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Enhancing drought resilience in Persian walnut genotypes: Insights from photosynthetic dynamics and phenotypic analysis

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Abstract

Objective: The Persian walnut (*Juglans regia* L.), known for its notable sensitivity to drought stress, exhibits considerable genetic diversity within Iran. This genetic variability provides a valuable resource for developing and selecting drought-tolerant rootstocks.

Methods: This study investigates the effects of water withholding on 115 walnut families from various climatic zones in Khorasan Razavi province, Iran, by analyzing polyphasic fast chlorophyll a fluorescence induction (OJIP), relative water content (RWC), and chlorophyll index (SPAD).

Results: We observed reductions in electron transport flux per reaction center (ET_0/RC), relative maximal variable fluorescence (F_M/F_0), maximum quantum efficiency of PSII (F_V/F_M), quantum yield of electron transport (Phi-E_0), and the performance index (PI-$_{ABS}$) in response to drought stress. In contrast, increases were observed in dissipated energy flux (DI_0/RC), quantum yield of energy dissipation (Phi-D_0), and specific energy fluxes per reaction center for energy absorption (ABS/RC), which are associated with photo-inhibition in plants subjected to water withholding. Using drought tolerance assessments and principal component analysis, we tentatively categorized the 115 walnut families into sensitive, tolerant, and moderately tolerant groups. Tolerant families, such as G107, B31, B66, B68, and B142, exhibited less variation in most traits from normal to drought-stress conditions, compared to sensitive and moderately tolerant families.

Conclusion: Our findings highlight the effectiveness of chlorophyll fluorescence characteristics, RWC, and SPAD for the rapid identification of drought-tolerant genotypes.

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Introduction

The Persian walnut (*Juglans regia L.*) is an important dry fruit tree used worldwide for various purposes, including nut production, timber, and byproducts like dyes and fragrances (Vahdati *et al*. 2018; Vahdati *et al*. 2020). These walnut trees are particularly vulnerable to drought stress, a situation worsened by decreasing rainfall patterns in areas such as Central Asia (Aletà *et al*. 2009; Arab *et al*. 2020). Iran, a significant center for walnut production, has a rich diversity of walnut trees that originated from seeds. Obviously, this means of production provides a great diversity and valuable resource for developing drought-tolerant varieties, as different genotypes exhibit unique physiological and morphological responses to this stress. Collecting and identifying resilient genotypes from natural populations is essential for breeding programs focused on improving drought tolerance.

Drought stress leads to several detrimental effects in plants, including reduced photosynthesis, chlorophyll breakdown, disrupted leaf water balance, stomatal closure, decreased growth and yield, and heightened susceptibility to pests (Aliniaeifard and van Meeteren 2016a, 2016b). The response of a plant's photosynthetic system to drought stress is influenced by the genotype and the severity of the drought (Xiao *et al*. 2008; Sousaraei *et al*. 2021). Non-destructive techniques, such as chlorophyll fluorescence analysis, have become important tools for identifying drought-tolerant genotypes, allowing for the assessment of plant health without causing damage (Adhikari *et al*. 2009). Measuring changes in the photosynthetic system is one of the effective methods for predicting drought tolerance in plants (Bresson *et al*. 2015; Demmig-Adams *et al*. 2018). The fast chlorophyll a fluorescence induction (OJIP) test is particularly useful for understanding how photosynthesis responds to stress, as it detects the damage to photosystem II (PSII) within the photosynthetic electron transport chain (Küpper *et al*. 2019). These techniques provide valuable insights into electron transfer rates and alterations in PSII functionality under stress conditions (Kalaji *et al*. 2016). Key indicators for evaluating PSII performance during abiotic stress include the maximum quantum efficiency of PSII (F_V/F_M), the apparent antenna size of an active PSII reaction center (ABS/RC), and the photosynthetic performance index based on absorbance (PI_{ABS}) (Sharma *et al*. 2015; Athar *et al*. 2016; Kalaji *et al*. 2016). Chlorophyll fluorescence evaluation has proven useful in identifying drought-tolerant genotypes in various crops, including walnuts (Arab *et al*. 2023), wheat (Guellim *et al*. 2020), tomatoes (Sousaraei *et al*. 2021), grapes (Su *et al*. 2015), mung beans (Bano *et al*. 2021), sunflowers (Çiçek *et al*. 2019), and forest spruce (Tomášková *et al*. 2021).

This study focused on assessing 115 native walnut genotypes from Khorasan Razavi province, Iran, using chlorophyll fluorescence, relative leaf water content (RWC), and the greenness index to uncover traits related to drought tolerance. The primary goals were to identify genotypes resilient to drought and to conduct a phenotypic evaluation of photosynthetic traits to guide future cultivation and breeding initiatives.

Materials and Methods

A total of 115 walnut genotypes were collected from five cities in the Khorasan Razavi province, Iran, encompassing arid and semi-arid regions. The characteristics of the collection sites are detailed in Table 1. Each population was situated in a distinct habitat with varied environmental conditions, including climate, geology, and topography. The mother trees were open-pollinated seedling trees aged from 75 to 500 years, originating from ecologically diverse regions such as Torbat-e Jam, Shadmehr, Gonabad, Bayg, and Torbat-e Hydarieh.

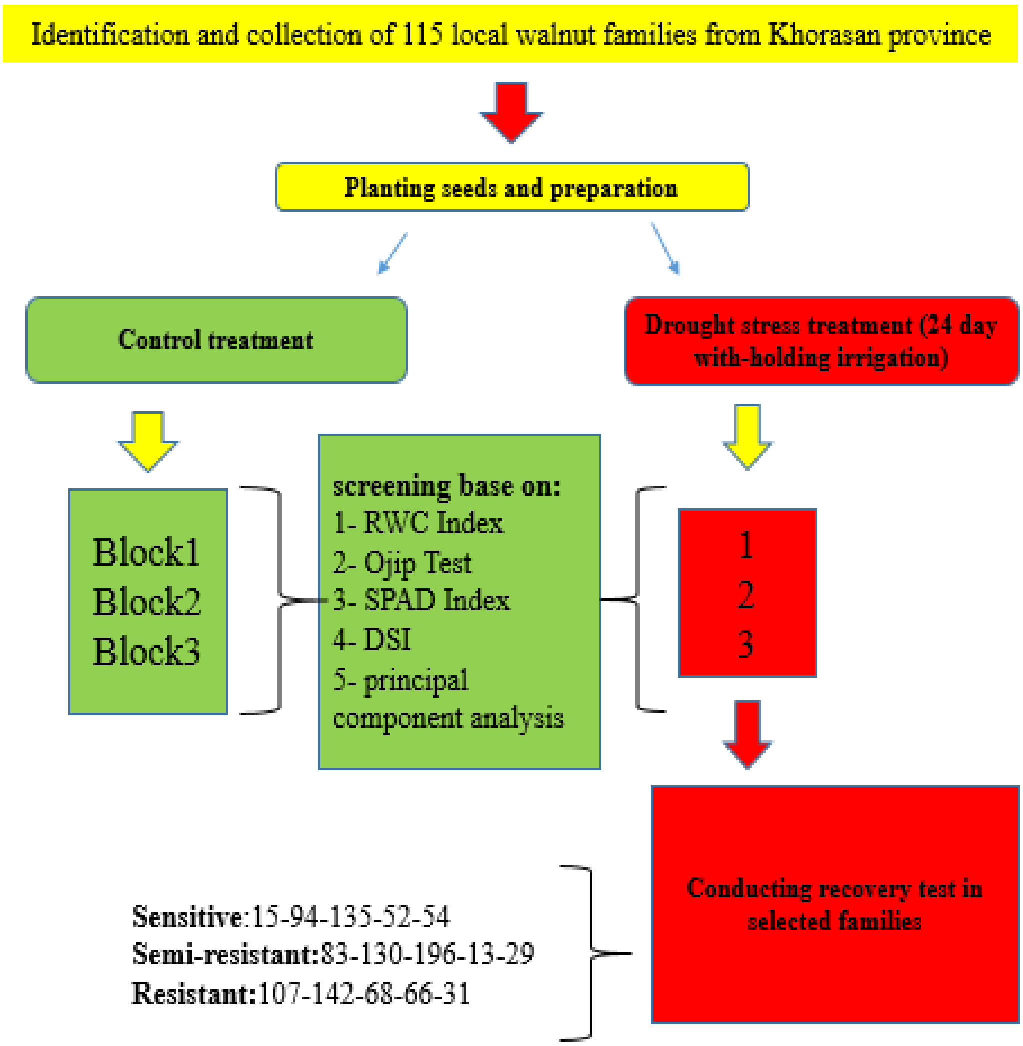
Table 1. Location, height, and total annual rainfall (mm/year) of five different geographical areas of Khorasan Razavi Province, Iran, where local landraces were collected (Iran Meteorological Organization 2021).

City	Genotype No.	Site location	Altitude (m)	Total annual rainfall (mm/ year)
Torbat e hydarieh	19	35°25′N 59°09′E	1351	214
Torbat e jam	11	35°14′N 60°37′E	906	110
Bayg	44	35° 22′N 59° 2′E	1529	182
Shadmehr	22	35° 10′N 59° 2′E	1206	95
Gonabad	19	34° 21′N 58° 41′E	1096	86

Experimental design

This study was conducted on saplings of 1-year-old half-sib families of 115 native genotypes from various regions of Khorasan Razavi province, characterized by hot and arid climatic conditions. Seeds obtained from the 115 families were cultivated in a common orchard to assess the photosynthetic performance of the progeny under water-deficit stress and well-watered conditions. The seeds (20 seeds per family) were collected from mature Persian walnut trees in the Khorasan Razavi province, Iran, stratified, germinated, and grown in 7-L polyethylene pots (15 × 40 cm) filled with a potting mix (2:1:1, soil: sand: leaf manure) under ambient greenhouse conditions (25 ± 2 ◦C). After nine months, the saplings were transplanted into 15-L polyethylene pots (20 × 50 cm) with an adequate volume for the 15-month-old seedling root growth. Two months before the drought stress experiment, the saplings were transferred to an environmentally controlled greenhouse at the Research

Greenhouse of the Department of Horticulture, University of Tehran, Pakdasht, Tehran, Iran. The plants were irrigated to maintain soil hydration and fertilized for two months with macro- and micro-nutrients (Figure 1). The saplings were divided into two groups (with three replications): well-watered (above 75% field capacity) and severely water-deficient (~25–35% field capacity). The field capacity and permanent wilting point were determined using the soil water retention curve. This experiment was conducted in the form of a completely randomized block design with three replications in each irrigation condition.



Relative water content (RWC)

Drought response of the plants was graded on a scale of 1–7 based on visual appearance. Also, leaf discs were collected, and RWC was calculated using the formula RWC = [(FW–DW)/(TW–DW)] × 100, where FW is the fresh weight, TW is the turgor weight, and DW is the dry weight.

Chlorophyll fluorescence

OJIP transients were recorded by using a portable fluorometer (Fluor pen FP 100-MAX, Photon Systems Instruments, Drasov, Czech Republic) after 20 min dark adaptation, in young fully-expanded walnut leaflets from the middle part of the saplings for each plot. Before performing the test, to make sure the reaction centers of the PSII were closed, we put saplings in the dark overnight so that the test would end before dawn. Minimum fluorescence (F_0) was measured at 50 µs, corresponding to the state when all PSII reaction centers are open, representing the O step. The leaflet samples were immediately exposed to a saturating light of ~3000 µmol m^{-2} s^{-1}, and fluorescence intensity at 2 ms (J-step, FJ), 30 ms (I-step, FI), and when all the active receptors of PSII reaction centers were deactivated, i.e. at the time of maximum fluorescence intensity (P step), were recorded for further calculations. In addition, to detect the amount of damage to the electron receptors in PSII, other calculation methods, presented by Strasser *et al*. (2000), were used. Some important characteristics, including the performance index (PI_{ABS}), PSII maximum photochemical efficiency (F_V/F_M), maximum quantum yield of energy trapping by PSII (Phi-D_0), quantum yield of intersystem electron transport (Phi-E_0), PSII antenna size (ABS/RC), trapped energy flux (TR_0/RC), electron transport flux per reaction center (ET_0/RC), and dissipated energy flux (DI_0/RC) were recorded to provide more structural information on the photosynthetic apparatus (Strasser *et al.* 2000, 2004). The definition and calculation of the most important chlorophyll fluorescence characteristics are described in Table 2.

Drought tolerance evaluation

To assess the drought tolerance, the average response of each family to drought stress for all measured traits was expressed using the drought stress index (DSI) described by Wójcik-Jagła *et al*. (2013) and calculated as follows: DSI = (value of the trait under water-deficit-stressed conditions)/(value of the trait under well-watered conditions) × 100.

Statistical analysis

Fourteen indicators were utilized for the analysis. Each trait was measured with three replications per family, and the data were processed using Microsoft Excel. Analysis of variance and mean comparisons were performed using SPSS Statistics version 21.0 (IBM Inc., Armonk, NY, USA). Means were compared using Duncan's multiple range test. Correlation coefficients were calculated, and principal component analysis (PCA) was performed, employing the *factoextra* package (Kassambara and Mundt 2017) in R.

Table 2. The formulae and definitions of chlorophyll fluorescence characteristics (Strasser *et al.* 2004) measured in this study, with their broad classification.

Phenotype category	Trait	Formula	Definition
Fluorescence traits	F_M/F_0		Relative maximal variable fluorescence
	F_V/F_0		Maximum efficiency of photochemistry
	F_V/F_M	$(F_M - F_0)/F_M$	Maximum yield of primary photochemistry
	Ψ_0	$\Psi0 = ET_0/TR_0 = 1- VJ$	Likelihood that a trapped exciton can move an electron further than plastoquinone (QA)
	φ_{E0}	$\Phi_{E0} = ET_0/ ABS = (1-(F_0/ F_M)) \times \Psi$	Quantum yield of electron transport
	φ_{D0}	$\Phi_{D0} = 1- \Phi_{p0} = (F_0/F_M)$	Quantum yield at time 0 for energy dissipation
	Φ_{pav}	$\Phi pav = \Phi_{p0}$ (1- Vav)	Average quantum yield of primary photochemistry
	PI_{ABS}	$(RC/ABS) \times (\varphi_{P0}/1- \varphi_{P0})$	Performance index of PSII normalized for equal absorption
	ABS/RC	$ABS/RC = M0 \times (1/VJ) \times (1/ \Phi_{p0})$	Energy absorption by antenna per reaction center
	TR_0/RC	$TR_0/RC = M_0 \times (1/VJ)$	Flux of excitons trapped per reaction center
	ET_0/RC	$ET_0/RC = M_0 \times (1/VJ) \times \Psi0$	Energy flux for electron transport per reaction center
	DI_0/RC	$DI_0/RC= (ABS/RC)- (TR_0/RC)$	Flux ratio of energy dissipation per reaction center
Drought score	DS		Drought scoring system based on the appearance characteristics of seedlings
RWC	RWC	(FW – DW)/ (TW – DW)	Leaf relative water content

Results

Analysis of variance of 115 Persian walnut families under different irrigation conditions for the physiological characteristics were presented in Supplementary Table 1. Significant differences were observed among families in terms of the studied traits in each of the normal and water-deficit stress conditions. Based on the assessment of SPAD index, leaf RWC, DSI index, PCA, and visual appearance, the families were tentatively categorized into three groups: sensitive, moderately tolerant, and tolerant. Significant differences were observed among the 15 selected families (five from each of the three categories) in terms of the studied traits in each of the normal and water-deficit stress conditions (Supplementary Table 2). Also, according to Figures 2 and 3, significant differences are seen among the 15 selected families in each of the normal, water-deficit stress, and recovery conditions for RWC, SPAD, F_V/F_M, and F_M/F_0. Considering the average of all families, drought stress reduced RWC, SPAD, and photosynthetic indices, except for Phi-D_0, Phi-Pav, ABS/RC, DI_0/RC, and TR_0/RC, which showed an increased value as expected; however, the increase in TR_0/RC was negligible (Table 3). The tolerant families exhibited smaller changes from normal to drought stress, compared to the sensitive families for RWC, SPAD, F_V/F_M, and F_M/F_0. Sensitive families had the

Table 3. Descriptive statistics of 115 walnut seedling attributes under non-stress and water-deficit stress conditions.

	Control						Drought stress					
Statistics	**Min**	**Max**	**Mean**	**Variance**	**Std. deviation**	**CV (%)**	**Min**	**Max**	**Mean**	**Variance**	**Std. deviation**	**CV %**
RWC	70.10	82.9	76.7	13.21	3.63	4.74	46.4	77.9	61.6	33.7	5.8	9.4
SPAD	40.02	55.9	47.7	20.9	4.57	9.58	15.7	37.9	28.1	17.5	4.2	14.9
Fm/Fo	4.03	5.4	4.5	0.122	0.35	7.78	1.212	3.951	2.9	0.33	0.57	19.5
Fv/Fo	3.03	4.45	3.53	0.122	0.35	9.92	0.212	2.951	1.9	0.33	0.58	29.4
Fv/Fm	0.73	0.84	0.78	0.0004	0.02	2.56	0.416	0.751	0.6	0.01	0.07	10.8
Psi_o	0.23	0.53	0.4	0.003	0.06	13.69	0.088	0.475	0.3	0.0036	0.06	20.6
Phi_Eo	0.18	0.43	0.31	0.003	0.05	17.67	0.038	0.348	0.2	0.0036	0.06	29.2
Phi_Do	0.16	0.261	0.22	0	0.02	9.5	0.246	0.585	0.3	0.01	0.06	19.8
Phi_Pav	927.4	985.2	951.2	169.6	13.02	1.37	918.6	988.7	968	133	11.5	1.2
Pi_Abs	0.4	1.998	0.9	0.124	0.35	38.2	0.008	0.918	0.29	0.04	0.2	65.4
ABS/RC	2.2	3.467	2.8	0.101	0.32	11.3	2.45	4.934	3.5	0.3	0.5	15.5
TRo/RC	1.73	2.714	2.2	0.05	0.22	10.2	1.45	2.916	2.3	0.06	0.25	11.0
ETo/RC	0.5	1.239	0.88	0.019	0.14	15.6	0.27	1.036	0.7	0.02	0.13	18.7
DIo/RC	0.4	0.86	0.62	0.012	0.11	17.3	0.6	2.409	1.2	0.17	0.4	33.2

largest decrease in RWC, SPAD index, F_V/F_M, and F_M/F_0, compared to the moderately tolerant and tolerant families (Figures 2 and 3). In these plant families, the DSI index ranged from 25 to 41%, but in tolerant families this index was higher than 86%. All plant families were able to recover their initial state after re-irrigation. The recovery rate in the tolerant families was higher than in the sensitive families. Sensitive families still had drooping and yellow leaves after 10 days of re-irrigation. Sensitive families experienced leaf shedding with leaves turning approximately 70% yellow, while tolerant families showed no change in leaf color or shedding.

The SPAD values ranged from 15 to 23 in the sensitive families, from 24 to 27 in the moderately tolerant families, and from 28 to 37 in the tolerant families. According to Table 3, the phenotypic coefficient of variation for the SPAD index was 9.58% under control conditions and 14. 9% under water-deficit stress conditions, showing the larger differentiation of the walnut families in the stress conditions.

RWC decreased in all genotypes due to drought stress, compared to the normal conditions. Among the selected tolerant families, the highest and lowest changes in RWC were observed in families B31 and B68, respectively (Figures 2 and 3). The phenotypic coefficient of variation in the families under drought stress and control conditions ranged from 9.42% to 4.73%, respectively (Table 3), again showing the larger differentiation of the walnut families in the drought stress conditions in terms of RWC.

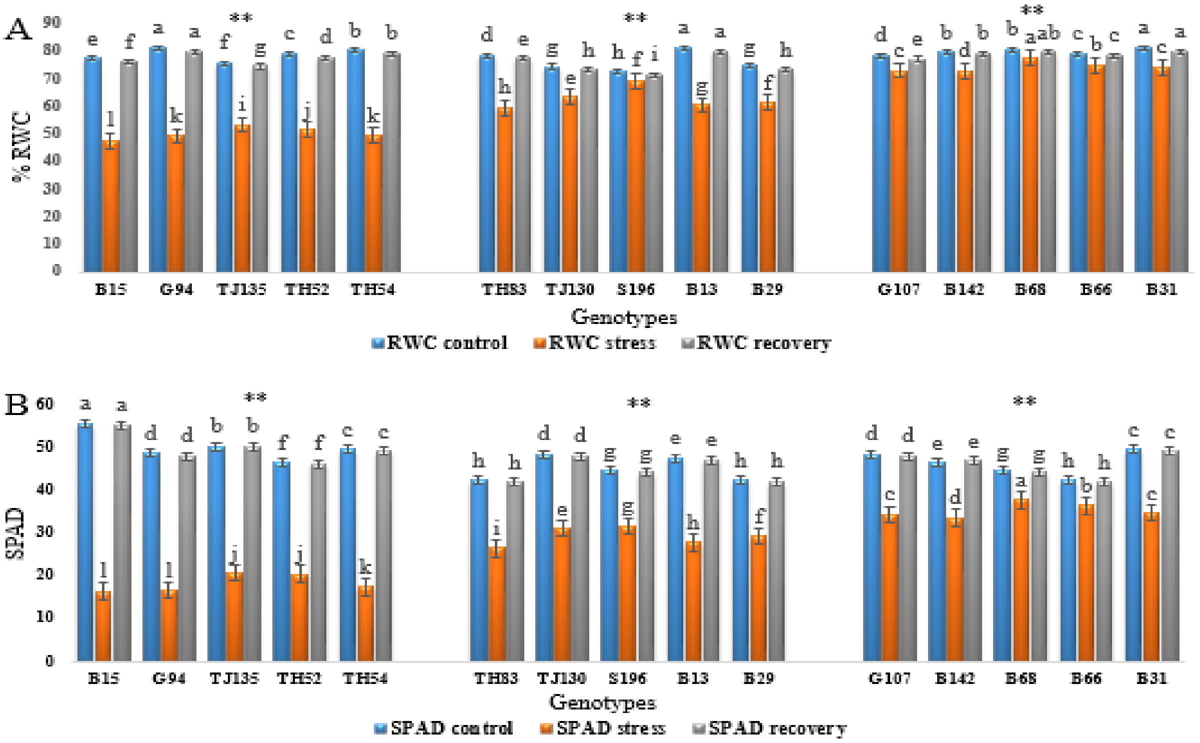


Figure 2. The results of changes in the traits RWC (A) and SPAD (B) in five selected sensitive (B15, G94, TJ135, TH52, TH54), moderately tolerant (TH83, TJ130, S196, B13, B29), and tolerant (G107, B142, B68, B66, B31) families of Persian walnut. The different letters represent significant differences according to Duncan's multiple range test within each environmental condition.

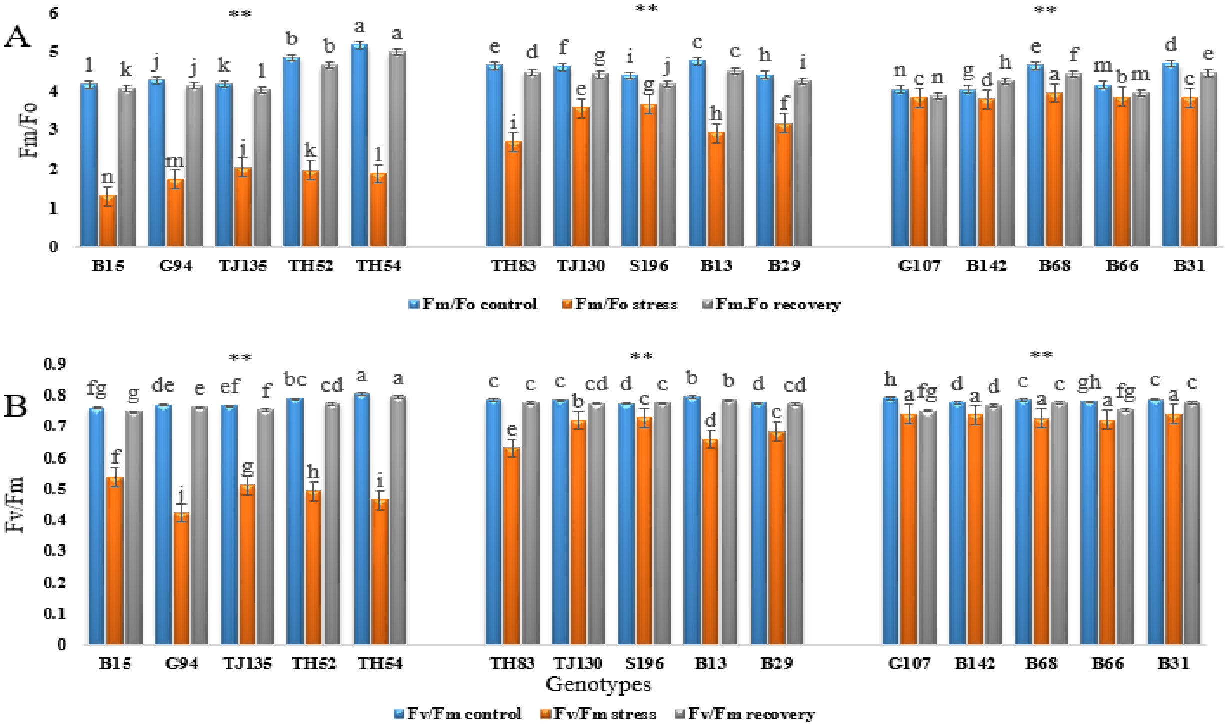


Figure 3. The results of changes in the traits of F_M/F_0 (A) and F_V/F_M (B) in five selected sensitive (B15, G94, TJ135, TH52, TH54), moderately tolerant (TH83, TJ130, S196, B13, B29), and tolerant (G107, B142, B68, B66, B31) families of Persian walnut. The different letters represent significant differences according to Duncan's multiple range test within each environmental condition.

The analysis of photosynthetic indices revealed a general decrease under drought stress. The phenotypic coefficient of variation for these parameters ranged from 1.2% to 33.2% under drought stress and from 2.56% to 38.2% in normal conditions (Table 3).

Principal component analysis

The results of PCA showed that the first and second components had the largest share of data dispersion in the control conditions with 50.6% and 18.2%, respectively (Figure 4A and 4C). Under drought stress conditions, the first (73.4%) and second (10.2%) components explained a large proportion of variation (Figure 4B and 4D). Based on the obtained data, some photosynthetic parameters such as TR_0/RC, DI_0/RC, and ABS/RC, although they were the main components of the PC1 and played a large role in the grouping of genotypes, had similar patterns and positive correlations in both normal and stress conditions. In contrast, although SPAD, RWC, and ET_0/RC were also the main components of the PC1, they played a significant role in discriminating between normal and water-deficit stress conditions, because their pattern was different in these conditions. RWC and SPAD were not discriminating in normal conditions; however, they were more effective in discrimination of the walnut families under water-deficit stress conditions (Figure 4A and 4B).

In normal conditions, the traits F_M/F_0 (0.79), F_V/F_0 (0.79), F_V/F_M (0.89) $Psi\text{-}_0$ (0.81), $Phi\text{-}E_0$ (0.88), $Phi\text{-}D_0$ (-0.89), Phi-Pav (-0.54), $PI\text{-}_{ABS}$ (0.95) and DI_0/RC (-0.87) were included in the first component and the traits ABS/RC (0.76), TR_0/RC (0.86) and ET_0/RC (0.83) were included in the second component. In the drought stress conditions, the traits RWC (0.88), SPAD (0.94), F_M/F_0 (0.95), F_V/F_0 (0.95), F_V/F_M (0.94) $Psi\text{-}_0$ (0.79), $Phi\text{-}E_0$ (0.93), $Phi\text{-}D_0$ (-0.87), Phi-Pav (-0.74), $PI\text{-}_{ABS}$ (0.91), ABS/RC (-0.78), ET_0/RC (0.68), DI_0/RC (-0.93) and DSI (0.92) were included in the first component, and only the TR_0/RC (0.91) index was included in the second component.

Based on PCA, families TH54, G94, S190, B39, TH52, B37 and B15had were considered as sensitive families. On the other hand, families B149, B142, S191, TH70, B66, B68, B34, G111, and B125 were determined as drought-tolerant families (Figure 4D).

Drought tolerance evaluation

The results showed that, tentatively, 20 families were classified as sensitive and 11 families as resistant. The remaining families fell into the moderately tolerant categories. Therefore, PCA and drought-stress assessment were able to tentatively identify tolerant and sensitive families.

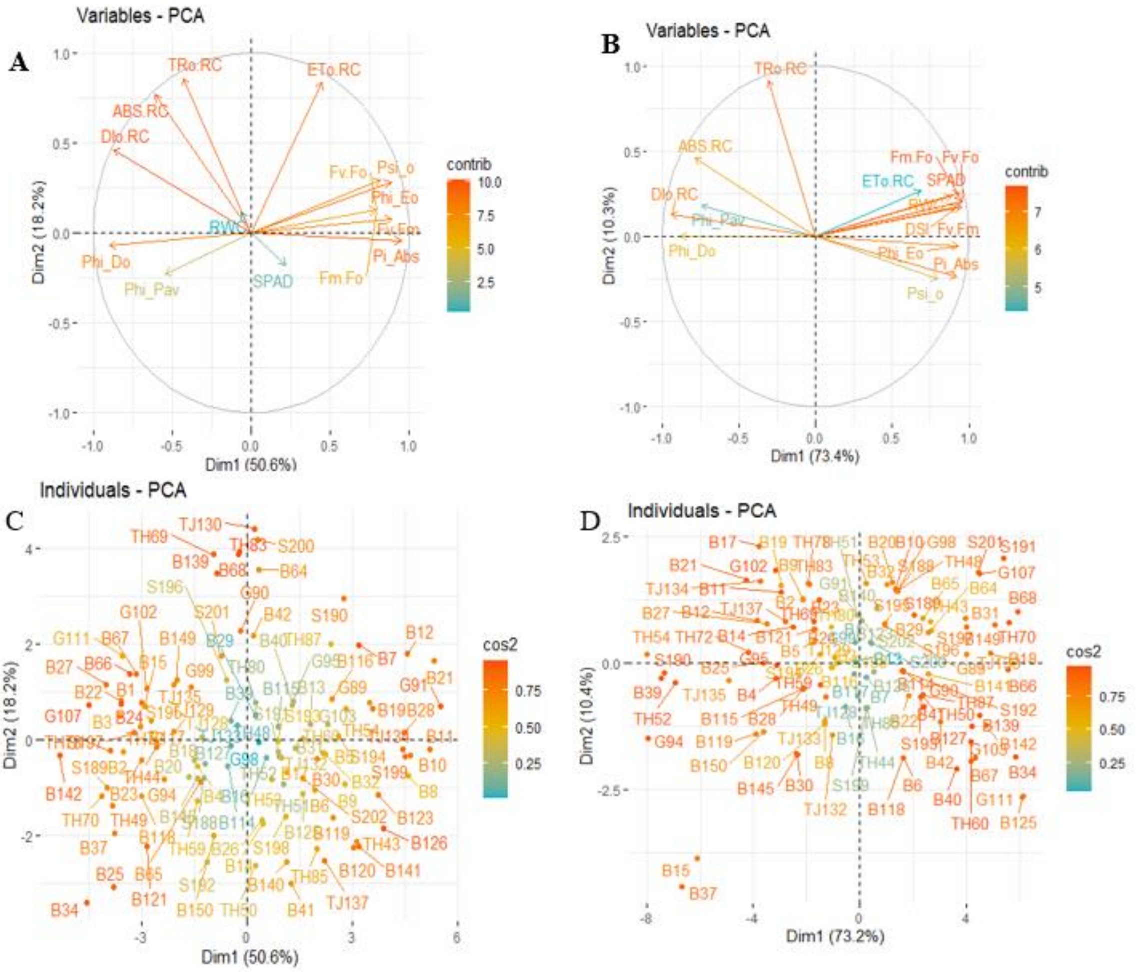


Figure 4. Principal component analysis of 115 families of Iranian walnut under control and drought stress conditions. Distribution of traits under control conditions (A), distribution of traits under drought stress conditions (B), distribution of walnut families under control conditions (C), and distribution of walnut families under drought-stress conditions (D). In the control and drought stress conditions, traits or families marked in red showed greater response in each environment.

Pearson correlation coefficients

The heatmap of correlation coefficients between walnut traits in the control and drought stress conditions are shown in Figure 5. Under control conditions RWC only had a significant, but weak, correlation with SPAD (-0.19, $p \leq 0.05$). Although SPAD showed a significant ($p \leq 0.05$) correlation of trait with TR$_0$/RC (-0.18), ABS/RC (-0.2) and DI$_0$/RC (-0.2), but these correlations were also weak. All photosynthetic characteristics were significantly correlated with each other, except for the correlation of TR$_0$/RC with Phi-Pav. Some of the strong correlations are as follows: F$_M$/F$_0$ with F$_v$/F$_M$ and Phi-D$_0$ (0.726 and -0.747, respectively); F$_v$/F$_0$ with F$_v$/F$_M$ and Phi-D$_0$ (0.726 and -0.747, respectively); F$_v$/F$_M$ with Phi-E$_0$, Phi-D$_0$, PI-$_{ABS}$, and DI$_0$/RC (-0.741, -0.931, -0.841, and 0.788, respectively); Psi-$_0$ with Phi-E$_0$, PI-ABS, and ET$_0$/RC (-0.945, -0.841, and -0.721, respectively; Phi-E$_0$

with Phi-D$_0$, PI-$_{ABS}$, and ET$_0$/RC (-0.749, -0.888, and -0.708, respectively); Phi-D$_0$ with PI-$_{ABS}$ and DI$_0$/RC (-0.820 and 0.791, respectively); PI-$_{ABS}$ with DI$_0$/RC (0.846); ABS/RC with TR$_0$/RC and DI$_0$/RC (0.958 and 0.883, respectively); and TR$_0$/RC with DI$_0$/RC (0.757).

Under drought stress, all measured traits were significantly correlated, except for the correlation of TR0/RC with RWC, SPAD, F$_M$/F$_0$, F$_V$/F$_0$, and F$_V$/F$_M$, and correlation of TR$_0$/RC with ET$_0$/RC and DSI. Contrary to the normal conditions, RWC and SPAD were significantly correlated (positively or negatively) with all of the photosynthetic characteristics, except for TR$_0$/RC; all of these correlations were either moderate or high, ranging from the absolute values of 0.550 to 0.967. RWC was also highly and significantly correlated with SPAD. The DSI index also showed significant correlations (positive or negative) with all of the studied characters, except for TR$_0$/RC; all of these correlations were either high or moderate with the range of 0.586 to 0.941 in absolute values (Figure 5).

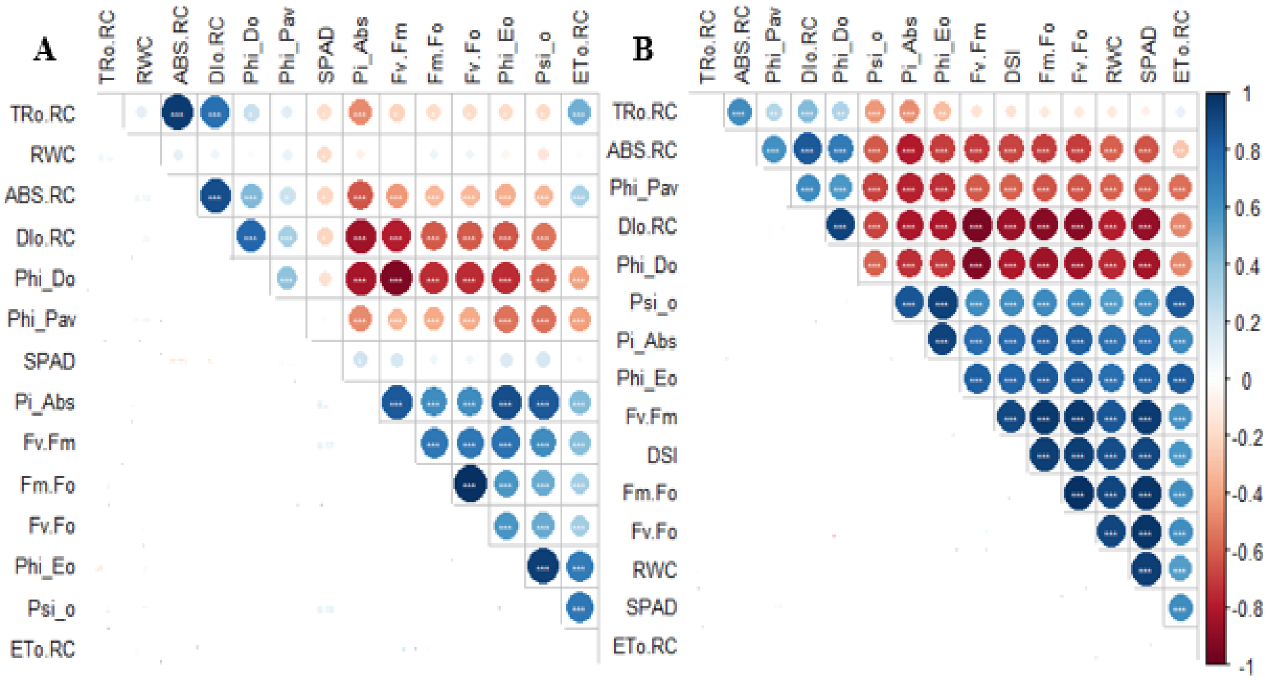


Figure 5. Correlation coefficient of photosynthetic traits (TR$_0$/RC, ABS/RC, DI$_0$/RC, Phi-D$_0$, Phi-Pav, PI-$_{ABS}$, F$_V$/F$_M$, F$_M$/F$_0$, F$_V$/F$_0$, Phi-E$_0$, Psi-$_0$, ET$_0$/RC), relative water content (RWC), and chlorophyll index (SPAD) in 115 walnut plant families under control (A) and drought stress (B) conditions. Dark red and blue colors indicate strong positive and negative correlation, respectively, among the measured traits. Also, significant levels at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$ are marked with *, **, and ***, respectively.

Discussion

Weather conditions are vital for the successful cultivation of fruit trees, as environmental stresses like drought and heat can significantly impact horticultural crops, particularly temperate fruit trees.

Walnut trees are especially susceptible to drought stress, which can result in decreased yield, heightened vulnerability to pests, and even tree mortality. These issues have limited cultivation efforts and led to substantial yield reductions in arid and low-water areas. By identifying and evaluating germplasm in these regions, we can harness valuable genetic resources to develop programs focused on improving drought-stress tolerance and enhancing the cultivation of drought-tolerant trees. To identify valuable genotypes that are tolerant to drought stress and well-suited to arid conditions, we collected seeds from 115 local genotypes of arid and semi-arid areas of Iran and subjected them to drought stress. We observed diverse responses in RWC among the Persian walnut families. RWC is a critical indicator of drought tolerance in plants (Fahimi Khoyerdi e*t al*. 2016) and often reflects the initial effects of drought stress, typically decreasing as drought conditions worsen. Tolerant plants maintain stable water content levels and show less sensitivity to fluctuations in RWC. Arab *et al*. (2023) reported a negative correlation between drought stress and RWC, noting varying responses among different genotypes. In our study of native walnut genotypes, we found that the RWC of tolerant families ranged from 77.9% to 82.9%, with the most resilient individuals hailing from regions located at altitudes between 1,200 and 1,300 meters. The variations in RWC observed among different plant families can be attributed to differences in leaf cell size and wall thickness as they adapt to drought stress (Liu *et al*. 2012). Maintaining a balanced photosynthetic system is crucial during abiotic stresses, as a strong photosynthetic capacity enhances stress resistance and helps plants cope more effectively with adverse conditions. Previous studies have demonstrated that plant genotypes from different altitudes exhibit varying photosynthetic efficiencies when subjected to drought stress (Tomášková *et al*. 2021; Viljevac Vuletić *et al*. 2022).

Our analysis of transient OJIP indices among walnut families from different regions exposed to drought stress revealed diverse results. The OJIP test, which assesses bioenergy determination, evaluates the function of reaction centers and examines both donor and acceptor centers of photosystem II (PSII). Under drought stress, we observed a decrease in indices such as the maximum initial photochemical efficiency of PSII, maximum quantum efficiency of PSII, trapped oxidation, quantum efficiency of electron transfer, and the photosynthetic performance index based on absorption. Conversely, indicators such as the quantum efficiency at zero time for energy loss, the size of the apparent antenna of active PSII, the maximum trapped oxidation in the two photosystems, and the ratio of energy loss flux in each reaction center increased. Maintaining photosynthetic balance is critical during abiotic stresses, as a robust photosynthetic system enhances stress resistance, enabling plants to better cope with stress. Studies have shown that the performance and efficiency of a plant's photosynthetic system decline under various stresses, including cold (Dong *et al*. 2020), drought (Cicek *et al*. 2019; Arab *et al*. 2023), heat (Zhou *et al*. 2015), salinity (Mathur *et al*. 2013), and nutrient deficiency (Hu *et al*. 2023).

The results of PCA and Pearson correlation showed that RWC, SPAD index, F_M/F_0, F_V/F_0, F_V/F_M and Pi-$_{ABS}$ were positively correlated under water-deficit stress conditions; However, RWC was not significantly correlated with the photosynthetic characteristics under normal conditions and the SPAD was only significantly, but weakly, correlated with three traits, including TR_0/RC, ABS/RC, and DI_0/RC. More important, based on PCA, we observed that under water-deficit stress conditions, RWC and SPAD were more discriminating among the walnut families than under normal conditions, indicating their efficiency in identify drought-tolerant families. Based on the results obtained in the resistant families under drought stress, these traits cause stability, water conservation, and greenness of plants against drought stress. In plant families TH70, S191, B149, B66, B68, and S201, which were identified as tolerant, these traits had lower reduction in RWC, SPAD, and photosynthetic characteristics as compared to the control conditions. However, in the families TH52, TH54, S190, B39, G94, B15, B37, and TG134, which were identified as susceptible families, SPAD, RWC, and some traits related to chlorophyll fluorescence were severely reduced under drought-stress conditions. The results of principal component analysis can confirm the efficiency of the SPAD, RWC, and some traits related to chlorophyll fluorescence, in selecting susceptible and tolerant families. The results of our experiment were consistent with previous reports on walnut (Arab *et al.* 2023), almond (Karimi *et al.* 2015) and pistachio (Fahimi Khoyerdi e*t al.* 2016).

The OJIP test is an excellent tool for understanding the adaptability and resilience of plant genotypes under stress, enabling us to identify tolerant varieties for breeding programs. In our study, we found significant variations in OJIP indices among walnut families, with some displaying higher photosynthetic efficiencies under drought stress. These robust families are promising candidates for further evaluation and could be useful in breeding programs aimed at enhancing drought tolerance in walnut trees. Our research underscores the importance of identifying and characterizing walnut families that are resilient to drought stress for sustainable cultivation in arid and semi-arid areas. By evaluating physiological characteristics such as RWC, SPAD index, stomatal density, chlorophyll content, and OJIP indices, we can effectively assess the stress tolerance and adaptability of walnut genotypes to drought conditions. The insights gained from this study provide valuable information for breeding programs focused on developing drought-tolerant walnut varieties, ultimately enhancing the resilience and productivity of walnut cultivation in water-limited environments.

Conclusion

Our study revealed that local walnut populations display a wide range of photosynthetic and physiological responses to drought stress. We assessed photosynthetic-related traits across 115 walnut families cultivated in a controlled greenhouse, examining both well-watered and water-stressed conditions. Our results indicated that characteristics such as RWC, SPAD, and various chlorophyll fluorescence metrics (F_V/F_0, ET_0/RC, F_V/F_M, PI-$_{ABS}$, Phi-E_0, Psi-$_0$, and F_M/F_0) decreased under water-deficit conditions. Conversely, indices like DI_0/RC, TR_0/RC, ABS/RC, Phi-Pav, and increased in these walnut families. Through principal component analysis and drought stress evaluation indices, we tentatively categorized the walnut families into three groups: drought-tolerant, moderately drought-tolerant, and drought-sensitive. The drought-tolerant group showed smaller changes across most measured characteristics from normal to water-deficit stress conditions. Notably, families G107, B31, B66, B68, and B142 from arid areas in Khorasan Razavi Province, Iran, demonstrated the highest photosynthetic efficiency under drought conditions. PCA of phenotypic data highlighted that chlorophyll fluorescence characteristics (F_V/F_M, F_M/F_0, F_V/F_0, Pi-$_{ABS}$, ABS/RC, TR_0/RC, and DI_0/RC), along with RWC and SPAD, can be effective biomarkers for identifying drought-tolerant genotypes of Persian walnut. This study emphasizes the critical role of genetically diverse resources, particularly the G107 and B142 families, which exhibit significant drought-tolerance potential. These families could play an essential role in breeding programs focused on enhancing walnut adaptability to drought stress.

Author Contributions

H. Ataee, M. Alizadeh, and K. Vahdati: Conceptualisation. H. Ataee, S. Sarikhani: Physiological and photosynthetic data collection and respective analysis. H. Ataee: Writing –original draft. H. Ataee, M. Alizadeh, S. Sarikhani, and K. Vahdati: Writing – review and editing. All authors have read and approved the manuscript for publication.

Data Availability

Data will be made available on request.

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Conflict of Interest

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

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