# Soluble sugars and proline accumulation play a role as effective indices for drought tolerance screening in Persian walnut (*Juglans regia* L.) during germination

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## Soluble sugars and proline accumulation play a role as effective indices for drought tolerance screening in Persian walnut (*Juglans regia* L.) during germination.

**Abstract** -- **Introduction**. Drought stress is the major factor affecting growth, development and production of walnut trees. In Iran, approximately 33 Mha of land is affected by salinization and drought stress. Finding genetic resources tolerant to drought stress at different growth stages is important for such semi-arid regions. Our aim was to understand better the adaptive mechanisms that enable different genotypes of walnut population to survive under drought stress, and to provide some useful clues for walnut tree breeding toward improved drought tolerance with utilization of existing drought-tolerant genetic resources. **Materials and methods**. To study the mechanism(s) involved in drought tolerance of some Persian walnut genotypes, drought stress was induced using polyethylene glycol-6000 to produce water potentials of 0 Mpa (control), –0.10 MPa, –0.50 MPa, –0.75 MPa, –1.00 MPa, –1.50 MPa and –2.00 MPa. The amount of proline and soluble sugar accumulation in four walnut genotypes ('Panegine$_{20}$', 'Lara', 'Serr' and 'Chandler') were determined after being exposed to the various water potential levels. **Results**. The rates of seed germination in all genotypes were significantly reduced by low external water potentials. Plants exposed to water stress had a higher amount of soluble sugars in roots and shoots of tolerant genotypes ('Panegine$_{20}$' and 'Chandler') and a lower amount of starch in their tissues. These results imply the important roles of soluble sugars as solutes conferring resistance to drought in these genotypes. The free proline levels were also increased in response to drought stress. They were higher in drought-tolerant genotypes than in sensitive ones ('Lara' and 'Serr'). Proline increased more in shoots than in roots. However, the soluble sugar and starch fluctuations were higher in the roots. **Conclusion**. Our results support a direct correlation between the degree of drought stress and proline content. As a consequence, proline concentrations could be used as a biochemical marker of drought stress level in walnut plants.

**Iran Islamic Republic / *Juglans regia* / genetic resources / drought stress / seedlings / sugars / proline**

## Les sucres solubles et l'accumulation de proline sont des indicateurs efficaces pour la sélection de noyers persans (*Juglans regia* L.) tolérants à la sécheresse pendant la germination.

**Résumé** -- **Introduction**. Le stress dû à la sécheresse est le principal facteur influant sur la croissance, le développement et la production de noyers. En Iran, environ 33 Mha de terres sont affectées par la salinisation et la sécheresse. Trouver des ressources génétiques tolérantes à la sécheresse à différents stades de croissance est important pour ces régions semi-arides. Notre objectif a été de mieux comprendre les mécanismes adaptatifs qui permettent aux différents génotypes de noyers de survivre en conditions de stress hydrique et de fournir quelques indications utiles pour une amélioration de ces arbres vis-à-vis de la tolérance à la sécheresse en utilisant des ressources génétiques existantes. **Matériel et méthodes**. Pour étudier les mécanismes de certains génotypes de noyers persans impliqués dans la tolérance à la sécheresse, un stress hydrique a été induit en utilisant du polyéthylène glycol-6000 pour produire des potentiels hydriques de 0 Mpa (témoin), –0.10 MPa, –0.50 MPa, –0.75 MPa, –1.00 MPa, –1.50 MPa et –2.00 MPa. Le niveau d'accumulation de proline et de sucres solubles dans quatre génotypes de noyer ('Panegine$_{20}$', 'Lara', 'Serr' et 'Chandler') a été déterminé après qu'ils ont été exposés aux différents niveaux de potentiels hydriques. **Résultats**. Pour les quatre génotypes étudiés, les taux de germination des semences ont été considérablement réduits par les bas potentiels hydriques. Les semis des génotypes tolérants ('Panegine$_{20}$' et 'Chandler') exposés au stress hydrique ont présenté les plus grandes quantités de sucres solubles dans les racines et les tiges, mais une moindre quantité d''amidon dans leurs tissus par rapport aux génotypes sensibles. Ces résultats suggèrent l'importance des sucres solubles comme solutés conférant, à ces génotypes, la résistance à la sécheresse. Les niveaux de proline libre ont également été augmentés en réponse à la sécheresse. Ils ont été plus élevés chez les génotypes tolérants à la sécheresse que chez les plus sensibles ('Lara' et 'Serr '). La teneur en proline a davantage augmenté dans les tiges que dans les racines. Toutefois, les fluctuations du sucre soluble et de l'amidon ont été plus élevées dans les racines. **Conclusion**. Nos résultats confirment qu'il existe une corrélation directe entre le degré de stress hydrique et la teneur en proline dans les plantules. En conséquence, les concentrations en proline pourraient être utilisées comme marqueurs biochimiques du niveau de stress hydrique chez le noyer.

**Iran République islamique / *Juglans regia* / ressource génétique / stress dû à la sécheresse / semis / sucres / proline**

## 1. Introduction

Iran is located in the mid-latitude belt of arid and semi-arid regions of the Earth in the Asian continent [1]. About 42 Mha of land in South Asia is affected by salinization and drought stress [2]. Approximately 33 Mha of these are in Iran, where about 55% of all agricultural lands are affected. Finding genetic resources tolerant to drought stress at different growth stages is important for such semi-arid regions.

The majority of walnut orchards in the world are propagated by seed or by grafting onto seedling rootstocks [3]. Hence, there is a high amount of genetic diversity in their rootstock-related traits. There are many elderly walnut trees in Iran that are usually planted on the borders of rivers, in the environs of other fruit tree orchards or interplanted with other fruit trees; some of these trees reach 300–700 years. Existence of such elderly walnut trees indicates the presence of noteworthy resistance genes for different types of stress in these tree genotypes that could grow in unfavorable environmental conditions [3].

Persian walnut (*Juglans regia* L.) is one of the most economically valuable tree species of northwest, northeast and central regions of Iran. Natural distribution of this species is quite sensitive to site water status. Walnut trees need large amounts of water for optimum growth and productivity, and are among the more sensitive plants to abiotic stresses; water management is a fundamental cultural practice affecting orchard performance [4]. Water stress in walnut can either be the result of too much or too little water and sometimes the combination of both at different times in the life of an orchard. It may be expressed by relatively acute symptoms such as leaf wilt, reduced shoot growth, sunburn and defoliation. Water stress may also be expressed by chronic symptoms such as shoot die-back, crown and root rot, and tree decline and eventual death [4, 5]. There is a straight inverse relationship between stress treatment and most of the vegetative growth and visual parameters (leaf area, leaf necrotic area, dry weight of different parts of the plant, shoot diameter, etc.) [6, 7].

Different species have different ways of adapting to drought [8]. Walnuts close stomata under high leaf-to-air vapor pressure deficit (VPD) or low leaf water potential, preventing the stem water potential ($\Psi_s$) from becoming lower than –1.4 MPa, when cavitation occurs in the xylem [9]. Hence, walnut has been defined as a "drought avoider" [10]. In a drying soil, net photosynthesis and leaf conductance to water vapor declined substantially, even under mild water stress. These responses were more strongly related to soil water status, as estimated by predawn leaf water potential, than to leaf water potential at the time of gas exchange measurement [11]. It has been shown that there are no differences among families in the pattern of gas exchange response to developing water stress; however, families differed in capacity for recovery of gas exchange from water stress following rehydration [11].

Plants resort to many adaptive strategies in response to abiotic environmental stresses such as dehydration and excessive osmotic stress. These adaptive mechanisms involve changes in physiological and biochemical processes. Among these, the accumulation of compatible solutes in response to drought stress has drawn much attention [12]. Adaptation to these stresses is associated with metabolic changes that lead to the accumulation of several organic solutes such as sugars, polyols, betaines and proline in plants [12]. The compatible solutes may be classified into two categories: (1) nitrogen-containing compounds such as proline and other amino acids, quaternary ammonium compounds and polyamines, and (2) hydroxyl compounds, such as sucrose, polyhydric alcohols and oligosaccharides [13]. Other functions related to proline accumulation have also been proposed, including stabilization of macromolecules, a source of carbon and nitrogen to be used after plants are relieved from water deficit [14, 15], as an antioxidant [16] and regulation of cellular redox status [17].

There are several reports on carbohydrate accumulation during reproductive development in cereals and temperate grasses in response to various abiotic stresses [18, 19]. Different authors point to

the role of soluble sugars in the protection of plants against stresses. Utilization of storage reserves in the endosperm of cereal seeds is tightly regulated and has a primary pivotal role in the interactions among sugars, ABA and gibberellin pathways responsible for plant responses to drought [20]. A central role of sugars depends not only on their direct involvement in the synthesis of other compounds and energy provision, but also on stabilization of membranes [21], acting as regulators of gene expression [22] and signal molecules [23, 24]. Soluble sugar content has proved to be a better criterion than proline content in screening durum wheat (*Triticum durum* Desf.) for drought tolerance [25].

Osmotic solutions are used to impose water stress reproducibly under *in vitro* conditions [26]. Polyethylene glycol molecules with a molecular weight 6000 (PEG 6000) are inert, non-ionic and virtually impermeable chains that have frequently been used to induce water stress and maintain uniform water potential throughout the experimental period [27, 28]. Molecules of PEG 6000 are small enough to influence the osmotic potential, but large enough to not be absorbed by plants [29] because PEG does not enter the apoplast and water is withdrawn from the cell and the cell wall. Therefore, PEG solutions mimic dry soil more closely than solutions of low $M_r$ osmotica, which infiltrate the cell wall with solutes [30].

Genetic variation in growth indices and biochemical and cellular responses to water stress of walnut, especially in germination and early growth of seedlings, have not been studied. However, there are economic incentives for identification of drought-adapted genotypes of walnut for planting in these extensive arid and semi-arid regions. Our preliminary experiments revealed that walnut seedlings are very tolerant to drought and especially to salt stresses at the germination stage [31, 32]. Therefore, it was decided to find out how much the accumulation of proline and soluble carbohydrates contributes to drought tolerance in various walnut genotypes at the germination stage. Therefore, to look into drought stress-induced biochemical changes and to elucidate adaptive mechanisms at the cellular level, different concentrations of PEG-6000 were used as an osmoticum to investigate the amount of carbohydrates and proline in walnut seedlings grown under normal and osmotic stress conditions.

Our aim was not only to understand better the adaptive mechanisms that enable walnut populations of different origin (different genotypes) to survive under drought stress, but also to provide some useful clues for walnut tree breeding toward improved drought tolerance by utilization of existing drought-tolerant genetic resources. The results may help in using the degree of proline accumulation as a screening index for drought tolerance in walnut genotypes.

## 2. Materials and methods

### 2.1. Plant materials

The half-sib seeds from four open pollinated walnut genotypes (*J. regia* L.), 'Panegine$_{20}$', 'Lara', 'Serr' and 'Chandler', were used. Among these genotypes, 'Panegine$_{20}$' is grown in the Kerman area in the Southeast of Iran. The other genotypes were obtained from the clonal collections of the Kamal Shahr Station of Seed and Plant Improvement Institute, South of Karaj County, Iran. The climate of Karaj County is characterized by 242 mm average annual rainfall, a relatively long (about 5–6 months) drought period per year, cold winters and 5 months of frost period (October–April), lat. 35.95 N, long. 51.60 E, alt. 1321 m above sea level. At the beginning, PEG-6000 was used to produce media of different osmotic potentials [31, 32]. Our results revealed that some of the walnut genotypes have high potential in coping with drought stress. From our preliminary study, two tolerant and two sensitive genotypes were selected to compare their responses to drought stress at the cellular level.

### 2.2. Preparation of osmotic media and seed germination

Polyethylene glycol (PEG-6000) (Duchefa Biochemic. Co., 2003 RV Haarlem, The Netherlands) was applied to induce drought stress. Six drought stress levels with osmotic potentials of (–0.10, –0.50, –0.75, –1.00,

–1.50 and –2.00) MPa were prepared by using PEG as described by Michel and Kaufmann [33]. Distilled water was used as control (0 MPa). Seeds were allowed to germinate at (25 ± 1) °C under a 12 h·d$^{-1}$ photoperiod at a light intensity of (350 ± 15) µmol·m$^{-2}$·s$^{-1}$ PPFD (Photosynthetic Photon Flux Density). Seeds were soaked in water for 10 days before being pre-treated with a fungicide (Captan 5%) before chilling treatment [34]. Seeds were then placed in a refrigerator for a period of 2–4 weeks at 4–6 °C to overcome their chilling requirements. At the end of the chilling period, seeds were placed in small size polyethylene pots (250 mL) containing pure medium size perlite granules. A 50-mL PEG solution with known osmotic potential was added to each pot. Pots were weighed and their surfaces were covered with plastic film to prevent surface evaporation; they were then placed in a growth chamber maintained at (25 ± 1) °C and 46% relative humidity. Pots were weighed every evening and distilled water was added to make up for the amount lost by evaporation. No nutrient solution was added to the pots. Seedlings were grown for a period of 29 days in these conditions.

## 2.3. Measurement of growth indices

When the primary root length of the control seedlings (0 water potential) was 4 cm long, the following growth indices were recorded: seed germination percent, root length, root dry weight and relative water content.

Root length was measured by Vernier calliper (LG Co.) by (125 × 0.02) mm or by (5 × 1.1000) inch accuracy.

For dry weight determination, samples were dried in an oven set at 72 °C for 48 h. Values of relative water content (RWC) were determined by the following equation: RWC = [(FW – DW) / (SW – DW)], where FW is the tissue fresh mass; DW, the tissue dry weight; and SW is the saturated weight (turgid mass). Dry weight was determined after drying the samples at 80 °C for 24 h. For SW determination, tissues were rehydrated by immersing them in distilled water in a big beaker sealed with parafilm. Full rehydration was achieved in 24–48 h in complete darkness at 2–4 °C.

## 2.4. Soil water status

Medium water content at field capacity was determined gravimetrically. After percolation of gravitational water, four 200-g medium samples were taken from four pots without seedlings and covered with a plastic film to prevent evaporative water loss. Medium water content at the permanent wilting point was measured gravimetrically on four medium samples taken from four randomly selected pots with seedlings. Medium available water was obtained by calculating the difference between medium water content at field capacity and medium water content at permanent wilting point.

## 2.5. Plant water status

Throughout the drought treatment, plant water status was determined by simultaneous measurements of $\Psi_w$ and leaf water content on 4 to 5 fully expanded leaves from each plant taken from the mid-section of shoots. Each excised leaf was immediately put inside a polyethylene bag for $\Psi_w$ measurement. All predawn and midday $\Psi_w$ measurements were made with a pressure chamber (Model 600, PMS Instruments, Corvallis, OR), according to Turner [35].

## 2.6. Proline analysis

Free proline accumulation was determined using the method of Bates *et al.* [36]. Root and shoot fresh tissues (0.5 g) were homogenized with 3% sulfosalicylic acid and, after 72 h, the homogenates were centrifuged at 3000 *g* for 20 min. The supernatants were treated with acetic acid and ninhydrin, boiled for 1 h and then absorbance of colored phases was read at 520 nm using a UV-visible spectrophotometer (Model Lambda 25, Perkin Elmer Co.). Proline concentration, in µg·g$^{-1}$ fresh weight, was calculated using L-proline for the standard curve.

## 2.7. Soluble carbohydrate analysis

Sugars were extracted from the root and shoot samples as follows: 0.1 g (dry weight) of samples were added to a centrifuge tube and homogenized with 2 mL of 80% ethanol solution in a vortex for 50 s. Then tubes

were centrifuged at 2700 *g* for 10 min. The insoluble residue was removed by centrifuge and the precipitate was re-extracted with 2 mL of 80% ethanol and re-centrifuged. The supernatants were pooled and dried at 35–45 °C. Also, supernatants were evaporated in an oven at 100 °C. After evaporation of supernatants, residues were washed with 20 mL tepid distilled water and placed in centrifuge tubes. Then 10 mL of 0.3 N BaOH and 10 mL of 5% Zn $(SO_4)_2$ solutions were added. The solutions were placed in a dish and precipitate was dissolved in 20 mL water and re-centrifuged. This solution was added to the previous until the terminal volume reached 50 mL. This solution, including monosaccharide and oligosaccharide, was used to assay soluble sugars. The absorbance of solutions was read at 485 nm using a spectrophotometer. Soluble sugars were determined using the phenol-sulfuric acid method [37]. Soluble sugar content was calculated using glucose for the standard curve.

### 2.8. Starch analysis

Starch content was determined using the phenol-sulfuric acid method [37]. The materials left on filters in soluble sugar analysis were dried, weighed and boiled with deionized water. The solutions above the sediments were used for starch analysis. Glucose was used as a standard for both soluble sugar and starch.

### 2.9. Statistical analysis

The experimental design for growth indices was factorial, including two factors (7 osmotic levels × 4 seedling genotypes), arranged in a completely randomized design with four replications and 12 seeds per replicate (for the germination test four replications and 50 seeds per replication were used). The first factor was the seven osmotic levels [(0.00 (control), –0.10, –0.50, –0.75, –1.00, –1.50 and –2.00) MPa] and the second was the four seedling genotypes ('Panegine$_{20}$', 'Lara', 'Serr' and 'Chandler'). The mean values of proline, soluble sugar and starch content were taken from the measurements of three replicates and the standard error of the means was calculated. One-way ANOVA was applied to determine the significance of the results between different treatments; Duncan's multiple range test was performed at the probability level of 5%. Analysis of variance and comparison of means were performed using SAS (SAS Institute Inc., Cary, NC) software. In order to screen the tolerant genotypes, we classified the genotypes by cluster analysis using SPSS. Cluster analysis of genotypes was performed by the UPGMA method using the square Euclidean distance for all trait means related to drought tolerance at –1 MPa osmotic level. The number of the groups was determined by comparing variations in the square Euclidean distances of each step of cluster analysis [38].

## 3. Results

### 3.1. Effects of drought stress on growth indices

The final seed germination percentage of walnut genotypes in response to osmotic stresses was significantly different (*table I*).

In normal conditions, 'Panegine$_{20}$' (86.26%) and 'Lara' (58.73%) had the highest and the lowest germination rates, respectively.

With the decrease (more negative) in water potentials, there was a significant decrease in the final germination percentage. Decreasing the water potential to –1.0 MPa reduced the germination of all families to less than 50% and, at –1.50 MPa, the germination decreased to less than 25% (*table I*).

Because the genotypes and osmotic level effects, as well as genotypes × osmotic level interaction, were highly significant ($P \leq 0.05$), the means of the genotypes × osmotic level interaction were compared by Duncan's multiple range test ($P \leq 0.05$, *tables I–IV*). Measuring growth indices indicated that, by increasing osmotic stress levels, root length was decreased (*figure 1*). Under normal conditions (distilled water), the offspring of 'Chandler' had the highest root length. Under high osmotic level of drought stress, 'Chandler' and 'Panegine$_{20}$' had the highest

**Table I.**
Final germination percentage (%) of control and drought-stressed walnut seeds, observed 29 days after seeds were allowed to germinate on substrates with different water potentials. Each value is the mean ± standard error of four tray measurements of 50 seeds that were used for each tray.

| Walnut cultivars | Water potential (MPa) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | –0.1 | –0.5 | –0.75 | –1 | –1.5 | –2 |
| Chandler | 74.38 ± 0.21 b | 72.38 ± 0.20 bc | 67.92 ± 0.18 bc | 59.34 ± 0.15 c | 46.15 ± 0.12 cd | 34.85 ± 0.09 d | 33.97 ± 0.05 d |
| Lara | 58.73 ± 0.09 c | 53.24 ± 0.08 de | 49.58 ± 0.06 cd | 38.86 ± 0.04 d | 22.61 ± 0.03 e | 18.15 ± 0.01 f | 15.42 ± 0.14 g |
| Panegine$_{20}$ | 86.26 ± 0.16 a | 84.26 ± 0.12 a | 67.34 ± 0.10 bc | 62.73 ± 0.08 c | 53.61 ± 0.03 c | 37.58 ± 0.01 d | 35.81 ± 0.09 d |
| Serr | 59.37 ± 0.16 c | 52.62 ± 0.13 c | 43.29 ± 0.09 de | 34.65 ± 0.06 d | 23.55 ± 0.04 e | 11.34 ± 0.01 fg | 9.57 ± 0.07 h |

Means followed by the same letter are not significantly different at $P \leq 0.05$, according to Duncan's multiple range test (four replications).

**Table II.**
Effect of different water potentials on [soluble sugar / starch] ratio in the roots of four walnut genotype seedlings. Results are shown as mean ± standard error.

| Walnut cultivars | Water potential (MPa) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | –0.1 | –0.5 | –0.75 | –1 | –1.5 | –2 |
| Chandler | 0.78 ± 0.01 s | 1.01 ± 0.05 p | 1.09 ± 0.01 n | 1.23 ± 0.00 l | 1.4 ± 0.01 j | 1.78 ± 0.02 h | 2.08 ± 0.03 a |
| Lara | 2.1 ± 0.02 a | 1.95 ± 0.00 b | 1.45 ± 0.00 i | 1.44 ± 0.00 i | 1.43 ± 0.00 i | 1.42 ± 0.00 i | 1.36 ± 0.00 k |
| Panegine$_{20}$ | 0.69 ± 0.00 t | 0.79 ± 0.00 s | 0.9 ± 0.00 q | 1.2 ± 0.01 m | 1.61 ± 0.00 g | 1.83 ± 0.01 e | 2.1 ± 0.00 a |
| Serr | 0.83 ± 0.00 r | 0.91 ± 0.00 q | 1.06 ± 0.01 o | 1.34 ± 0.05 k | 1.7 ± 0.02 f | 1.72 ± 0.01 d | 1.91 ± 0.00 c |

Means followed by the same letter are not significantly different at $P \leq 0.05$, according to Duncan's multiple range test (three replications).

**Table III.**
Effect of different water potentials on [soluble sugar / starch] ratio in the shoots of four walnut genotype seedlings. Results are shown as mean ± standard error.

| Walnut cultivars | Water potential (MPa) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | –0.1 | –0.5 | –0.75 | –1 | –1.5 | –2 |
| Chandler | 0.68 ± 0.04 p | 1.15 ± 0.00 m | 1.26 ± 0.00 k | 1.39 ± 0.02 j | 1.54 ± 0.03 f | 1.73 ± 0.05 e | 2.08 ± 0.04 c |
| Lara | 2.21 ± 0.03 a | 1.44 ± 0.02 i | 1.45 ± 0.04 i | 1.44 ± 0.06 i | 1.44 ± 0.02 i | 1.43 ± 0.00 i | 1.42 ± 0.03 i |
| Panegine$_{20}$ | 0.65 ± 0.02 p | 0.75 ± 0.00 o | 0.94 ± 0.00 n | 1.24 ± 0.01 k | 1.68 ± 0.00 e | 1.87 ± 0.02 d | 2.14 ± 0.05 b |
| Serr | 1.36 ± 0.00 j | 1.42 ± 0.00 i | 1.42 ± 0.02 i | 1.44 ± 0.04i | 1.46 ± 0.00 h | 1.49 ± 0.00 g | 1.52 ± 0.07 f |

Means followed by the same letter are not significantly different at $P \leq 0.05$, according to Duncan's multiple range test (three replications).

**Table IV.**
Percent of relative water content (%) in roots of control and drought-stressed walnut seedlings, measured 29 days after seeds were allowed to germinate on substrates with different water potentials. Each value is the mean ± standard error of four measurements each with four seeds per salinity treatment.

| Walnut cultivars | Water potential (MPa) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | –0.1 | –0.5 | –0.75 | –1 | –1.5 | –2 |
| Chandler | 91.57 ± 0.06 a | 91.34 ± 0.06 a | 90.45 ± 0.08 a | 88.46 ± 0.06 a | 87.72 ± 0.14 a | 86.38 ± 0.04 a | 84.67 ± 0.04 a |
| Lara | 79.43 ± 0.08 c | 75.32 ± 0.09 d | 70.26 ± 0.05 d | 68.89 ± 0.09 d | 61.18 ± 0.07 d | 56.26 ± 0.03 d | 51.68 ± 0.11 d |
| $Panegine_{20}$ | 84.43 ± 0.09 b | 82.97 ± 0.03 b | 80.53 ± 0.05 b | 77.68 ± 0.07 b | 74.19 ± 0.05 b | 71.92 ± 0.06 b | 68.23 ± 0.06 b |
| Serr | 84.13 ± 0.08 b | 81.45 ± 0.07 bc | 76.23 ± 0.04 c | 72.98 ± 0.09 c | 68.46 ± 0.09 c | 64.83 ± 0.16 c | 59.48 ± .014 c |

Means followed by the same letter are not significantly different at $P \leq 0.05$, according to Duncan's multiple range test (four replications).

root length in response to different osmotic stress levels (*figure 1*). Furthermore, root dry weight in the offspring of most genotypes decreased significantly ($P \leq 0.05$) in response to increase in osmotic stress levels. Whereas, in tolerant genotypes, differences between means of root dry weight by increase in osmotic potential were not significant (*figure 2*). 'Lara' seedlings were severely affected by drought stress, while 'Chandler' seedlings were the least affected. The increase in osmotic drought level was accompanied by a substantial decrease in root relative water content and differences between genotypes at different osmotic levels were highly significant (*table IV*).

## 3.2. Effects of drought stress on plant water status

The decline in relative water content in the walnut seedlings at different osmotic potential was paralleled by a substantial decrease in water potential ($\Psi_w$), especially in tolerant genotypes (*table IV*, *figure 2*). Values of $\Psi_w$ decreased during the day and subsequently recovered and re-equilibrated at night, showing a pattern of progressive decline during the drought treatment. During the last day (29th day) of the drought treatment, $\Psi_w$ decreased in all plants subjected to drought stress. But in '$Panegine_{20}$' and 'Chandler' progeny, there was a quick reduction in $\Psi_w$ from –1.8 MPa in control plants to –4.9 at –2.0 MPa of osmotic treatments (*figure 2*).

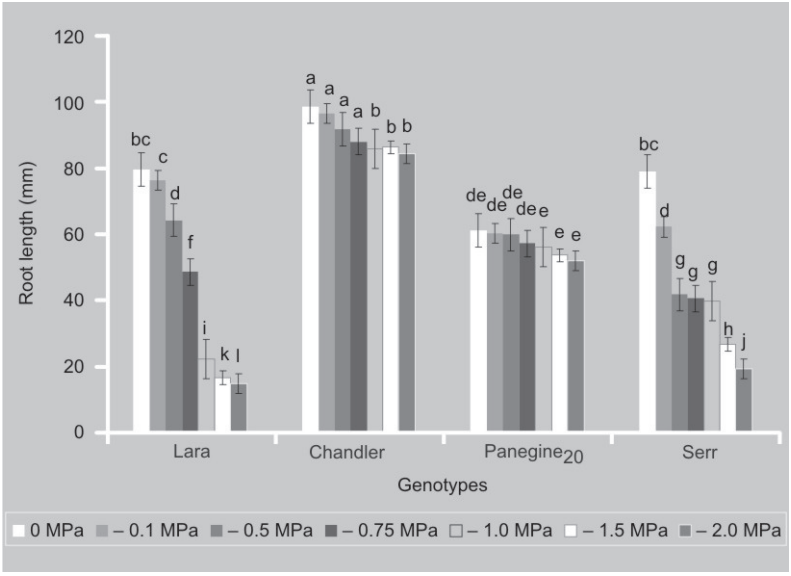


**Figure 1.**
Root length of control and drought-stressed walnut seedlings, observed 29 days after seeds were allowed to germinate on substrates with different water potentials. Each value is the mean ± standard error of four measurements on four seeds per drought treatment. Means followed by the same letter are not significantly different at $P \leq 0.05$, according to Duncan's multiple range test (four replications).

## 3.3. Effects of drought stress on proline content

Proline content increased significantly ($P \leq 0.05$) in relation to the severity of drought stress, in particular in roots of tolerant genotypes (*figure 3*). There was a rapid increase in proline at leaf water potentials lower than –1.5 MPa. Proline content of both genotypes was elevated linearly with increase in water deficit. At water potential –2.0 MPa, root proline content increased 2.13 times in '$Panegine_{20}$' and 1.70 times in 'Chandler'. Shoot proline

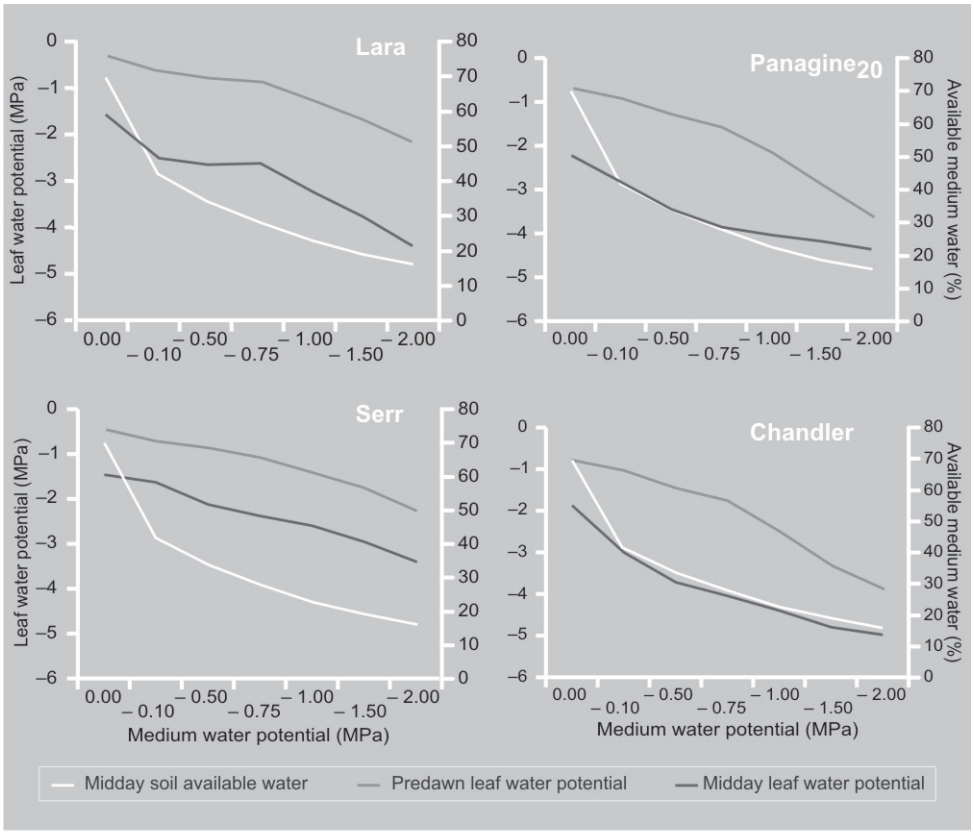


**Figure 2.**
Patterns of predawn leaf water potential, midday leaf water potential and soil available water measured in walnut seedlings during drought treatments.

content increased 2.10 times in 'Panegine$_{20}$' and 1.74 times in 'Chandler' progeny as compared with the control plants. Increase in proline content in 'Panegine$_{20}$' was higher than in 'Chandler' and it was higher in roots than in shoots (*figure 3*).

### 3.4. Effects of drought stress on soluble sugar and starch content

Imposition of different polyethylene glycol treatments on all genotypes of walnut seedlings significantly ($P \leq 0.05$) increased total soluble sugar content (*figure 4*). As compared with control, a drastic increase was observed in shoots and roots. Root soluble sugar content increased 1.65 times in 'Panegine$_{20}$' progeny and 1.70 times in 'Chandler', and shoot soluble sugar content increased 1.73 times in 'Panegine$_{20}$' and 1.60 times in 'Chandler' as compared with control plants. But starch content significantly decreased ($P \leq 0.05$) in roots and shoots of both genotypes. Root starch content decreased 49.46% in 'Panegine$_{20}$' and 38.18% in 'Chandler'. However, shoot starch content decreased 52.79% in 'Panegine$_{20}$' and 47.42% in 'Chandler' as compared with control plants (*figure 5*).

### 3.5. Cluster analysis

Based on the results of cluster analysis, seedlings of the tested genotypes were classified into two groups: the 'Serr' and 'Lara' genotypes were in the first group and the 'Chandler' and 'Panegine$_{20}$' genotypes were in the second group (*figure 6*). Both the cluster analysis and measured growth traits indicated that seedling genotypes of 'Chandler' and 'Panegine$_{20}$' were the most drought-tolerant (*figure 7*). The 'Serr' and 'Lara' genotypes were found to be sensitive genotypes.

### 3.6. Correlation coefficients among some physiological properties

Root length showed a significant correlation with relative water content. The soluble sugar content, proline, starch and soluble sugar to starch ratio in shoots were positively correlated with the same parameters in roots. A significant positive correlation was also observed between proline content, starch content and soluble sugar (*table V*). In addition, under drought stress, root dry weight showed a poor negative correlation with the soluble sugar to starch ratio (*table V*).

## 4. Discussion

Decreasing the water potential in the substrate decreased the germination rate, indicating that water stress inhibits germination [31]. Germination percentage was reduced by more than 50% when the water potential was –0.75 MPa (*table I*). Seeds from all genotypes germinated at an osmotic potential of –1 MPa, although the seed germination at osmotic potential of –1.5 MPa was reduced by more than 80%. Among the four genotypes tested for drought tolerance, 'Panegine$_{20}$' had the highest germination percentage (*table I*).

Plant adaptations to drought stress are complex and affected by internal constitutive drought tolerance mechanisms and external environmental factors such as water availability, or their interaction. An early morphological response of plants to drought stress is the avoidance mechanism through adjustment of plant growth rate such as a reduction in root height and root dry weight. Moreover, plants can exploit the limiting water resource in a more efficient way by increasing the proportion of water-absorbing root biomass relatively to the water-losing leaf biomass [39, 40]. This is in agreement with the results of our experiment since plant growth was significantly inhibited in the four walnut genotypes when subjected to the drought stress. Moreover, significant differences between the four genotypes were observed in root length and root dry weight under different osmotic

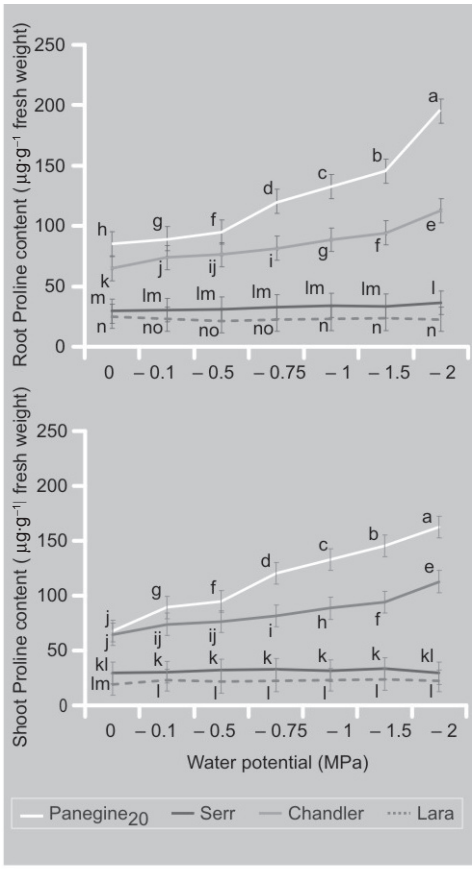


**Figure 3.**
Effect of different water potentials on proline content in roots and shoots of four walnut genotype seedlings. Results are shown as mean ± standard error, obtained from three replicates. Means followed by the same letter are not significantly different at $P \leq 0.05$, according to Duncan's multiple range test.

potentials. The 'Lara' and 'Serr' genotypes had smaller root length and root dry weight under lower osmotic potentials, but 'Chandler' and 'Panegine$_{20}$' genotypes were less affected by drought stress. Our results are consistent with previous studies [39, 41]. Root length growth showed the same trend as seed germination except for 'Chandler' and 'Panegine$_{20}$', whose root lengths showed no significant difference ($P \leq 0.05$) between control and different osmotic potential (*figure 1*). Decreases in root length with osmotic potential were observed from –0.5 MPa to –2 MPa (*figure 1*). Root length of 'Chandler' and 'Panegine$_{20}$' plants was greater under osmotic potentials of ≥ –1.5 MPa than root length of other genotypes. Our findings on higher reduction of both germination and root length of walnut by polyethylene glycol treatment in comparison with the different osmotic potential are in agreement with those reported by Berg and Zeng [42]. In our study, high level of osmotic drought stress

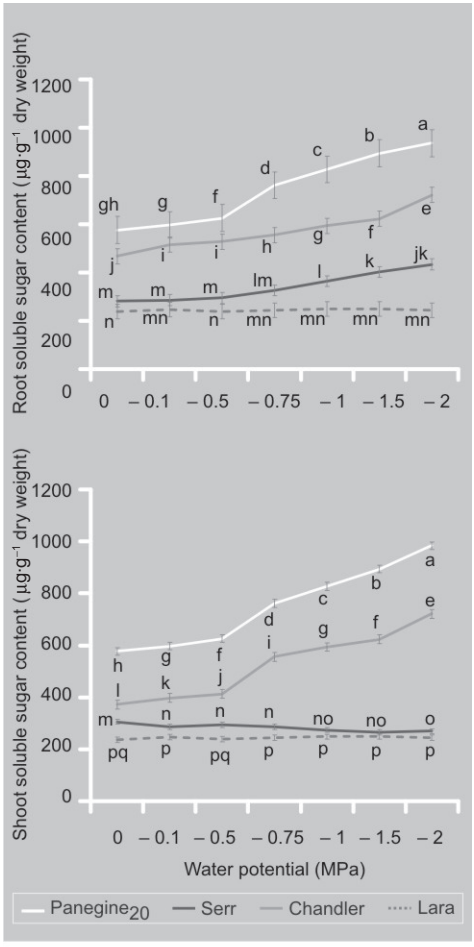


**Figure 4.**
Effect of different water potentials on soluble sugar content in the roots and shoots of four walnut genotype seedlings. Results are shown as mean ± standard error, obtained from three replicates. Means followed by the same letter are not significantly different at $P \leq 0.05$, according to Duncan's multiple range test.

resulted in a small decrease in root length and dry weight of tolerant genotypes, which to a certain degree might happen due to the decline in relative water content (RWC) of roots under drought. The decrease in RWC of stressed walnut genotypes that we observed could result from the high osmotic potential of the external polyethylene glycol solution, which caused osmotic stress and dehydration at the cellular level. However, the reasonably high values of RWC (> 70%) recorded in drought-stressed seedlings in the genotypes we used ('Chandler' and 'Panegine20') revealed the operation of mechanisms that limit excessive water loss.

The ability of the walnut seedling to transfer water from its tissues, both under control and drought conditions, causes a greater lowering of $\Psi_w$. During the drought treatment, available soil water decreased from field capacity to near the permanent wilting point (*figure 2*), which is equal to –1.53 MPa in walnut plants; our results are in agreement with Rosati *et al.* [8] and Cochard *et a*l. [9]. The ability of walnut tissues to lose water to transpirational flux caused the concentration of cell solutes to decrease w with increasing drought stress (*table IV*, *figure 2*).

Proline accumulation in leaves and, mainly, in roots is considered as a drought-sensitive trait in walnut that may be used to select plants with different degrees of tolerance. The increase in proline in severe drought stress in our experiment was consistent with these facts that evidence the transport of proline to the root tip, where it accumulates during stress, as has been reported [43]. The results showed that 'Panegine$_{20}$' and 'Chandler' plants increased proline content more than sensitive genotypes. This increase was very significant ($P \leq 0.05$) at –1.5 MPa of osmotic drought stress that accompanied predawn leaf water potential of –2.13 MPa to –2.87 MPa, midday leaf water potential of –4.17 MPa to –4.79 MPa and available medium water of 21%. It is possible that these differences are due to up-regulation of proline biosynthesis enzymes such as proline dehydrogenase (PDH) in drought-stressed 'Panegine$_{20}$' seedlings. These results prove that proline accumulation by 'Panegine$_{20}$' seedlings is due to up-regulation of the proline biosynthesis pathway rather than inhibition of the catabolic process, and in order to keep proline at a high level 'Panegine$_{20}$' progeny consume more energy and substances, but 'Chandler' progeny keep their proline constant at a high level because of suitable management and resist drought stress.

The mean differences in proline content in both tolerant genotypes were significant ($P \leq 0.05$) between all treatments. It means that 'Chandler' progeny had another cellular mechanism to keep their osmotic potential at a high level under severe drought stress. Our results indicate that proline accumulation by the repressed catabolic pathway under oxidative stress helps plants to decrease oxidative damage. Usually the magnitude of proline accumulation is relatively dependent on the levels of carbohydrates [17]. Larher *et al.* [44] mentioned that

sucrose was a positive effector for proline accumulation.

The increase in sugar concentration may be a result of the degradation of starch [45]. From the changes in total soluble sugar content in roots and shoots of tolerant genotypes, it was observed that soluble sugar content increased at the early drought stage in drought-stressed tissues in both tolerant genotypes. The current hypothesis is that sugars act as osmotica and/or protect specific macromolecules and contribute to the stabilization of membrane structures [46]. In general, soluble sugar content tends to be maintained in the leaves of drought-stressed plants, although rates of carbon assimilation were partially reduced. The maintenance of soluble sugar content may be achieved at the expense of starch, which drastically declines [47]. Increase in soluble sugar contents through inversion of some carbohydrates may contribute to enhanced desiccation tolerance and allows metabolic activity to be maintained. This was in agreement with the results observed in another study [48]. Starch plays an important role in accumulation of soluble sugars in cells. Starch depletion in grapevine leaves was noted by Patakas and Noitsakis [49] in response to drought stress, too. Increase in concentration of soluble sugars at high osmotic potential was simultaneously paralleled with decrease in the starch concentration. It means that the raised soluble sugar fraction was accompanied by a sharp decrease in the starch fraction as the water potential dropped. This change increased the soluble [sugar/starch] ratio in roots and shoots of both tolerant genotypes. The increase in the root soluble [sugar/starch] ratio is sharp at water potential –2.00 MPa,

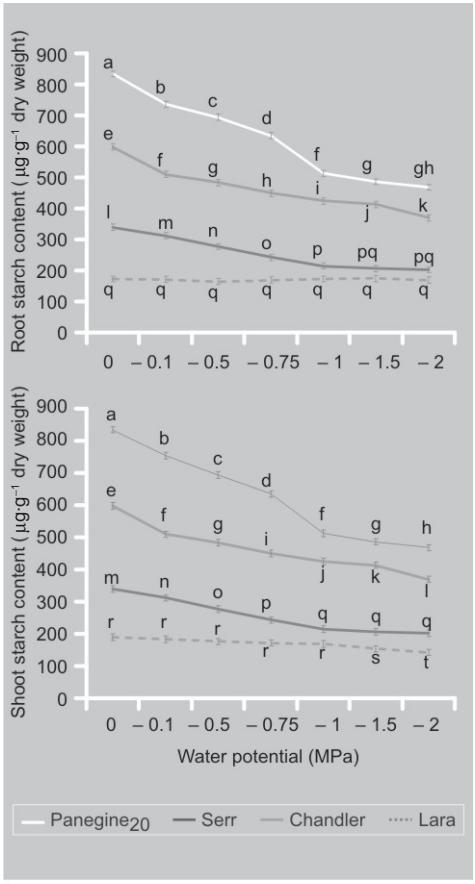


**Figure 5.**
Effect of different water potentials on starch content in the roots and shoots of four walnut genotypes. Results are shown as mean ± standard error, obtained from three replicates. Means followed by the same letter are not significantly different at $P \leq 0.05$, according to Duncan's multiple range test.

but this ratio increased gradually in shoots of all genotypes (*table III*). The tolerance mechanism in water deficit may be associated with accumulation of osmoprotectants such as proline and soluble sugars.

Among amino acids, the accumulation of proline is frequently reported in many plants or tissues in response to a genotype of abiotic stresses [17]. In the maize primary


**Figure 6.**
Dendrogram of the UPGMA clustering algorithm using square Euclidean distance based on all trait means related to drought tolerance at –1 MPa osmotic level. Groups 1 and 2 include sensitive and tolerant genotypes.

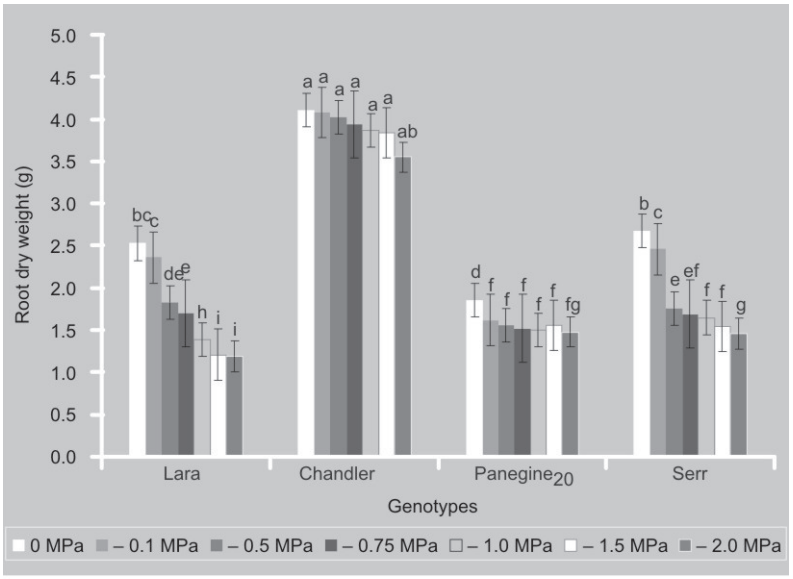


**Figure 7.**
Effects of osmotic potential on the root dry weight of four walnut genotype seedlings. Each bar represents the mean ± standard error of three measurements for four plants per treatment. Means followed by the same letter are not significantly different at $P \leq 0.05$, according to Duncan's multiple range test.

root, for example, the proline level increases as much as a hundredfold under a low water potential [50]. However, the precise role of proline accumulation is still elusive. Whether it is to act as an osmoregulator [51], an osmoprotector [52], or a regulator of the redox potential of cells [53] has not been identified.

In our study, the increase in soluble sugar content in roots of 'Panegine$_{20}$' progeny was higher than that of 'Chandler' progeny; also, the increase in soluble sugar content in shoots of 'Panegine$_{20}$' progeny was higher than that of 'Chandler' progeny. Therefore, it seems that the accumulation rate was correlated with drought tolerance and our results were consistent with this. Root soluble sugar increased linearly in both genotypes and soluble sugar increased enormously in roots and shoots at water potential –0.75 MPa in both tolerant genotypes. Shoot starch content decreased linearly in both tolerant genotypes and root starch content decreased markedly at water potential –0.75 MPa. The accumulation of sugars in response to drought stress is also quite well documented [12, 32, 51].

Cluster analysis classified seedlings of the tested genotypes into two separate groups. The results of cluster analysis suggest that different walnut cultivars have different tolerance to drought stress. This analysis demonstrated variability among walnut cultivars to tolerate different osmotic potential. The results show that critical osmotic potential in walnut for drought tolerance is –1 Mpa, that screens tolerant genotypes from sensitive according to the related growth indices. Based on our results, the tolerant genotypes of walnut trees ('Chandler' and 'Panegine$_{20}$') could be considered as moderately drought-tolerant plants.

In conclusion, the results discussed here support our hypothesis that proline accumulation during seed germination in drought stress is a part of a physiological response of walnut tree to the imposition of an intense drought stress. Under drought stress conditions, walnut seeds have a high level of free proline content and activate osmotic adjustment mechanisms not only in shoots or leaves, but also in roots, in this way increasing their capacity to extract water from dry medium [31, 32]. Our results support a direct correlation between the degree of drought stress and proline content. As a consequence, proline concentrations could be used as a biochemical marker of drought stress level in walnut plants. Under stress conditions, we postulate that the depletion of starch with induced plasmolysis will reduce the volume of cytoplasm. Accumulation of soluble sugars may be to counter the osmotic stress. How metabolic flux of soluble sugars was altered under the stress conditions and how it should involve modulation of many enzyme activities in carbohydrate metabolic pathways remains to be investigated.

## Acknowledgements

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**Table V.**
Correlation coefficients among some growth and physiological properties in *Juglans regia* L. genotypes studied under progressive drought stress treatment.

| Parameters studied | Root length | Root dry weight | Final germination % | Relative water content | Root proline content | Shoot proline content | Root soluble sugar content (RSS) | Shoot soluble sugar content (ShSS) | Root starch content (RSta) | Shoot starch content (ShSta) | [RSS / RSta] | [ShSS / ShSta] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Root length | 1.000 | | | | | | | | | | | |
| Root dry weight | 0.826*** | 1.000 | | | | | | | | | | |
| Final germination percentage | 0.823** | 0.504 | 1.000 | | | | | | | | | |
| Relative water content | 0.980*** | 0.895*** | 0.710** | 1.000 | | | | | | | | |
| Root proline content | 0.754** | 0.282 | 0.911*** | 0.613* | 1.000 | | | | | | | |
| Shoot proline content | 0.755** | 0.282 | 0.913*** | 0.615* | 0.999*** | 1.000 | | | | | | |
| Root soluble sugar content (RSS) | 0.739** | 0.249 | 0.838*** | 0.604* | 0.985*** | 0.983*** | 1.000 | | | | | |
| Shoot soluble sugar content (ShSS) | 0.753** | 0.297 | 0.920*** | 0.606* | 0.996*** | 0.995*** | 0.978*** | 1.000 | | | | |
| Root starch content (RSta) | 0.859*** | 0.450 | 0.937*** | 0.739** | 0.981*** | 0.982*** | 0.963*** | 0.981*** | 1.000 | | | |
| Shoot starch content (ShSta) | 0.867*** | 0.456 | 0.922*** | 0.753** | 0.978*** | 0.978*** | 0.967*** | 0.975*** | 0.998*** | 1.000 | | |
| [RSS / RSta] | – 0.035 | – 0.379 | – 0.131 | – 0.025 | 0.203 | 0.206 | 0.316 | 0.138 | 0.127 | 0.163 | 1.000 | |
| [ShSS / ShSta] | – 0.047 | – 0.373 | – 0.171 | – 0.099 | 0.191 | 0.189 | 0.323 | 0.158 | 0.111 | 0.139 | 0.650* | 1.000 |

***, ** and *: significant at $P \leq 0.001$, $P \leq 0.01$ and $P \leq 0.05$, respectively.


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## Los azúcares solubles y la acumulación de prolina son indicadores eficaces para la selección de nogales persas (*Juglans regia* L.), tolerantes a la sequía durante la germinación.

**Resumen** -- **Introducción**. El estrés causado por la sequía es el factor principal que influye el crecimiento, el desarrollo y la producción de nogales. En Irán, cerca de 33 Mha de tierras están afectadas por la salinización y por la sequía. Es importante para estas regiones semiáridas encontrar recursos genéticos que toleran la sequía en diferentes estadios de crecimiento. Nuestro objetivo fue comprender mejor los mecanismos de adaptación que permiten los diferentes genotipos de nogales sobrevivir en condiciones de estrés hídrico. También pretendimos aportar, mediante el empleo de los recursos genéticos existentes, algunas indicaciones útiles para una mejora de estos árboles con respecto a la tolerancia de la sequía. **Material y métodos**. Con el fin de estudiar los mecanismos de ciertos genotipos de nogales persas implicados en la tolerancia de la sequía, se indujo un estrés hídrico mediante el empleo del polietilenglicol-6000 para producir potenciales hídricos de 0 Mpa (testigo), –0.10 MPa, –0.50 MPa, –0.75 MPa, –1.00 MPa, –1.50 MPa y –2.00 MPa. El nivel de acumulación de prolina y de los azúcares solubles en cuatro genotipos de nogales ('Panegine$_{20}$', 'Lara', 'Serr' y 'Chandler') se determinó tras su exposición a diferentes niveles de potenciales hídricos. **Resultados**. Para los cuatro genotipos estudiados, los valores de germinación de las semillas se redujeron considerablemente por los bajos potenciales hídricos. Las siembras de los genotipos tolerantes ('Panegine$_{20}$' y 'Chandler') expuestos al estrés hídrico, en relación con los genotipos sensibles, presentaron las cantidades más grandes de azúcares solubles en las raíces y en las ramas, pero una cantidad menor de almidón en sus tejidos. Los resultados en cuestión sugieren la importancia de los azúcares solubles y solutos, que confieren a estos genotipos la resistencia a la sequía. Los niveles de prolina libre aumentaron igualmente en respuesta a la sequía. Fueron más elevados en los genotipos tolerantes a la sequía que en aquellos que eran más sensibles ('Lara' y 'Serr'). El contenido en prolina aumentó considerablemente más en las ramas que en las raíces. No obstante, las fluctuaciones de azúcar soluble y del almidón fueron más elevadas en las raíces. **Conclusión**. Nuestros resultados confirman que existe una correlación directa entre el grado de estrés hídrico y el contenido en prolina en las plántulas. Por consecuente, las concentraciones de prolina podrían emplearse como marcadores bioquímicos del nivel de estrés hídrico en el nogal.

**Iran República Islámica / *Juglans regia* / recursos genéticos / estrés de sequia / plantulas / azucares / prolina**