi-Ouzou (Algeria) (Table 2). The geographical and climatic data and the physico-chemical characteristics of the soil are shown in Tables 2 and 3. In the laboratory, the soil was sieved and then air-dried after removal of debris and stones.

Table 2. Geographical and climatic data for ITFSA soil collection station

Longitude	Latitude	Altitude	Bioclimatic stage	Pluviometry	Temperatures (°C)		
					m	t	M
36°44'47"N	4°01'40"E	230	Subhumide	488mm	14,9	21,4	26,3

Table 3. Physico-chemical characteristics of the soil used

Texture	pH	electrical conductivity (ds/m)	Organic matter (%)	N(%)	C(%)	C/N	$CaCO_3$ (%)	K_2O (meq/100gMS)	P_2O_5 (ppm)
Silty-clay	7.14	0.540	2.79	0.020	1.42	71	0.32%	0.53	105.40

Application of Drought stress:

The seeds were disinfected with 1% sodium hypochlorite for 5mn, and then carefully rinsed with distilled water to remove any trace of sterilizing agent before germination (35). We sowed the seeds in pots (14 cm high and 16.5 cm in diameter) containing a layer of gravel at the bottom, followed by 1 kg of substrate made up of a mixture of 1/3 horticultural potting soil, 1/3 topsoil (ITFSA) and 1/3 washed sand. The pots were placed in a semi-controlled greenhouse located at the Mouloud Mammeri University (UMMTO, Tizi-Ouzou, Algeria) at 36°41'49''N latitude, 4°03'21''E longitude and 147 m altitude at a temperature (day/night, 24/12 °C) and relative humidity varying between 65% and 75%.

All pots were watered regularly (20ml/pot) 3 times a week for 30 days. After one month, we divided the pots into two batches of 30 pots each: a control batch and a batch subjected to water stress. The control batch was watered regularly (3 times a week) and we applied water stress by stopping watering to the batch subjected to water stress. The pots were moved and rearranged daily to give a random distribution of growing conditions in the greenhouse based on a completely randomized design. After 2 months of cultivation, morphological, physiological and biochemical parameters were measured in control and stressed plants.

Parameters Measured:

Morphological parameters:

Growth trait (Soot length, fresh and dry weight):

A sample of 30 plants (one plant per pot) for each treatment was collected at the end of the treatment. The lengths of the aerial parts were measured with a caliper. The values obtained are the mean of 30 replicates. Above-ground biomass was estimated by weighing the fresh and dry matter (FW,DW), after standing for 48 hours in an oven at 80°C until a constant weight was obtained (36) using a precision balance (KERN PCB250-3).

Measurement of leaf area (LA):

To measure leaf area, we used the Mesurim pro software. Leaves were taken from a sample of 30 plants, one leaf per plant per treatment.

Physiological parameters:

Relative water content (RWC):

To determine the RWC, 30 plants were sampled, one leaf per plant. The leaves were quickly weighed to obtain their fresh weight (FW) and then immersed in 10ml of distilled water. The samples were kept in a cool place for 24 hours to ensure complete hydration. After hydration, the leaves were weighed to obtain the turgidity weight (TW). They were then placed in an oven at 80°C for 72 hours to obtain a dry weight (DW). The relative water content was determined using the formula of Seelig et al. (37): RWC= (FW-DW)/ (TW-DW) ×100.

Electrolyte leakage (EL):

EL was determined in five plants/treatment (control and stressed) on the basis of one leaf per plant using the method of Ibrahim and Quick (38), after two months of cultivation. Randomly selected leaves were rinsed with water, then cut into 1cm-long portions and placed in 10ml of distilled water in hermetically sealed tubes and incubated for 2 hours at 25°C. The electrical conductivity of the solution (L1) was then measured using a conductivity meter (HANNA EC214). The samples were then autoclaved at 100°C for 30 minutes, and the final conductivity (L2) was measured after cooling to 25°C. Electrical conductivity (EL) was calculated using the formula: EL = (L1/L2) ×100.

Biochemical parameters:

Determination of soluble sugars:

The sugars were extracted using the method of Hedge and Hofreiter (39) adopted by Khan et al. (40) from dry leaf powder ground in 10ml ethanol (80%). After 10min of centrifugation at (4000rpm) and shaking, the supernatant was recovered. A 2nd extraction was performed with ethanol, followed by centrifugation (3000rpm for 10min). The total extract was adjusted to 50ml with distilled water. The assay was carried out on 1ml of extract combined with 2ml of Anthrone reagent at 100°C for 7min. A spectrophotometer reading (Shimadzu UV.101.02) at 630nm was then taken. The total carbohydrate content was expressed in mg.g-1 DW, using glucose as the standard.

Proline determination:

Proline was assayed in five plants/treatments after two months of cultivation using the method of Troll and Lindsley (41) reproduced by Khan et al. (40). Extraction was carried out at 85°C for 30 minutes in a mixture of 0.1g of dry leaves and 10ml of methanol (40%). Then 1ml of acetic acid and 1ml of Ninhydrin reagent were added to 1ml of extract. The mixture was boiled for 30 minutes. After cooling, 5ml toluene was added, causing two phases to form. Na2SO4 was added to the upper phase. Optical densities were read at 528nm on a 2ml volume of the upper phase. Proline content was expressed in mg.g-1 DW using Proline as the standard.

Statistical Analysis:

The data were processed using R software (version 3.6.2 2019) by means of a 1-factor analysis of variance (ANOVA) when the data follow a normal distribution or its equivalent non-parametric Kruskal walis test in the opposite case, supplemented by the Newman&Keuls post hoc test when a significant difference with an error threshold of 5% is revealed. Finally, a PCA is performed to determine the correlations between the various parameters measured.

Results:

Effect of drought stress on morphological parameters (Shoot length, LA, FW, DW):

The statistical test revealed that water stress affects morphological parameters (Shoot length, Leaf area, fresh and dry weight) with significant differences ($P < 0.001$***). The Newman&Keuls test classified the treatments into two homogeneous groups: group a, control plants, with the highest value, and group b, stressed plants, with the lowest value.

Group a, representing the values of the morphological parameters of the control plants of stem length, leaf area, fresh and dry biomass of the aerial parts, with values estimated at 76cm±6.81; 1.47cm2 ±0.31; 1.421g ±0.23; 0.169g ±0.026 respectively (Fig.1 A, B, C, D).

Group b includes the lowest plant growth parameter values 56cm±4.11; 0.62cm2 ±0.28; 0.35g ±0.05; 0.077g ±0.01 (Fig1. A, B, C, D).

Stopping watering had a negative effect on the parameters studied (Shoot length, LA, FW, DW), causing an estimated regression of 26.72%; 57.86%; 76.36; 54.44% respectively.

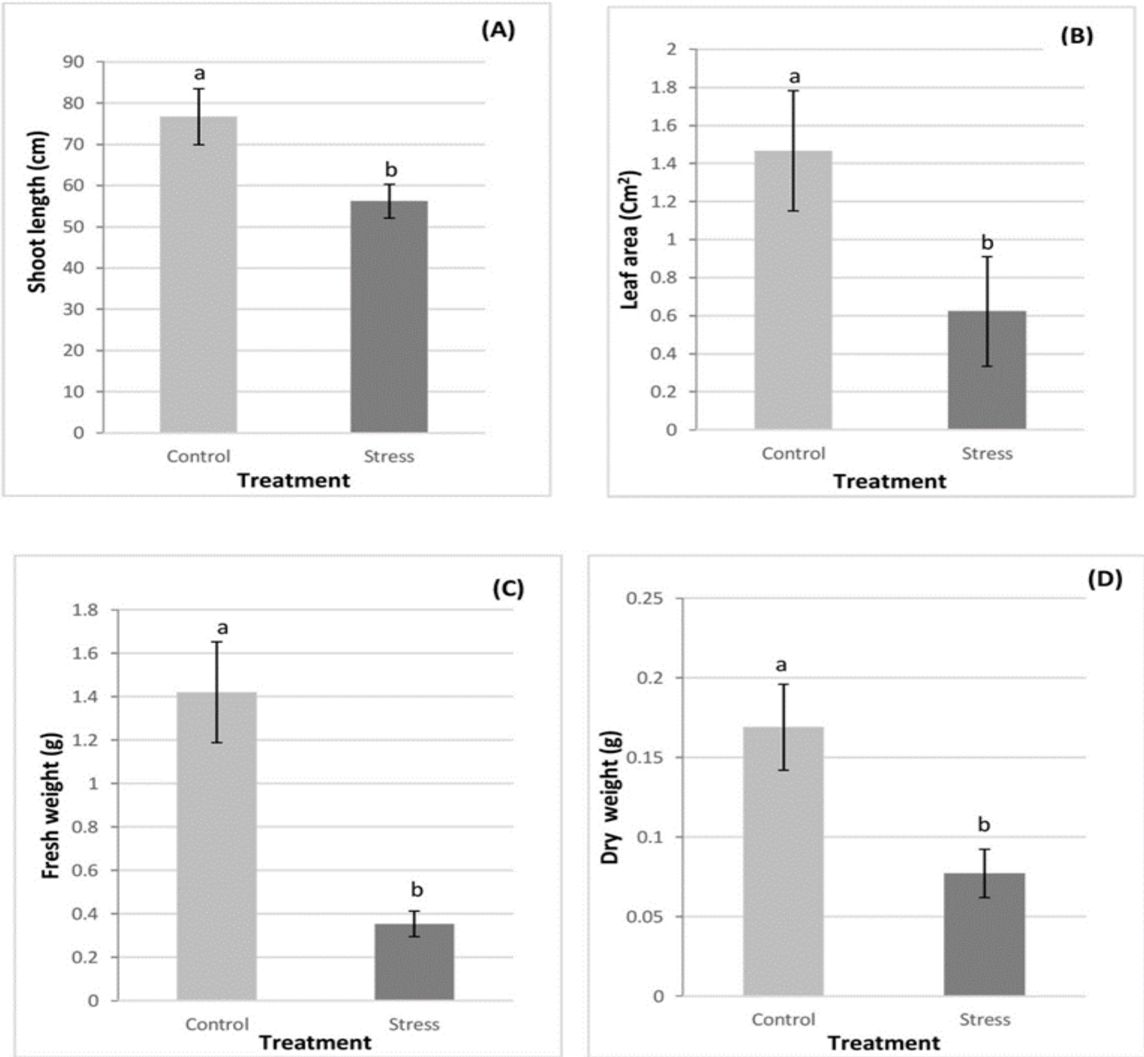

Fig 1. Shoot length (A), LA (B) and FW, DW (C, D) of *P. sativum* subsp. *arvense* under Drought stress (Stress). The letters "a, b" show significant differences according to the analysis of variance (P < 0,001*), completed with the Newman&Keuls test.**

Effect of drought stress on physiological parameters (RWC, EL):

The effect of water stress on relative water content (RWC) and electrolyte leakage (EL) was highly significant (P < 0.001***) according to the non-parametric Kruskal walis test.

Changes in relative water content (RWC) showed that water stress caused a 21.48% drop in leaf water percentage compared with the control. The Newman&Keuls test classified the RWC of the stressed leaves in group b with a percentage of 52.16%±6.68 compared with the control leaves classified in group a with a percentage of 75.09%±5.16 (Fig. 2A). In contrast to electrical leakage, where the highest value was recorded in the leaves that had undergone a watering stop (57.82%±3.49) and were classified in group a, while the control presented the lowest value (18.82%±1.53), and was statistically classified in group b (Fig. 2B), with an almost threefold rate of increase.

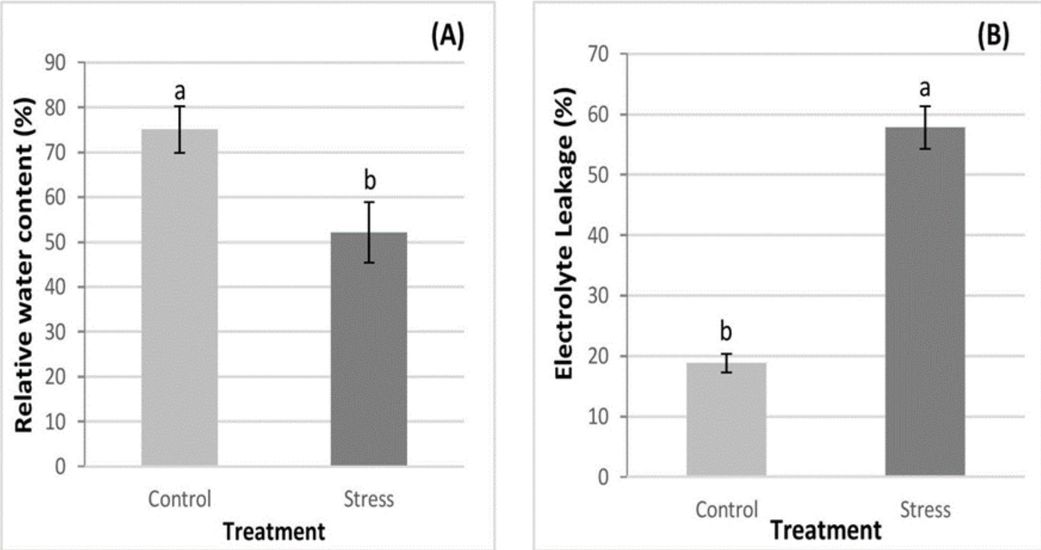

Fig 2. RWC (A), EL (B) of *P. sativum* subsp. *arvense* leaves under drought stress (Stress). The means of 30 replicates for RWC and 5 replicates for EL, the letters "a, b" show significant differences according to the analysis of variance (P < 0.001*), completed by the Newman&Keuls test.**

Effect of drought stress on biochemical parameters (soluble sugars, proline):

Stopping watering resulted in an increase in soluble sugar and proline content. The Kruskal walis test revealed a highly significant difference ($P < 0.001$***), and the Newman&Keuls test at the 5% threshold classified 2 homogeneous groups a (Stress) and b (Control).

Soluble sugars and proline content increased in the leaves of stressed seedlings (Fig.3 A,B). We found a threefold increase in proline (14±2.08 compared with 48mg/g DW±2.51) compared with the control. The leaves of the Sefrou seedlings exposed to stress underwent an increase in soluble sugars in response to the water deficit compared with the control. The high accumulation of sugar was obtained with a quantity of 20.9 mg/g DW±2.81 compared with the results from the control plants with 3.78 mg/g DW±1.05. The rate of increase was five times that of the control leaves.

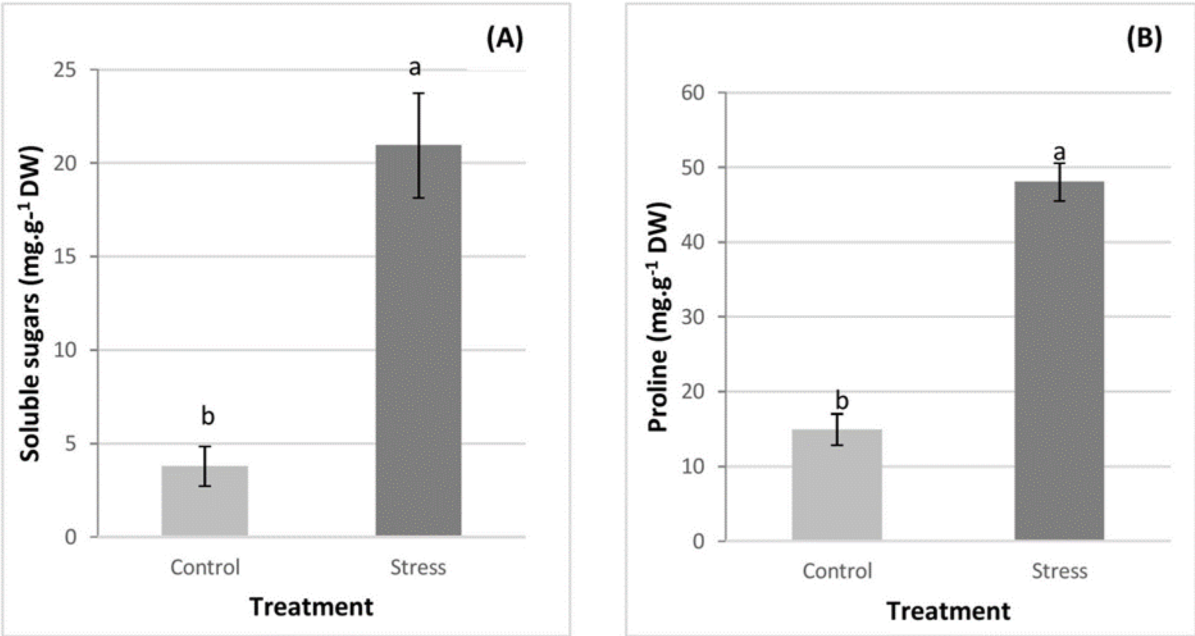

Fig 3. Soluble sugars (A) and Proline (B) content of *P. sativum* subsp. *arvense* leaves under drought stress (Stress). The letters "a, b" show significant differences according to the analysis of variance (P < 0.001*), completed with the Newman&Keuls test.**

PCA analysis:

The results of the PCA confirm the previous results by revealing the existence of a positive correlation between the RWC and morphological parameters and a negative correlation between the RWC and morphological parameters, with electrolyte leakage and soluble sugar and proline content (Fig.4). Thus, a decrease RWC causes a decrease in growth (decrease in morphological parameters) but an increase in soluble sugar and proline content and electrolyte leakage.

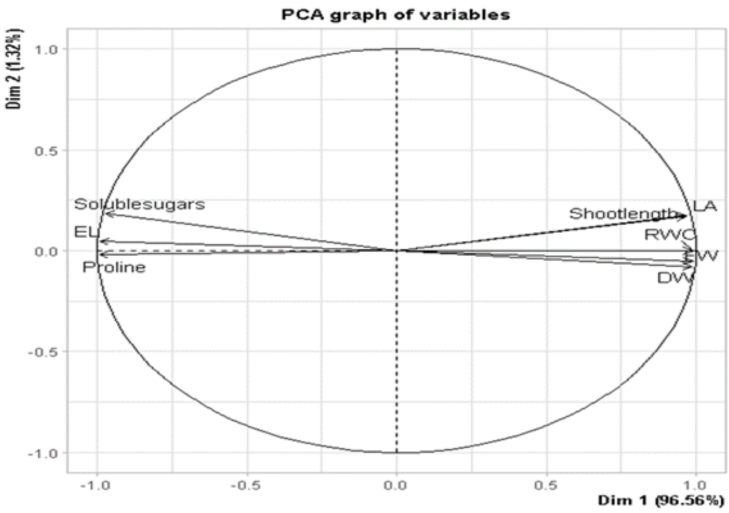

Fig 4. PCA Of The Different Quantitative Parameters Studied Under Drought Stress *In P. Sativum* Subsp. *Arvense*

Discussion:

Drought stress applied by stopping watering for 30 days showed statistically very highly significant effects (P < 0.001) for all parameters evaluated in *P.sativum* subsp. *arvense*: a decrease in RWC, a decrease in morphological parameters (thus a reduction in growth) and an increase in electrolyte leakage, soluble sugars and Proline content.

The decrease in morphological parameters observed has previously been noted by numerous authors in this species (42,19,11,43). According to Okcu et al. (44), Petroviÿ et al. (45), Pereira et al. (46) and Tamindžić et al. (47), low growth is due to reduced shoot growth and not root growth in *Pisum sativm*. Embiale et al. (10) even observed a reduction in growth as early as the sixth day after watering was stopped (20% reduction) and a 53% reduction after just 12 days of watering in the *Pisum sativum* cultivar 'Brukitu'. The 57% reduction in leaf area obtained confirms the results of Arafa et al. (48) and Sutulienè et al. (49), who reported a 50% reduction after 15 days of watering, by comparing these results; our cultivars appear to be more tolerant in terms of stress duration (30 days).

One of the first reactions of plants to drought deficit is to reduce leaf area in order to reduce water loss through transpiration (50). In many species exposed to water stress, this results in a significant modification of plant architecture to save water, at the cost of yield loss (51). Growth reduction is caused in many legume species (52,53,54,55,56). Decreased growth in plants exposed to water stress is attributed to loss of turgor and reduced cell enlargement and growth (44,57,54,47), reduced net photosynthesis (58) through stomatal closure (57,54) and the production of toxic reactive oxygen species (59). Impaired mitosis and reduced cell elongation and expansion at root level result in reduced plant height, leaf area and crop growth in *Helianthus annuus* L. (60,61). Reduced growth is also attributed to decreased hormone and enzyme secretion and ionic imbalance (62). Reduced plant dry weight under water stress could be the result of unbalanced stomatal conductance leading to reduced carbon assimilation per unit leaf area and low biomass production (63,64).

Decreased relative water content is one of the first symptoms of water deficiency in plant tissue (water status) (65). The negative correlation between decreasing RWC and increasing EL observed are in agreement with results obtained in *Vicia sativa* (Mahali) (66,67) and *Pisum sativum* (10,68,11). An increase in electrolyte leakage of 30% after 15 days of watering cessation was observed by Dziurka et al. (69) in *Pisum sativum*.

Sreenivasulu et al. (70) have shown that there is a positive correlation between water stress and cell membrane damage; cell membranes undergo increased permeability and loss of integrity under environmental stress (71). Indeed, it is generally accepted that maintaining cell membrane integrity and stability under water stress conditions is a major component of plant drought tolerance (72,66,73), and electrolyte leakage is even recommended as a valuable criterion for identifying stress-tolerant varieties in several species (67).

The accumulation of sugars and proline (5 times more and 3 times more in the stressed respectively) induced after 30 days of watering cessation in this variety of Pisum sativum was previously observed in Pisum after 20 days of watering cessation (approximately 1.5 times more sugars and 3 times more proline in the stressed than in the controls) (43) and a doubling of proline and sugar was noted by Latif, (42) and Al-quraan et al. (19) in which species of Pisum sativum after 14 days of stress. In pea, the increase in sugar concentration under drought conditions may be the result of an increase in starch and/or sucrose degradation rates (73). The proline content of leaves from plants exposed to severe osmotic stress can increase up to 100-fold, and its concentration can reach 80% that of controls in many species (74,75,76).

Conclusion

The present study concludes that the severe stress applied considerably reduces vegetative growth, although the reduction rates are low compared with the results of previous studies on Pisum sativum subjected to mild stress. The induced accumulation of soluble sugars and proline may be a criterion of tolerance to the physiological impact of stress by protecting plants from oxidative stress. In order to provide further information on this cultivar, a complementary field study is required.

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