Natural variation in photosynthesis and water use efficiency of locally adapted Persian walnut populations under drought stress and recovery

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ABSTRACT

Persian walnut is a drought-sensitive species with considerable genetic variation in the photosynthesis and water use efficiency of its populations, which is largely unexplored. Here, we aimed to elucidate changes in the efficiency of photosynthesis and water content using a diverse panel of 60 walnut families which were submitted to a progressive drought for 24 days, followed by two weeks of re-watering. Severe water-withholding reduced leaf relative water content (RWC) by 20%, net photosynthetic rate (P_n) by 50%, stomatal conductance (g_s) by 60%, intercellular CO_2 concentration (C_i) by 30%, and transpiration rate (T_r) by 50%, but improved water use efficiency (WUE) by 25%. Severe water-withholding also inhibited photosystem II functionality as indicated by reduced quantum yield of intersystem electron transport (φ_{Eo}) and transfer of electrons per reaction center (ET_0/RC), also enhanced accumulation of Q_A (V_J) resulted in the reduction of the photosynthetic performance (PI_{ABS}) and maximal quantum yield of PSII (F_V/F_M); while elevated quantum yield of energy dissipation (φ_{Do}), energy fluxes for absorption (ABS/RC) and dissipated energy flux (DI_0/RC) in walnut families. Cluster analysis classified families into three main groups (tolerant, moderately tolerant, and sensitive), with the tolerant group from dry climates exhibiting lesser alterations in assessed parameters than the other groups. Multivariate analysis of phenotypic data demonstrated that RWC and biophysical parameters related to the chlorophyll fluorescence such as F_V/F_M, φ_{Eo}, φ_{Do}, PI_{ABS}, ABS/RC, ET_0/RC, and DI_0/RC represent fast, robust and non-destructive biomarkers for walnut performance under drought stress. Finally, phenotype-environment association analysis showed significant correlation of some photosynthetic traits with geoclimatic factors, suggesting a key role of climate and geography in the adaptation of walnut to its habitat conditions.

1. Introduction

Persian walnut (*Juglans regia* L.) has a broad global distribution in the temperate regions and plays an important role in the economy of various countries for its edible nuts, fresh kernels, wood production, and numerous benefits to human health (Habibi et al., 2022; Vahdati et al., 2018; Vahdati et al., 2020). Nonetheless, water deficit is considered to be one of the main environmental factors strongly limiting Persian walnut growth and production around the world (Aletà et al., 2009; Arab et al., 2020; Famula et al., 2019; Knipfer et al., 2020; Liu et al.,

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2019; Momayyezi et al., 2022). Climate change is expected to exacerbate water limitations in semi-arid regions, especially central Asia (Vahdati et al., 2021; Arab et al., 2019). Therefore, understanding the physiological responses of diverse and locally adapted populations to drought stress is an essential prerequisite toward increasing the efficiency of rootstock and cultivar breeding programs (Arab et al., 2020). To this end, identifying relevant and reliable traits as biomarkers of Persian walnut tree performance under drought stress enables more efficient screening and selection of promising genotypes, facilitating targeted breeding efforts and accelerating the development of drought-resistant walnut cultivars.

Drought stress causes various adverse effects including negative leaf water balance, chlorophyll degradation, reduction in photosynthesis, growth, and yield, and may ultimately cause plant death (Osakabe et al., 2014). Plant species and genotypes within a specific species have developed different mechanisms for responding to drought stress and subsequent re-watering (Sousaraei et al., 2021; Xiao et al., 2008). Drought stress is not only assigned to stomata closure and decreased transpiration but also to reduce the water content and the water potential of plant tissues (Farooq et al., 2012). The relative water content (RWC), and water use efficiency (WUE) are considered appropriate parameters generally used to study plant water status to drought stress (Hatfield and Dold, 2019; Khoyerdi et al., 2016). Lotfi et al. (2010) showed that walnut cultivars that had a high leaf RWC were also better able to resist drought stress, providing further evidence that RWC is a suitable indicator of plant water status. Liu et al. (2019) have reported that the intrinsic water-use efficiency (WUEi) in walnut seedlings exposed to water deficit increases as the result of stomatal closure to prevent excessive water loss. Furthermore, Knipfer et al. (2020) found that WUEi in two drought-tolerant clonal walnut rootstocks (RX1 and Vlach) increased compared to WUE in a drought-sensitive clone (VX211) under drought stress, suggesting this trait is an appropriate screening index for future studies.

It is well-documented that inhibition of photosynthesis is one of the primary physiological consequences of drought stress. Carbon assimilation declines due to both stomatal and non-stomatal limitations (Cochard et al., 2002; Nikinmaa et al., 2013; Rosati et al., 2006; Varone et al., 2012). Stomatal closure is commonly the first response to drought stress (Chaves, 1991; Yordanov et al., 2000). However recent studies argue that reduced CO_2 diffusion from the atmosphere to the site of carboxylation due to both stomatal closure and reduction of mesophyll conductance, is the main obstacle decreasing photosynthesis under drought (Chaves et al., 2009; Flexas et al., 2006; Flexas et al., 2013; Chaves & Oliveira, 2004). In addition, a non-stomatal limitation such as metabolic impairment, which is connected with deficiency of Calvin cycle intermediates, is also observed under moderate water deficit conditions (Flexas and Medrano, 2002). Drought stress also can cause damage to the pigments responsible for photosynthesis in both photosystems (PSI and PSII), leading to a decrease in their ability to absorb light and, hence, a reduced capacity for photosynthesis (Ashraf and Harris, 2013). Several studies demonstrated that PSI is more sensitive to drought than PSII (Banks, 2018; Kalaji et al., 2016). However, the functional activity and structural stability of PSII, which includes the oxygen-evolving complex (OEC), is considered as one of the most sensitive components of the photosynthetic apparatus to drought stress (Kalaji et al., 2016). Under mild-to-moderate drought stress, stomatal closure reduces the photosynthetic rate and leads to excess energy that, if not dissipated as heat, can cause severe damage to photosystem II (PSII) functionality by the generation of reactive oxygen species (ROS) (Flexas and Medrano, 2002). There are conflicting reports on the impact of drought stress on PSII activity, and the specific sites of PSII damage caused by drought stress remain poorly understood (Bano et al., 2021). Several studies indicated that drought stress leads to damage in the reaction center and oxygen-evolving complex of PSII. Additionally, it results in a decrease in electron transport rate (ETR) and inhibition of PSII activity, due to the loss of the D1 protein (Bano et al., 2021; Kalaji et al.,

2016). In contrast, some researchers argue that PSII damage occurs only under severe drought stress conditions. They propose that the down-regulation of PSII activity, ETR, and even photo-inhibition of PSII act as potential protective mechanisms in response to drought stress (Bano et al., 2021; Flexas et al., 2009). Therefore, evaluating the functionality of the photosynthetic system can aid in predicting the ability of crops to withstand drought stress.

Plants photosynthetic responses to drought stress and subsequent rewatering are complex and depend on plants' genotype/species together with the severity/duration of water deficit and plant growth stage (Farooq et al., 2012; Flexas et al., 2009; Kalaji et al., 2016). For example, Liu et al. (2019) reported that the decrease in net photosynthetic rates depends on the intensity and duration of drought stress, and is more significant in Juglans nigra L. than Juglans mandshurica Maxim. or Juglans regia L. cv. Jizhaomian, indicating that these latter species are more tolerant to drought. Furthermore, it has been observed that walnut genotypes differ in their photosynthetic capacity due to variations in the structure and function of their leaves (Momayyezi et al., 2022). Therefore, it is crucial to study the photosynthetic behavior of locally-adapted populations and screen for drought-tolerant genotypes in both stressful and normal environments. Gas exchange measurements using an infrared gas analysing system, IRGA (LI-6400, Li-Cor, Inc., Lincoln, NE, USA) can provide real-time information on photosynthesis-related parameters, including net CO_2 assimilation rate (P_n), transpiration rate (T_r), stomatal conductance to water vapor (g_s), and internal CO_2 concentration (C_i) (Liu et al., 2019; Momayyezi et al., 2022). However, these measurements alone do not provide sufficient information about the functionality of the photosynthetic apparatus, particularly the light reaction parts. To overcome this challenge, chlorophyll (Chl) fluorescence analysis (OJIP-test) can be employed as a quick, reliable, powerful, and simple tool to assess the status of PSII and evaluate photosynthetic electron transport (Kalaji et al., 2016). Chlorophyll fluorescence analysis offer a non-invasive approach to determine the plant's photosynthetic apparatus performance in response to drought (Mathur et al., 2013).

The OJIP test is named for specific points on the induction curve of chlorophyll fluorescence (Kalaji et al., 2016). The initial O–J portion of the fluorescence curve is associated with the closure of PSII reaction centers in response to the reduction of the primary quinone electron acceptor (QA). In the J–I part of the curve, the secondary electron acceptors QB, plastoquinone (PQ), cytochrome (Cyt *b*6f), and plastocyanin (PC) are also reduced. Finally, the increase in chlorophyll fluorescence in the I–P part of the curve is attributed to the reduction of electron transporters in PSI including ferredoxin, intermediary acceptors, and NADP. The OJIP-test is a widely applied tool for estimating the effects of drought on PSII as well as to differentiate genotypic responses under stress conditions in different plant species (Kalaji et al., 2016; Sousaraei et al., 2021; Woo et al., 2008). However, the maximal quantum yield of PSII (F_V/F_M) and the photosynthetic performance (PI_{ABS}) are the most frequently used parameters to measure PSII status under abiotic stresses (Kalaji et al., 2016). But there is conclusive evidence that advises against a reliance on F_V/F_M and PI_{ABS} as the most important parameters (Banks, 2018). Alternate parameters, such as energy fluxes for absorption (ABS/RC) and trapped (TR_0/RC) per reaction center (RC), electron transport rate (ET_0/RC), dissipated energy flux (DI_0/RC), quantum yield of electron transport (φ_{Eo}), quantum yield of energy dissipation (φ_{Do}), relative maximal variable fluorescence (F_M/F_0), and efficiency of donation electron to PSI (F_V/F_0), provide detailed information for identifying drought sensitivity in plants (Banks, 2018; Kalaji et al., 2016; Strasser et al., 2004; Sousaraei et al., 2021). Moreover, contrasting findings are available about the application of these indices to discriminate drought tolerant genotypes in different plant species. However, the application of this technique has received less attention for assessment of genetic variations among locally-adapted Persian walnut populations under drought stress and re-watering conditions.

Wild and locally-adapted germplasm can be a valuable resource to

increase genetic variability and enhance tolerance to abiotic and biotic stressors and walnut productivity (Momayyezi et al., 2022). Therefore, the selection and use of drought-tolerant genotypes are the first, basic, most reliable, practical, and also the absolute strategy in the executive management of an orchard in semi-arid and arid regions. Persian walnut is a typical drought-sensitive plant but has developed adaptive physiological mechanisms to withstand drought stress, and various responses have been reported among locally adapted populations (Aletà et al., 2009; Arab et al., 2020). Our previous studies of walnut germplasm demonstrated that over the past evolutionary history, under the heterogeneous climate and soil conditions of Iran, local populations of walnut accumulated a wealth of genetic resources adapted to drought stress (Arab et al., 2020). Thus, we assessed gas exchange measurements and chlorophyll fluorescence techniques to determine the functional diversity of 60 locally-adapted Persian walnut families with differential tolerance to drought. Our main objectives were: (1) to evaluate phenotypic plasticity in photosynthetic traits in locally-adapted Persian walnut families under water-withholding and re-watering conditions; (2) to identify drought-tolerant walnut families based on their physiological and photosynthetic response; (3) to assess the suitability of various water relations and photosynthetic parameters as biomarkers in screening for drought tolerance of walnut genotypes at the seedling stage; and (4) to examine correlations between photosynthetic traits and climatic variables in the origins of the mother trees, which are spread across diverse climatic zones in Iran. Overall, our results will facilitate the selection and breeding drought-tolerant rootstocks and cultivars for future walnut production in drought-prone regions.

2. Materials and methods

2.1. Plant material and climate data

Plant materials used for this study consisted of a diverse panel of 60 Persian walnut mother trees from 8 local populations from different geographical regions in Iran (Table S1). Each of the 8 populations was located in a distinct habitat with very diverse environmental conditions (e.g., climate, geology, and topography). The mother trees were 50- to 500-year-old open pollinated seedling trees from ecologically-distinct growing regions including Kerman, Fars, Ilam, Semnan, Yazd, Markazi, West Azerbaijan, and Hamedan. A detailed list of plant materials is presented in Table S1. GPS coordinates and elevation were used to determine climatic parameters (WorldClim; Hijmans et al., 2005) of the sampled areas from 1971 to 2000, while soil survey data (SoilGrids; Hengl et al., 2017) were used to describe the native habitat conditions of the mother trees. Our environmental data included a total of 245 variables, comprising 115 climatic and 130 soil variables. The climate dataset also included annual average potential evapotranspiration (PET), and an aridity index derived from precipitation and PET data (Table S2; Zomer et al., 2008). Principal component analysis on the climate and soil variables was conducted separately to remove collinearity among the variables.

2.2. Experimental design

To evaluate the photosynthetic performance of progeny under water-stressed and re-watering conditions, seeds sampled from the 60 families were grown in a common garden. Briefly, seeds were collected across Iran from mature Persian walnut trees, stratified, germinated and grown in 7-L polyethylene pots (Diameter × Height: 15 × 40 cm) in a potting mix (2:1:1 (v/v/v), soil:sand:leaf manure) under ambient greenhouse conditions (25 ± 2 °C) (Lotfi et al., 2010). At the end of the growing season, 9-month-old saplings were transplanted into 15-L polyethylene pots (Diameter × Height: 20 × 50 cm, adequate volume for 15-month-old seedling root growth) filled with potting mix (2:1:1 (v/v/v), soil:sand:leaf manure). The physical and chemical characteristics of the potting soil include; texture, specific gravity, amount of organic and mineral substances is given in Table S3. Two months before the onset of the drought stress experiment, saplings were moved to an environmentally controlled greenhouse (day/night temperature of ~25/20 °C, photoperiod of ~16 h, and relative humidity of ~35–50%) at the Research Greenhouses of the Department of Horticulture, University of Tehran, Pakdasht, Tehran, Iran. Saplings were continuously irrigated to keep the soil fully hydrated and fertilized with macro- and micro-nutrients each month.

To study the impact of water-withholding and re-watering on locally-adapted families (600 total saplings) photosynthetic performance, six to eight uniform saplings (~15-month-old) from each family were randomly assigned to either the control or the water deficit group (3–4 saplings per treatment), thereafter, were subjected to three irrigation treatments in the greenhouse: (i) well-watered (regular irrigation; above 75% field capacity (FC), (ii) severely water-stressed (withheld water; ~25–35% FC), and (iii) re-watered following severe stress (regular irrigation; above 75% FC) (Fig. S1). The field capacity (FC) and permanent wilting point (PWP) were determined using the soil water retention curve (SWRC). The results showed that when the soil moisture decreases to about 20% and 10%, respectively, the soil of the pots will be in the state of FC and PWP (Fig. S2). Experiments were laid out in a factorial completely randomized design with two factors (family and water treatment) and three replications.

2.3. Drought scoring (DS) index and relative water content (RWC)

Plants were graded for drought response on a range of 1–9 according to their visual appearance. Plants rated 1 were absolutely healthy plants with dark green leaves, while a rate of 9 indicates dying plants with necrotic leaves. Leaf discs were collected from fully expanded leaves of each plant. Ten uniform leaf discs were weighed immediately to compute the fresh weight (FW), and submerged in 25-ml closed tubes filled with distilled water for 24 h at 4 °C in darkness and again weighed to obtain turgor weight (TW). The leaf discs were then dried in an oven at 70 °C for 24 h, and the dry weight (DW) was recorded. Finally, the RWC or relative turgidity of each sample was calculated as RWC = [(FW–DW)/(TW–DW)] × 100.

2.4. Photosynthetic gas exchange measurements

Leaf gas exchange of 60 families (very drought tolerant to very sensitive) were measured under severe water stress (24 days after water-withholding) and recovery (two weeks after re-watering). Photosynthetic gas exchange was recorded with an infrared gas analysing system, IRGA (LI-6400, Li-Cor, Inc., Lincoln, NE, USA) on two fully-expanded leaflets at CO_2 concentration of 400 μmol CO_2 mol^{-1}, average temperature of 25 °C, light intensity of 1200–1500 μmol m^{-2} s^{-1} photosynthetic photon flux density and relative humidity of 55%. The infra-red gas analyser system (IRGA) was manually adjusted and the levels of CO_2 and H_2O references were fixed before measurements. Leaves with proper size were kept in the chamber for 1–3 min until the photosynthesis rate shown on the Li-Cor monitor was constant. Net CO_2 assimilation rate (P_n in μmol CO_2 m^{-2} s^{-1}), transpiration rate (T_r in mmol H_2O m^{-2} s^{-1}), stomatal conductance to water vapor (g_s in mol H_2O m^{-2} s^{-1}), and internal CO_2 concentration (C_i in μmol CO_2 mol^{-1} air) were recorded from two plants per treatment from 9 a.m. till 3 p.m. Intrinsic and instantaneous WUE were calculated as the ratio of the net CO_2 assimilation rate to stomatal conductance (P_n/g_s, intrinsic WUE in μmol CO_2 mol H_2O^{-1}) and transpiration rate (P_n/T_r, instantaneous WUE in μmol CO_2 mmol H_2O^{-1}), respectively. Carboxylation efficiency of Rubisco (CE) was estimated as the ratio of net CO_2 assimilation rate to internal CO_2 concentration (P_n/C_i) (Farquhar and Sharkey, 1982).

2.5. Chlorophyll fluorescence analysis

Chlorophyll-a fluorescence (OJIP) transients were recorded in young

fully-expanded walnut leaflets from the middle part of the sapling for each treatment after 20 min dark adaptation, using a portable fluorometer (Fluorpen FP 100-MAX, Photon Systems Instruments, Drasov, Czech Republic). Before taking readings, plants were allowed to dark-adapt overnight to ensure closure of almost all reaction centers of PSII, and the lights were extinguished in the greenhouse until measurements were concluded pre-dawn (between 1 and 5 a.m.). Briefly, the OJIP steps measured indicate fluorescence intensities at 50 μs referred to as F_0 (minimum fluorescence intensity) and occurs at the O step when all PSII reaction centers are open. 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, F_J), 30 ms (I-step, F_I), and at the maximum fluorescence intensity (approximately 200 ms, F_M) when all PSII RCs are closed (P step) were recorded for further calculations. In addition, other OJIP parameters were calculated according to the methods given by Strasser et al. (2004) to detect the injury site on the electron acceptor site of photosystem II (PSII). Some important parameters including the performance index (PI$_{ABS}$), PSII maximum photochemical efficiency (F_V/F_M), maximum quantum yield of energy trapping by PSII (φ_{Po}), quantum yield of intersystem electron transport (φ_{Eo}), 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 parameters are described in Table 1.

Three tolerant and sensitive families were selected for imaging the maximum quantum yield of PSII (F_V/F_M) using a FluorCam FC 1000-H (Photon Systems Instruments, Drásov, Czech Republic) which included a CCD camera and four fixed LED panels. Measurements were conducted on fully developed mature leaves attached to the sapling which were dark-adapted for 20 min after severe water stress and subsequent re-watering. F_V/F_M was calculated using a custom-made protocol by exposure of the samples to short flashes in darkness and following a saturating light pulse (3900 μmol m^{-2} s^{-1}) (Aliniaeifard et al., 2014; Genty et al., 1989). Maximum fluorescence (F$_m$') was recorded in light-adapted steady state and then NPQ was calculated using the ratio between the two maximum fluorescence (Fm/Fm').

2.6. Drought tolerance evaluation

To assess the drought tolerance of different populations, the response of families to drought stress for all measured traits was expressed using the drought stress index described in Wójcik-Jagła et al. (2013) and calculated as follows: DSI = (value of trait under water-stressed condition)/(value of trait under well-watered condition) × 100 (Wójcik-Jagła et al., 2013). The drought recovery index (DRI) was calculated using the same equation but substituting the value of trait under recovery condition in place of the value of trait under drought condition.

2.7. Statistical analysis

Statistical analyses were conducted using Minitab (Minitab, Inc., State College, PA, USA) and R (R Development Core Team, 2017). The normality of each trait was tested using the Shapiro-Wilk approach. General linear model (GLM) was applied to test the effect of families (F), water stress or re-watering treatment (T), and their interaction (F × T) on each photosynthetic-related trait under mild and severe drought stress, and subsequently re-watering. Significant differences between regions were calculated with Tukey's test using the TukeyHSD function of the multcompView package (Graves et al., 2015) and results were drawn as box plots using ggplot2. The phenotypic values of traits were then used for multivariate analysis. Multivariate statistical analyses comprising correlation analysis, and principal component analysis (PCA) were conducted via factoextra package (Kassambara and Mundt,

Table 1
Calculations and definitions of chlorophyll fluorescence (Strasser et al., 2004) and gas-exchange parameters measured in this study with their broad classification.

Phenotype category[a]		Phenotype	Calculation	Definition
Fluorescence parameters	(A)	F_0	$F_0 = F_{50μs}$	Minimum Fluorescence, when all PSII RCs are open
		F_J	$F_J = F_{2m}$	Fluorescence intensity at the J-step (2 ms)
		F_I	$F_I = F_{60ms}$	Fluorescence intensity at the I-step (60 ms)
		F_M	$F_M = F_{1s}$	Maximum fluorescence, when all PSII RCs are closed
		F_V	$F_V = F_M - F_0$	Variable fluorescence
		V_J	$V_J = (F_J - F_0)/(F_M - F_0)$	Variable Fluorescence at the J-step
		V_I	$V_I = (F_I - F_0)/(F_M - F_0)$	Variable fluorescence at the I-step
	(B)	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
	(C)	M_0	$4(F_{300μs} - F_0)/(F_M - F_0)$	Rate of reaction center closure
		N	(Area/($F_M - F_0$)) × M_0 × ($1/V_J$)	Turn-over number Q_A reduction events between time 0 and F_M
		Ψ_0	$\Psi_o = ET_0/TR_0 = 1 - V_J$	Likelihood that a trapped exciton can move an electron further than QA-
		φ_{Eo}	$\Phi_{Eo} = ET_0/ABS = (1-(F_0/F_M)) \times \Psi$	Quantum yield of electron transport
		φ_{Do}	$\Phi_{Do} = 1 - \Phi_{p0} = (F_0/F_M)$	Quantum yield at time 0 for energy dissipation
		Φ_{pav}	$\Phi_{pav} = \Phi p_0 (1 - V_{av})$	Average quantum yield of primary photochemistry
		PI$_{ABS}$	(RC/ABS) × ($\varphi_{Po}/1 - \varphi_{Po}$)	Performance Index of PSII normalized for equal absorption
	(D)	ABS/RC	ABS/RC = M_0 × ($1/V_J$) × ($1/\Phi p_0$)	Energy absorption by antenna per reaction center
		TR$_0$/RC	TR$_0$/RC = M_0 × ($1/V_J$)	Flux of excitons trapped per reaction center
		ET$_0$/RC	ET$_0$/RC = M_0 × ($1/V_J$) × Ψ_0	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
Gas-exchange parameters	(E)	P_n		Net photosynthetic rate (μmol CO_2 m^{-2} s^{-1})
		T_r		Transpiration rate (mmol H_2O m^{-2}s^{-1})
		g_s		Stomatal conductance (mol H_2O m^{-2} s^{-1})
		Ci		Intercellular CO_2 concentration (μmol CO_2 mol^{-1} air)
		Ca		Atmospheric CO_2 concentration
		WUEi	P_n/g_s	WUEintri in μmol CO_2 mmol H_2O^{-1}
		WUE	P_n/T_r	WUEinst in μmol CO_2 mmol H_2O^{-1}

Table 1 (*continued*)

Phenotype category[a]		Phenotype	Calculation	Definition
		CE	P_n/C_i	An estimate of carboxylation efficiency of Rubisco
Drought score and water relations	(F)	DS		Drought scoring system based on the appearance characteristics of seedlings
		RWC	$(FW - DW)/(TW - DW)$	Leaf relative water content (*RWC*)

[a] A: Primary fluorescence measurements; B: Fluorescence ratios; C: Derived parameters; D: Energy flux parameters; E: Gas-exchange parameters; F: Drought score.

2017) in R. Heatmap hierarchical cluster analysis (HCA) was depicted using the pheatmap package in R. Correlation network plot based on Pearson coefficients derived from the climate data and physiological parameters was constructed using ggraph package (Pedersen et al., 2017) under R program/environment.

3. Results

3.1. Natural variation in water use efficiency and photosynthetic traits among Persian walnut populations

In the present study, drought-related traits (water relations, gas exchange and chlorophyll fluorescence parameters) were measured in 60 walnut families (600 15-month-old sapling) grown under well-watered, water stressed, and subsequent re-watering conditions. A brief description of each measured photosynthetic trait is provided in Table 1. In a common garden experiment, our germplasm collection containing locally adapted Persian walnut families showed a wide range of phenotypic variability for water relation and the photosynthetic traits measured under water-stress and re-watering conditions. Most of the traits studied showed normal distribution under both water stressed and re-watering conditions (Fig. 1 and Fig. S3). The chlorophyll fluorescence parameters of drought-tolerant walnut families are generally less affected than those of drought-sensitive ones. Nevertheless, most walnut families quickly recovered after re-watering (Fig. 1). Families also responded differently to these conditions (Fig. S3).

Saplings water status was determined based on leaf relative water content (RWC) and water use efficiency (WUE). RWC and WUE showed significant variations by the effects of families, moisture regimes and their interactions in water stress (P ≤ 0.01) and re-watering (P ≤ 0.05) conditions. RWC significantly reduced by about 20% and 5% in water stressed and re-watering conditions, in comparison with well water condition in the entire population studied (Table 2). Although, RWC was
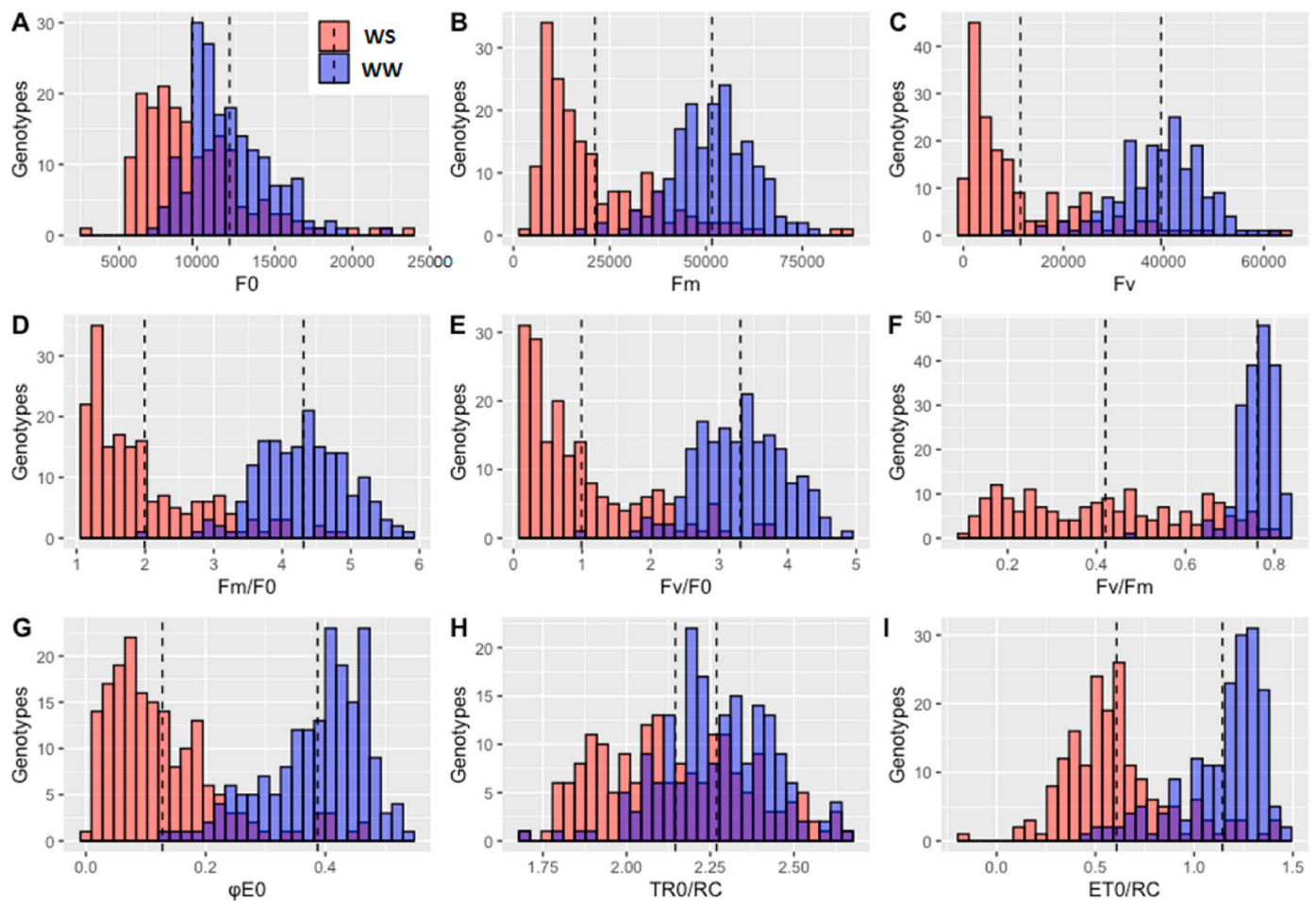

Fig. 1. Distribution of photosynthetic related traits in 15-month-old plants of 60 Persian walnut families under well-watered (blue), water-stressed (red) conditions and the overlap between them (purple). (A) F_0, (B) F_M, (C) F_V, (D) F_M/F_0, (E) F_V/F_0, (F) F_V/F_M, (G) φ_{Eo}, (H) TR_0/RC, and (I) ET_0/RC. Traits are indicated on the x-axis and number of families on the y-axis. Dashed vertical lines indicate the mean of each distribution.

Table 2

Descriptive statistics and the significance of *P* value of gas exchange parameters based on a general linear model for families (F; 60 walnut families), treatment (T) and their interactions (F × T) under severe water-stress and subsequent re-watering.

Conditions	Traits	Well-water (WW)	Water-stress (WS)	*P* value			Reduction or Enhancement (%)
		Mean ± SD	Mean ± SD	F	T	F × T	
Severe drought	RWC	80 ± 4	64 ± 6	**	**	**	−20
	P_n	5.1 ± 3.67	2.71 ± 2.02	**	**	**	−47
	g_s	0.042 ± 0.033	0.017 ± 0.013	**	**	**	−59
	WUE	138.6 ± 35.7	172.71 ± 47.30	**	**	**	25
	Ci	128.9 ± 31.09	92.78 ± 36.62	**	**	**	−28
	CE	0.04 ± 0.023	0.033 ± 0.043	**	**	**	−17
	T_r	2.12 ± 1.42	1.01 ± 0.79	**	**	**	−52
	WUEi	2.85 ± 1.15	3.54 ± 1.57	**	**	**	24
	Ci/Ca	0.35 ± 0.09	0.24 ± 0.1	**	**	**	−31
Recovery	RWC	75 ± 5	71 ± 7	**	**	**	−5
	P_n	4.49 ± 2.72	3.87 ± 1.7	**	**	**	−14
	g_s	0.039 ± 0.03	0.028 ± 0.012	**	**	**	−28
	WUE	135.9 ± 39.31	138.95 ± 14.59	**	*	**	2
	Ci	128.3 ± 31.72	133.7 ± 57.35	**	*	**	4
	CE	0.033 ± 0.015	0.034 ± 0.017	**	ns	**	3
	T_r	2.1 ± 1.44	1.51 ± 0.68	**	**	**	−28
	WUEi	2.77 ± 1.25	2.7 ± 0.68	**	**	**	−2
	Ci/Ca	0.35 ± 0.09	0.34 ± 0.1	**	*	**	−3

* Significant at $P < 0.05$, ** Significant at $P < 0.001$, ns: no significant.

on average 0.64 for families under drought, 0.71 for families under re-watering in the entire population; there was a significant decline in the sensitive walnut families under water-stress condition. As shown in Table 2, compared to well-water condition, WUE and WUEi under water-stress enhanced by 25% and 24% respectively (Table 2). Also, the values of RWC, WUE and WUEi after re-watering of stressed plants returned to close the well-water condition (Table 2).

All gas exchange parameters were significantly affected by families, moisture regimes and their interactions under both severe water-stress and re-watering conditions (**$P \leq 0.001$) except for the effects of moisture regimes on CE under re-watering condition (Table 2). In the entire population studied, the g_s, T_r, P_n, C_i/C_a, C_i and CE parameters significantly decreased by about 59%, 52%, 47%, 31%, 28% and 17% in water stress condition, respectively, compared to the well-water condition (Table 2). The results also showed a moderate reduction in the value of g_s (by 28%), T_r (by 28%) and P_n (by 14%) parameters under re-watering condition, while the C_i and CE parameters slightly increased in comparing to well-water condition (Table 2).

Chlorophyll fluorescence-related parameters including F_0, F_J, F_I, F_M, F_V, V_J, F_V/F_M, F_M/F_0 and F_V/F_0) except V_I were affected by families,

moisture regimes, and their interaction in water-stress condition ($P < 0.001$). As expected, in the entire population studied, water stress resulted in a significant reduction of F_0 (by 20%), F_J (by 45%), F_I (by 57%), F_M (by 59%), F_V (by 71%), F_V/F_0 (by 70%), F_M/F_0 (by 54%) and F_V/F_M (by 45%) but V_J value were significantly increased in water-stressed by about 45% as compared to control conditions (Table 3). In re-watering condition, except for F_I and F_V parameters which were not affected by moisture regimes, other chlorophyll fluorescence parameters were significantly affected by families, moisture regimes, and their interaction ($P < 0.001$). These parameters were altered differently during re-watering. The value of F_0, F_J and V_J were increased, while the value of F_V, F_V/F_0, F_M/F_0 and F_V/F_M were decreased in comparison with well-water condition (Table 4). Chlorophyll fluorescence parameters of different families were very variable and varied under drought stress and recovery conditions.

Quantum yields and specific energy fluxes parameters, including Ψ_0, φ_{Do}, φ_{Eo}, Φpav, PI_{ABS}, ABS/RC, TR_0/RC, ET_0/RC and DI_0/RC were significantly influenced by families, moisture regimes, and their inter-action in water-stress condition. Our finding showed that the value of PI_{ABS} by 88%, φ_{Eo} by 66%, Ψ_0 by 44%, ET_0/RC by 47%, and TR_0/RC by 6% were reduced as a result of the water-stress in the entire population studied (Table 3). While, some other parameters, including DI_0/RC (4.88-fold), φ_{Do} (1.44-fold), and ABS/RC (1.14-fold), were significantly increased in water-stressed samples as compared to well-watered conditions (Table 3). Our results also demonstrated that all quantum yields and specific energy fluxes except Φpav were affected by families, moisture regimes, and their interaction in re-watering condition ($P < 0.001$). In the entire studied population, the value of PI_{ABS} and φ_{Eo} in re-watering condition was lower than by about 26% and 7% respectively compared to well-water condition, while the value of DI_0/RC, φ_{Do} and ABS/RC were more than by about 24%, 14% and 7% respectively in comparison with well-water condition (Table 4).

The effect of severe water-withholding and subsequent re-watering on overall photosynthetic functionality of the three most tolerant and three most sensitive families was assessed by evaluating the spatial pattern of fluorescence emission through pseudo-color images of F_V/F_M (Fig. 2; equations in Table 1). Water stress led to lower F_V/F_M in the sensitive families than in tolerant families (Fig. 2). Susceptible families were able to recover significant amounts of photosynthesis after re-watering (Fig. 2).

In plants re-watered 14 days after experiencing water-withholding, chlorophyll fluorescence parameters recovered to the value of the well-water condition in most of the families except in the sensitive ones. These results indicate efficient recovery in walnuts. Overall, our findings showed that drought stress had a more severe negative effect on both gas exchange and chlorophyll fluorescence parameters in sensitive families. On the other hand, significant families-treatment interaction effects ($P \leq 0.001$) for most of the photosynthetic traits indicates the photosynthetic responses of different families is dependent on the conditions of the moisture regimes. Therefore, in selecting drought tolerant walnut genotypes the performance of genotypes under both moisture conditions should be considered.

3.2. Heatmap and hierarchical cluster analysis of Persian walnut populations using water relations and photosynthetic traits

A cluster heat map analysis was performed to classify walnut families according to their phenotypic plasticity of water status, photosynthesis, and chlorophyll fluorescence traits. According to the potential characteristics of the families, the studied 60 walnut families under water-stress and subsequent re-watering assigned into the three main groups. Under drought condition, 4 (Ke23, Ma2, Se2 and Ya1), 23, and 33 families were assigned to the "tolerant (A)", "sensitive (B)", and "moderately tolerant (C)" groups, respectively (Fig. 3). Our finding also demonstrated that the studied traits categorized into four and six clusters under water stress and re-watering conditions, respectively (Fig. 3).

Table 3

Descriptive statistics and the significance of *P* value of chlorophyll fluorescence parameters based on a general linear model for families (F; 60 walnut families), treatment (T) and their interactions (F × T) under severe water-stress.

Phenotype category	Traits	Well-water (WW)			Water-stress (WS)			*P* value			Reduction or Enhancement (%)
		Min	Max	Mean ± SD	Min	Max	Mean ± SD	F	T	F × T	
Fluorescence parameters											
Primary fluorescence measurements	F_0	7250	22,108	12,089 ± 2553	2592	23,343	9713 ± 3334	**	**	**	−20
	F_J	14,110	50,002	31,206 ± 5873	3181	52,733	17,219 ± 10,062	**	**	**	−45
	F_I	15,703	71,654	47,133 ± 9778	3260	84,691	20,110 ± 14,331	**	**	**	−57
	F_M	18,531	77,506	51,570 ± 1037	3420	87,195	21,204 ± 15,165	**	**	**	−59
	F_V	8745	61,088	39,481 ± 8578	818	65,120	11,472 ± 12,408	**	**	**	−71
	V_J	0.355	0.798	0.496 ± 0.092	0.404	1.091	0.72 ± 0.11	**	**	**	45
	V_I	0.677	0.953	0.886 ± 0.029	0.68	1.04	0.88 ± 0.047	**	ns	**	−1
Relative ratios	F_M/F_0	1.894	5.821	4.310 ± 0.643	1.15	4.8	1.99 ± 0.86	**	**	**	−54
	F_V/F_0	0.894	4.821	3.310 ± 0.463	0.15	3.8	0.99 ± 0.86	**	**	**	−70
	F_V/F_M	0.472	0.828	0.762 ± 0.042	0.13	0.79	0.42 ± 0.19	**	**	**	−45
Derived parameters	M_0	0.728	1.986	1.126 ± 0.228	0.88	2.48	1.54 ± 2.68	**	**	**	37
	N	456.4	7119.6	1004.5 ± 575.4	664	28,750	5061 ± 5191	**	**	**	404
	Ψ_0	0.202	0.645	0.504 ± 0.092	0.05	0.6	0.28 ± 0.11	**	**	**	−44
	φ_{Eo}	0.13	0.53	0.387 ± 0.082	0.013	0.47	0.13 ± 0.09	**	**	**	−66
	φ_{Do}	0.172	0.528	0.238 ± 0.042	0.21	0.89	0.58 ± 0.19	**	**	**	144
	Φ_{pav}	933.4	985.3	957.5 ± 10.07	947.8	995.2	978.51 ± 8.54	**	**	**	2
	PI_{ABS}	0.118	3.365	1.296 ± 0.684	0.001	1.96	0.15 ± 0.31	**	**	**	−88
Energy flux parameters	ABS/RC	2.424	4.779	2.989 ± 0.317	2.64	16.23	6.39 ± 3.13	**	**	**	114
	TR_0/RC	1.71	2.66	2.269 ± 0.163	1.69	2.65	2.14 ± 0.22	**	**	**	−6
	ET_0/RC	0.477	1.468	1.143 ± 0.212	0.11	1.41	0.61 ± 0.26	**	**	**	−47
	DI_0/RC	0.416	2.524	0.721 ± 0.212	0.55	14.12	4.241 ± 3.26	**	**	**	488

* Significant at *P* < 0.05, ** Significant at *P* < 0.001, ns: no significant.

In the "tolerant" group that were classified in group A, the RWC, WUE, PI_{ABS}, φ_{Eo}, ET_0/RC, Ψ_0, F_V/F_0, F_M/F_0, F_V/F_M, F_M, F_I, F_V, F_J, TR_0/RC and F_0 indices had the highest values, and the V_J, M_0, Φ_{pav}, N, φ_{Do}, ABS/RC, DI_0/RC, DS and C_i indices had the lowest values under water-stress condition (Fig. 3). Getting into more detail, various chlorophyll fluorescence ratios (F_V/F_0, F_M/F_0, F_V/F_M), which depict the PSII functional status were significantly decreased in the drought-sensitive group under water-stress. Our results further revealed that the water-stress caused a substantial reduction in PI_{ABS}, φ_{Eo}, ET_0/RC, Ψ_0, and TR_0/RC in the drought-sensitive group. Moreover, the φ_{Do}, ABS/RC, and DI_0/RC were slightly decreased in the drought-tolerant group. We also found a significant reduction in the relative variable chlorophyll fluorescence at J (V_J) and the rate of QA reduction (M_0) or closure of the PSII reaction center in the drought-tolerant group under drought stress (Fig. 3). In contrast, V_I was slightly higher in the drought-tolerant group. These results indicated that the main changes in fluorescence occurred at O, J, I, and P steps only in the drought-sensitive group, indicating that probably the sites of damage are the antennae, oxygen-evolving complex (OEC), intersystem electron acceptors, and energy dissipation.

Under re-watering condition, walnut families were classified into the "strong (A)", "weak (B)", and "moderate (C)" recovery groups including 14, 7, and 39 (16 and 23) families, respectively (Fig. 4). Our finding demonstrated that chlorophyll fluorescence ratios (F_V/F_0, F_M/F_0, F_V/F_M), PI_{ABS}, φ_{Eo}, Ψ_0, RWC, WUE, T_r and CE returned to their initial normal status in most families assigned to groups A and C, which had higher recovery potential (Fig. 4). Our results further showed that the primary fluorescence parameters (F_0, F_M, F_I, F_J, and F_V) and ET_0/RC were substantially lower in the weak recovery group. Furthermore, the energy flux parameters (ABS/RC, TR_0/RC, and DI_0/RC), φ_{Do}, M_0, V_J, and V_I were significantly higher in the weak recovery group and a subgroup of moderate recovery group. These findings indicate that most walnut families have recovered well after re-watering, except the weak recovery group. In addition, the electron transport chain has a higher efficiency in the strong recovery group. Among the families arranged in the strong recovery group (A), families Ma2 and Ya1 also demonstrated the highest recovery potential. These families exhibited high values for P_n, g_s, PI_{ABS}, Ψ_0, φ_{Eo}, WUE_i, WUE, F_V/F_M, F_V/F_0, F_M/F_0, while showing the minimum values for DS, φ_{Do}, DI_0/RC, V_J, M_0, V_I, ABS/RC and TR_0/RC. In addition, our results showed that most of the families with weak recovery potential originated from the humid areas (Fig. 4).

3.3. Correlation and principal component analysis of water relations and photosynthetic traits in Persian walnut populations

Significant correlations among DSI and DRI of photosynthetic traits in both water-stressed and re-watering environments were detected (Fig. 5; Figs. S4–S5). Drought scoring index (DS) was negatively correlated with the DSI of gas exchange parameters including T_r, g_s, C_i, and P_n ($r = 0.37**$, $r = 0.36**$, $r = 0.28**$ and $r = 0.26**$, respectively), but positively correlated with the DSI of RWC, WUE, and WUE_i ($r = 0.69***$, $r = 0.34**$ and $r = 0.38**$; Fig. 5). On the other hand, DS was positively correlated with some parameters obtained from analysis of chlorophyll fluorescence such as F_V/F_M, F_M/F_0, F_V/F_0, φ_{Eo}, and PI_{ABS} ($r = 0.35**$, $r = 0.33**$, $r = 0.32*$, $r = 0.30*$ and $r = 0.27*$; Fig. 5). Moreover, RWC was positively correlated with DSI of F_V/F_M, φ_{Eo}, F_V/F_0, F_M/F_0, TR_0/RC, ET_0/RC, and PI_{ABS} ($r = 0.4**$, $r = 0.39**$, $r = 0.36**$, $r = 0.35**$, $r = 0.34**$, $r = 0.31**$, and $r = 0.30*$) but negatively correlated with DSI of φ_{Do}, DI_0/RC, and ABS/RC ($r = 0.39**$, $r = 37**$, and $r = 0.36**$; Fig. 5). Correlations among the photosynthetic traits under recovery conditions were almost in line with the DSI of the studied traits (Fig. S5). Most of the phenotypes obtained from analysis of chlorophyll fluorescence are ratios or otherwise estimated based on primary phenotypes (Table 1).

Table 4

Descriptive statistics and the significance of *P* value of chlorophyll fluorescence parameters based on a general linear model for families (F; 60 walnut families), treatment (T) and their interactions (F × T) under re-watering.

Phenotype category	Traits	Well-water (WW)			Water-recovery (WR)			*P* value			Reduction or Enhancement (%)
		Min	Max	Mean ± SD	Min	Max	Mean ± SD	F	T	F × T	
Fluorescence parameters											
Primary fluorescence measurements	F_0	8030	18,726	12,403 ± 2123	5743	22,888	13,806 ± 2841	**	**	**	11
	F_J	22,043	44,573	31,355 ± 4623	13,151	53,936	32,781 ± 6554	**	*	**	5
	F_I	33,616	68,111	49,693 ± 6883	18,844	78,677	49,555 ± 9732	**	ns	**	0
	F_M	39,208	74,483	55,926 ± 7143	20,678	86,967	55,085 ± 10,339	**	ns	**	−2
	F_V	29,877	57,870	43,523 ± 5280	14,935	66,257	41,278 ± 8065	**	**	**	−5
	V_J	0.36	0.548	0.436 ± 0.031	0.37	0.59	0.46 ± 0.044	**	**	**	6
	V_I	0.79	0.91	0.856 ± 0.024	0.79	0.96	0.86 ± 0.026	**	**	**	0
Relative ratios	F_M/F_0	3.81	5.89	4.55 ± 0.36	2.29	5.27	4.03 ± 0.46	**	**	**	−11
	F_V/F_0	2.81	4.89	3.55 ± 0.36	1.29	4.27	3.03 ± 0.46	**	**	**	−15
	F_V/F_M	0.74	0.83	0.78 ± 0.017	0.56	0.81	0.75 ± 0.03	**	**	**	−4
Derived parameters	M_0	0.708	1.44	1.01 ± 0.101	0.8	1.56	1.11 ± 0.15	**	**	**	10
	N	382.4	1517.4	693 ± 196.6	438.1	2202.5	768.8 ± 285.9	**	**	ns	11
	Ψ_0	0.452	0.64	0.56 ± 0.031	0.4	0.63	0.54 ± 0.04	**	**	*	−4
	φ_{Eo}	0.342	0.531	0.44 ± 0.03	0.26	0.49	0.41 ± 0.04	**	**	**	−7
	φ_{Do}	0.17	0.263	0.22 ± 0.17	0.19	0.44	0.25 ± 0.03	**	**	**	14
	Φ_{pav}	914.4	968.7	942.9 ± 9.81	919.33	978.18	947.7 ± 11.4	**	**	ns	1
	PI_{ABS}	0.761	3.675	1.59 ± 0.38	0.28	2.43	1.17 ± 0.41	**	**	**	−26
Energy flux parameters	ABS/RC	2.37	3.58	2.98 ± 0.2	2.63	3.98	3.2 ± 2.67	**	**	**	7
	TR_0/RC	1.97	2.69	2.32 ± 0.12	2.08	2.74	2.39 ± 0.14	**	**	**	3
	ET_0/RC	1.04	1.46	1.31 ± 0.083	0.92	1.51	1.28 ± 0.09	**	*	*	−2
	DI_0/RC	0.4	0.94	0.66 ± 0.09	0.5	1.72	0.82 ± 0.18	**	**	**	24

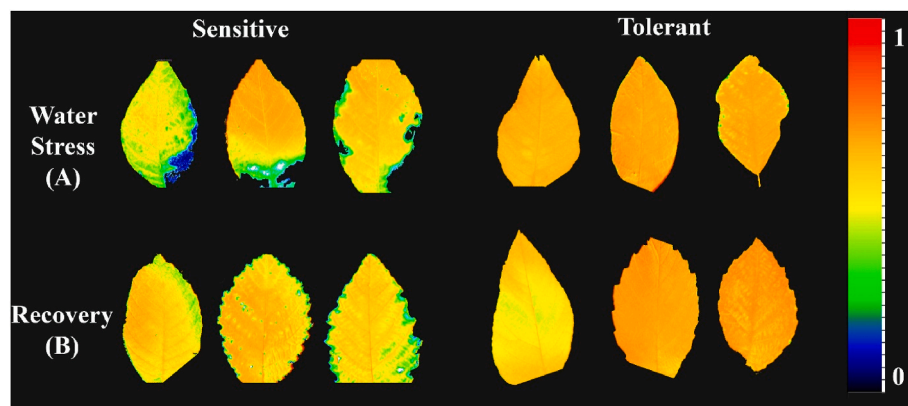* Significant at $P < 0.05$, ** Significant at $P < 0.001$, ns: no significant.

Fig. 2. Pseudo-color images of F_V/F_M (as described in Table 1) displayed by leaves sampled from Persian walnut sapling exposed to 24 days of water stress (A) and subsequent re-watering (B). Representative images of three tolerant and sensitive families are shown.

Therefore, these phenotypes are dependent on each other and many significant correlations are expected. Taken together, we observed a strong negative and positive correlation between various parameters and the DS index under water stress and re-watering conditions and these findings can be employed for identifying promising drought-responsive traits.

Principal Component Analysis (PCA) was applied on all photosynthetic traits of the 60 Persian walnut families during water-stress and re-watering to further explore the key parameters and to provide an integrated view of the relationships among traits within populations. PCA using DSI of the studied traits identified ten significant principal components (PCs) and the first six components cumulatively explained more than 85% of the total variation for the photosynthetic traits across the panel under severe drought stress (Fig. 6A). The first principal component (PC1), explaining more than 47% of the total variation, was positively associated with phenotypic variation of F_V/F_0 (96%), F_V/F_M (96%), φ_{Eo} (94%), PI_{ABS} (86%), and ET_0/RC (78%) (Fig. 6A). This component was also negatively correlated with φ_{Do} (96%), DI_0/RC (88%), and ABS/RC (85%). As a result, these traits that showed a high correlation with the PC1 are the most sensitive indices for the detection of the effects of drought on walnut seedlings. Since PC1 exhibits a high and positive correlation with both F_V/F_M and PI_{ABS}, it can be interpreted
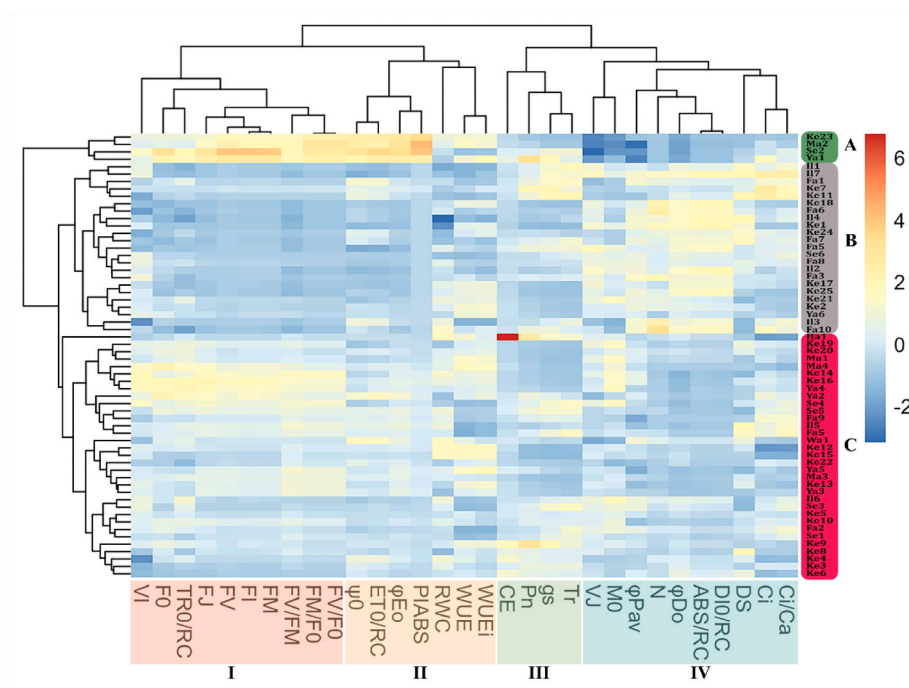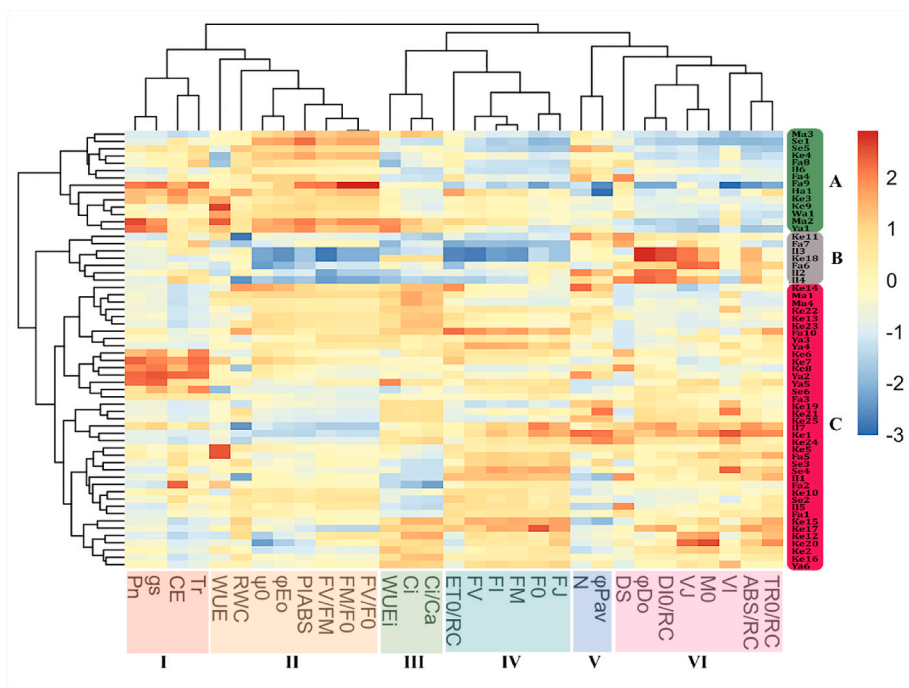
Fig. 3. Heatmap and hierarchical clustering of phenotypic plasticity (DSI) for physiological and photosynthetic parameters in the 60 Persian walnut families under water stress conditions (after 24 days of water-withholding). Clustering analysis of walnut families (left) showed three main groups where the groups A, B and C represent tolerant, sensitive and moderately tolerant families under the water stress treatment respectively. The clustering analysis of studied parameters (top) showed four major groups: group I includes some primary fluorescence measurements and their ratios, group II include the water relations and derived fluorescence parameters, while group III include the gas exchange parameter, and group IV include a combination of gas exchange and chlorophyll fluorescence parameters. See Table 1 for the definition of measured traits. By the province where the families are collected, the codes are as follows: Kerman: Ke, Fars: Fa, Ilam: Il, Semnan: Se, Yazd: Ya, Markazi: Ma, West Azerbaijan: Wa, and Hamedan: Ha.

Fig. 4. Heatmap and hierarchical clustering of phenotypic plasticity (DRI) for physiological and photosynthetic parameters in the 60 Persian walnut families under recovery conditions (after 14 days of re-watering). Clustering analysis of walnut families (left) showed three main groups where the groups A, B and C represent families with strong, weak and moderate recovery under the re-watering treatment respectively. The clustering analysis of studied parameters (top) showed six major groups: group I includes the gas exchange parameters, group II include the water relations and some derived fluorescence parameters, group III also include some the gas exchange parameter, group IV include some primary fluorescence measurements, group V a few derived fluorescence parameters, and group VI include mainly the energy flux parameters. See Table 1 for the definition of measured traits. By the province where the families are collected, the codes are as follows: Kerman: Ke, Fars: Fa, Ilam: Il, Semnan: Se, Yazd: Ya, Markazi: Ma, West Azerbaijan: Wa, and Hamedan: Ha.

as a component that reflects the responsiveness or tolerance to water stress. The second principal component (PC2), explaining more than 15% of the total variation, was associated mainly with gas exchange parameters; positively with T_r (95%), P_n (86%), and g_s (86%), and negatively with WUE (68%), and RWC (30%) (Fig. 6 A). In the PCA using DRI of traits, the first five components together accounted for more than 85% of the total variation of the photosynthetic traits under recovery (Fig. 6B). The first component (PC1), accounting for more than 35% of the total variation, was positively associated with the genotypic variation of PI_{ABS} (97%), F_V/F_M (94%), and φ_{Eo} (92%). PC1 was also negatively correlated with DI_0/RC (95%), φ_{Do} (94%), ABS/RC (88%), M_0

(87%), and V_J (80%). The second component (PC2), accounting for more than 19% of the total variation, was negatively associated with the genotypic variation of F_I (98%), F_M (98%), and F_V (94%). Overall, the results show that drought tolerance and recovery mechanisms are somewhat different.

3.4. Photosynthetic related traits correlate with geographical patterns of climatic variation

The studied walnut populations were divided into eight main regions based on climate clustering and their geographical origin. Significant
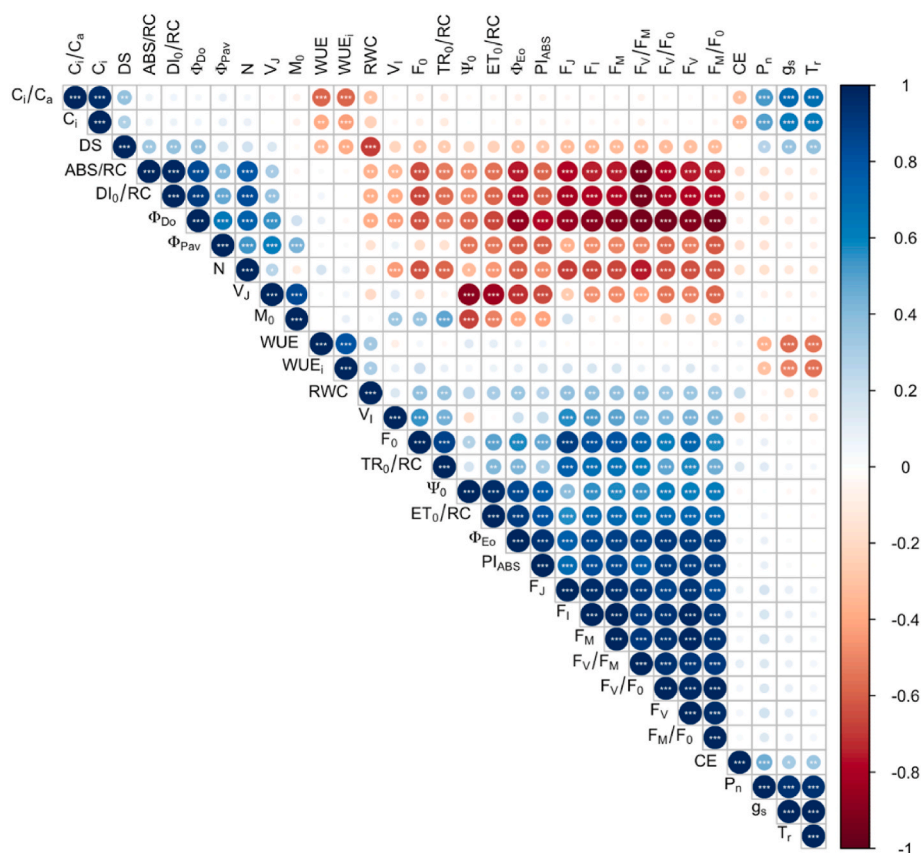
Fig. 5. Correlation coefficient of plasticity in trait value (DSI), between all gas exchange and chlorophyll fluorescence phenotypes in the 60 Persian walnut families grown in a common garden under water stress condition. The color spectrum, bright blue to bright red represents highly positive to highly negative correlations. Stars in circle indicate the significance of correlations (*P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001). See Table 1 for the definition of measured traits.

phenotypic differentiation among walnut populations from these regions for photosynthetic related traits was found under well-watered, water-stressed, and water-recovery conditions. WUE was significantly higher in the Markazi (means = 4.72 and 4.84 μmol CO_2 mmol H_2O^{-1}) and Yazd (means = 4.49 and 4.18 μmol CO_2 mmol H_2O^{-1}) populations under both water-stress and re-watering conditions compared to Ilam (means = 1.64 and 1.62 μmol CO_2 mmol H_2O^{-1}), Fars (means = 2.21 and 2.13 μmol CO_2 mmol H_2O^{-1}) and West Azerbaijan (means = 3.1 and 2.92 μmol CO_2 mmol H_2O^{-1}) populations. We also detected significant regional differentiation in chlorophyll fluorescence measurements (Figs. 7 and 8). The lowest reduction of F_V/F_M and PI_{ABS} value under water-stress conditions were recorded in walnut populations that collected from Yazd and Markazi provinces, respectively (Fig. 7 A and C). In the recovery conditions, except for the Ilam population, the F_V/F_M was not affected by populations of different regions, however, the F_V/F_M of populations from Yazd and Markazi provinces were very similar to the families that were grown under normal conditions (Fig. 7 B). Similar results were also observed in relation to performance index in recovery conditions between different walnut populations (Fig. 7 D). Our results showed the ABS/RC trait of walnut populations collected from Ilam and Fars provinces increased under drought stress compared to well-water condition, but other walnut populations collected from other provinces were slightly affected by water-stress (Fig. 7 E). The values of ABS/RC in stressed families of Fars population after re-watering returned to the corresponding value in well-water condition but it was not affected in families in Ilam population after re-watering (Fig. 7 F). Changes in TR_0/RC parameter among different populations of walnuts was not much affected by moisture regimes in water stress and re-watering conditions (Fig. 8 A and B). ET_0/RC was reduced significantly in all populations except in the case of West Azerbaijan population under

water-stress condition in comparison with well-watered condition. Under subsequent re-watering it returned to the first state like plants that were well watered in all walnut populations (Fig. 8 C and D). The DI_0/RC was increased in Fars, Ilam and Kerman populations under water-stress, while it was slightly different between well-watered and water-stressed walnut populations that collected from Hamedan, Markazi, Semnan, WeAzer and Yazd provinces. Although the DI_0/RC value of Fars, Ilam and Kerman populations was improved under re-watering condition but it also was higher in well-watered compared water-stressed families (Fig. 8 E and F).

In order to provide an overview of the phenotype-environment associations, we built a network of the photosynthetic related traits measured in a common garden, and the climate of the family's habitat. We applied PCA to describe multivariate variation in climatic environments reported for the provinces of family origin. As shown in the correlation network plot using DSI of the studied traits under severe drought (Fig. 9; Fig. S6), strong positive and slightly negative correlations were observed between WUE with altitude, PC1 of climate variables and PC2 of soil variables, and WUE with annual precipitation (BIO12), respectively. C_i showed strongly significant positive correlation with annual mean temperature (BIO1), and maximum temperature of warmest month (BIO5). RWC was negatively correlated with PC2 of climate variables, precipitation of the wettest quarter (BIO16), and precipitation of the coldest quarter (BIO19), while it was positively correlated with PC3 of climate variables (Fig. 9). Our results revealed that F_M/F_0, F_V/F_0, and F_V/F_M were negatively correlated with mean temperature of the coldest quarter (BIO11), BIO16, and BIO19 (Fig. 9). Another notable result of this study was the positive correlation of ABS/RC with BIO16 and BIO19 (Fig. 9). In addition, the correlation network plot using DRI of photosynthetic traits under recovery (Fig. S7) shows
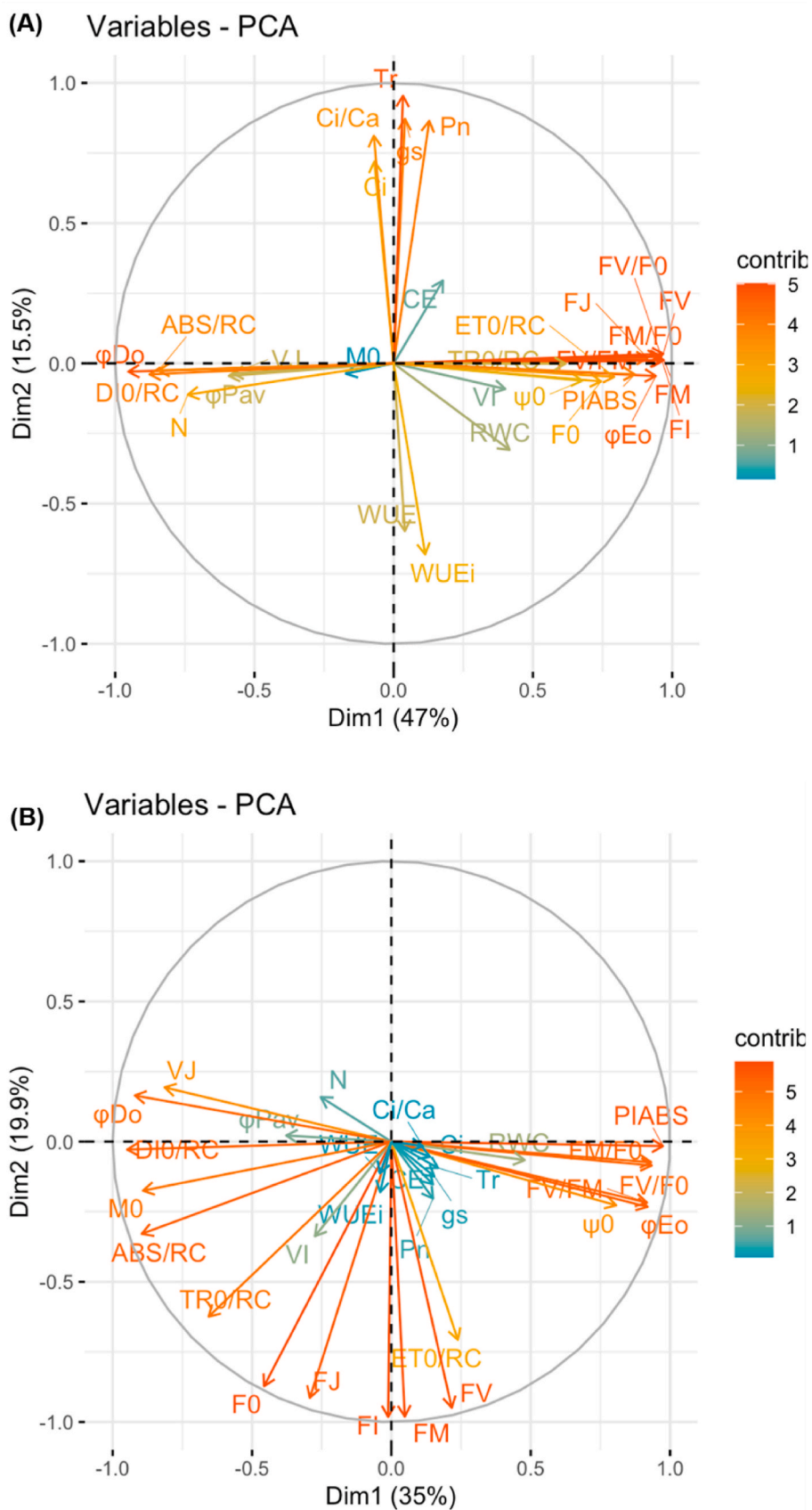
Fig. 6. Principal component analysis of all the photosynthetic traits in the 60 Persian walnut families, with the first two components illustrating variation in plasticity in trait value under drought (A) and re-watering (B) conditions. The traits coloured by red contributed more to the variation explained by PC1 and PC2, than those coloured by blue. See Table 1 for the definition of measured traits.
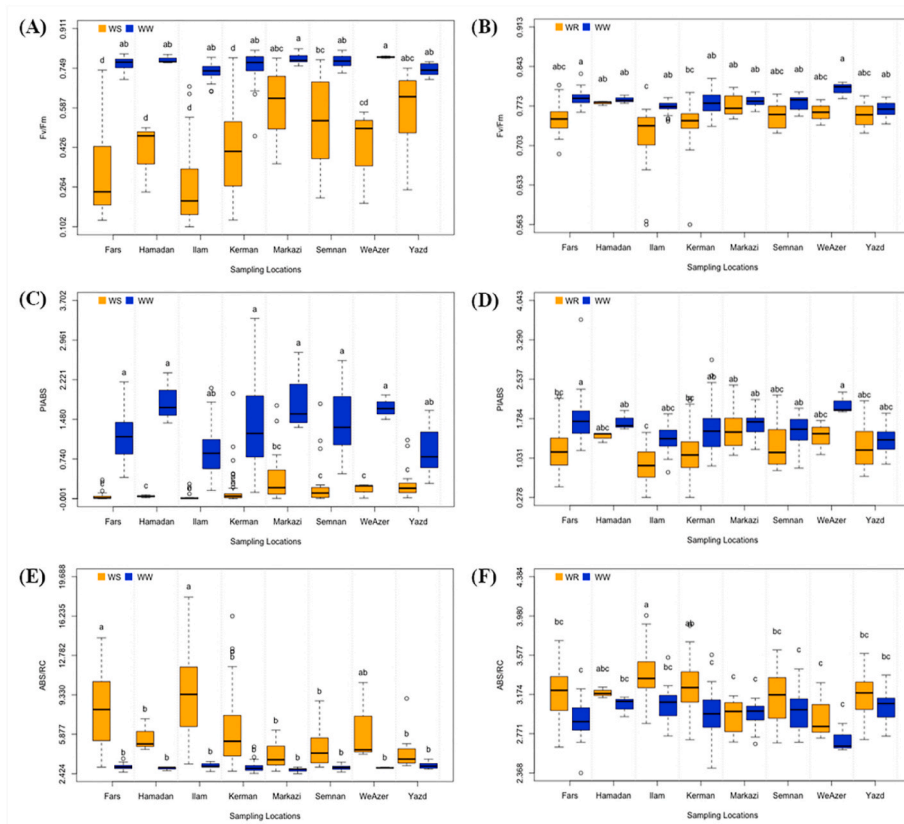
Fig. 7. Box plots of the regional differentiation of the chlorophyll fluorescence parameters in the 60 Persian walnut families. (A) and (B) F_V/F_M, (C) and (D) PI_{ABS}, (E) and (F) ABS/RC. WW: well-watered; WS: water-stressed; WR: water recovery. Different letters indicate statistically significant differences at the level of p < 0.01 (Tukey's test).

ABS/RC, φ_{Do}, and DI_0/RC have positive correlations with BIO16, and BIO19, but, RWC, F_M/F_0, F_V/F_0, and F_V/F_M correlate with them negatively.

4. Discussion

Walnut is one of the most economically important nut trees for its edible nuts and wood but greatly threatened by drought stress worldwide, especially in arid and semi-arid regions. Therefore, introducing drought-tolerant walnut rootstocks and cultivars, either by direct selection of superior genotypes from locally-adapted populations or the use of this valuable genetic resources in cross-breeding programs, is the most important step towards walnut tree orchard water management. To gain insights about physiological responses underlying drought tolerance of walnut, we investigated the phenotypic variability of 60 locally-adapted walnut families for drought-responsive physiological traits (photosynthetic and water relations parameters) under well-water, water withholding and re-watering conditions, and conducted phenotype-environment association using 245 climate and soil variables. Our finding revealed that the differential drought tolerance in locally-adapted walnut families was related to their plant water status and PSII quantum efficiency. In the present study, we identified four promising drought-tolerant families that can accelerate the release of drought-tolerant walnut rootstocks and varieties in the future, directly or by using them in breeding programs. Ultimately using these valuable genetic resources will reduce water consumption and increase yield (higher water use efficiency) in walnut orchards.

4.1. Natural variation in water use efficiency among Persian walnut populations

The RWC is one of the most critical physiological and reliable indices that can be used to assess drought tolerant of different genotypes (Khoyerdi et al., 2016). We found that water-withholding negatively correlated with the RWC, although response patterns varied between families. In line with our results, it has been indicated that drought reduce RWC in several species of fruit trees, such as apple (Liu et al., 2012) almond (Karimi et al., 2015) and pistachio (Behzadi Rad et al., 2021; Khoyerdi et al., 2016). Under the re-watering condition, the RWC value also returned to normal, although different families responded differently. Hence, the difference between families in terms of RWC value in drought stress and recovery conditions can be due to leaf cell size and wall thickness (Liu et al., 2012), root structure or the content of osmotic regulators (Khoyerdi et al., 2016). We found a stronger correlation between RWC and drought score (DS) among walnut populations under drought stress than recovery. The possible reason is that families with higher g_s and T_r values had lower tolerant indices due to reduced RWC values. Water use efficiency (WUE), a parameter of crop quality and performance under drought stress is an important selection criterion in genetic improvement programs (Gago et al., 2014; Osakabe et al., 2014). Our results indicated that WUE increased under drought stress. The WUE response in leaves is directly related to the physiological processes controlling the gradients of CO_2 and H_2O, such as leaf:air vapor pressure deficits (Hatfield and Dold, 2019; Gago et al., 2014). Since WUE at the leaf level is a complex trait dependent upon physiological responses, consistent with previous studies on different crop species (Hatfield and Dold, 2019), we also observed a wide variation in the WUE among the studied families. In agreement with the previous studies of Liu et al. (2019) and Karimi et al. (2020), we found a
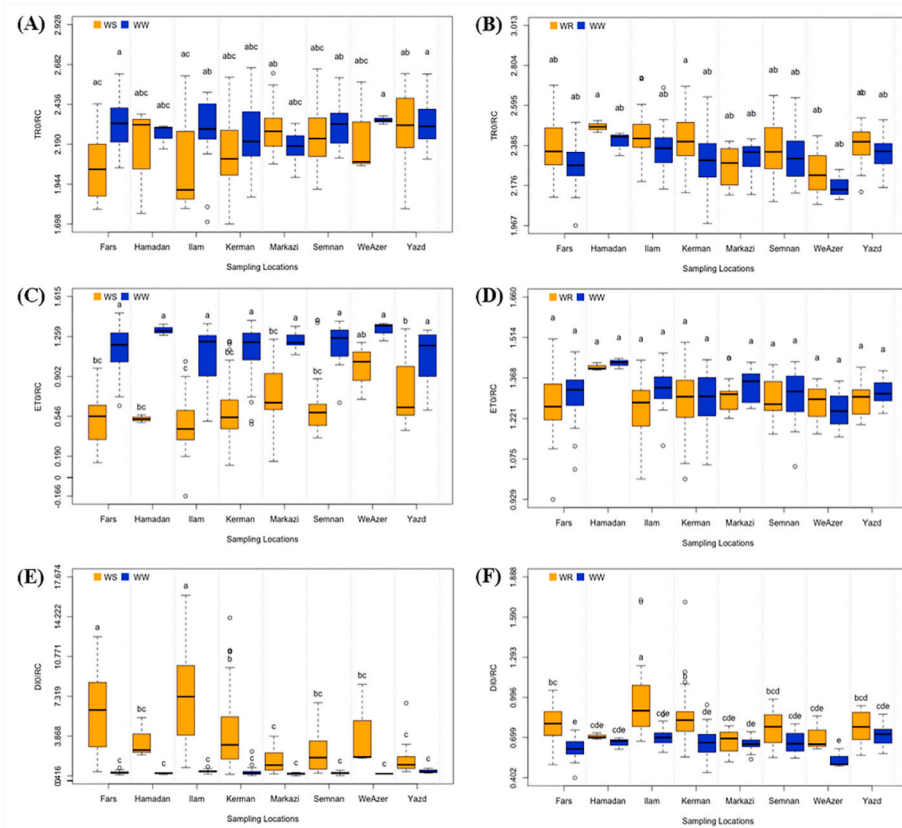
Fig. 8. Box plots of the regional differentiation of the chlorophyll fluorescence parameters in the 60 Persian walnut families. (A) and (B) TR_0/RC, (C) and (D) ET_0/RC, (E) and (F) DI_0/RC. WW: well-watered; WS: water-stressed; WR: water recovery. Different letters indicate statistically significant differences at the level of $p < 0.01$ (Tukey's test).
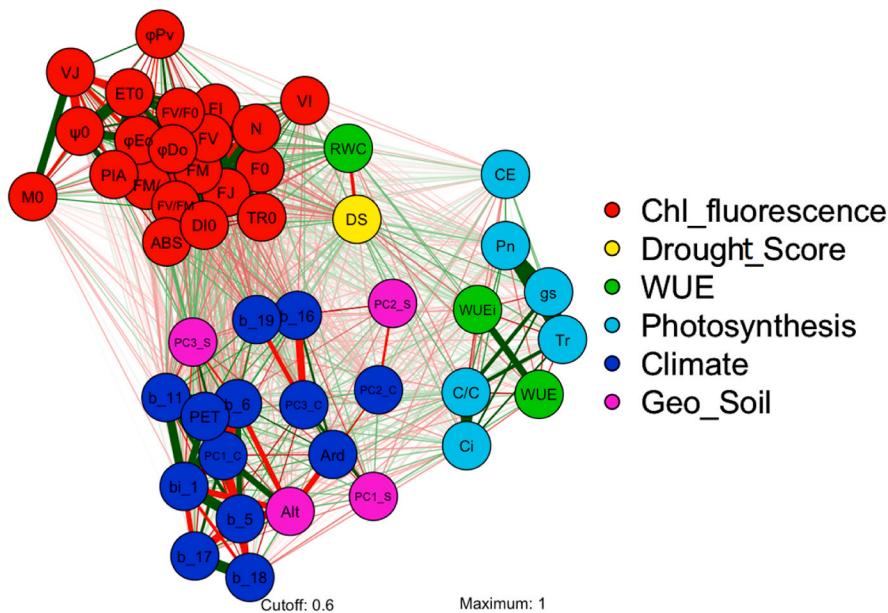

Fig. 9. Correlation network based on Pearson coefficients derived from the plasticity in trait value (DSI) data obtained from 60 Persian walnut families grown in a common garden under water stress condition. Traits associated with chlorophyll a fluorescence are shown as red dots, drought tolerance score yellow dots, water use efficiency green dots, gas exchange aqua dots, climate blue coloured and soil pink coloured. Green and red connecting lines represent positive and negative correlations, respectively. Line width is proportional to the strength of the correlation. See Table 1 for the definition of measured traits.

significant increase in WUE and WUEi in most families when placed under drought stress, especially those that originated form arid regions such as Markazi and Yazd. This is because stomatal closure under drought stress results in a simultaneous decrease in CO_2 uptake and water loss. Consequently, water use efficiency increases due to lower

stomatal conductance combined with sustained photosynthesis.

4.2. Natural variation in photosynthetic traits of Persian walnut populations

Morphophysiological responses to drought stress have been intensively investigated in Persian walnut (Knipfer et al., 2020; Jinagool et al., 2018; Vahdati et al., 2009; Rosati et al., 2006). However, the relevance of natural variation of photosynthetic traits for facing local limitations in water availability has not been documented in walnut. Photosynthetic response to drought stress is well characterized for Persian walnut but evidence about the plants' recovery ability remains scarce. In our study, gas exchange and chlorophyll fluorescence traits demonstrated a wide range of variation, suggesting that selection and genetic improvement of these traits are possible in walnut. Drought and the subsequent re-watering treatments significantly decreased the P_n, g_s, and T_r traits especially in drought sensitive families, in agreement with previous reports in walnut (Liu et al., 2019; Rosati et al., 2006). Our results also showed that P_n positively correlated with g_s, T_r, C_i, and CE under drought stress and re-watering conditions. We also found a more negative correlation between C_i and CE in drought stress conditions than in re-watering conditions. Our findings confirm previous studies' results that demonstrate photosynthesis is limited in walnut under drought stress due to stomatal and non-stomatal factors such as excessive heat (Arab et al., 2020; Rosati et al., 2006). It is generally believed that drought stress can precipitate a sequence of physiological responses involving stomatal closure, reduced g_s rates, consequent prevention of carbon fixation, and eventually reduced photosynthetic rate (Maleki et al., 2021; Nikinmaa et al., 2013). Our results indicate that reduction of P_n, g_s and T_r differed under drought stress among walnut families, which was consistent with previous finding (Karimi et al., 2018, 2020; Liu et al., 2019; Rosati et al., 2006). Under drought stress, the large decrease in g_s and C_i accompanied by increased water stress, suggests that stomatal closure probably is the main reason for the decline of P_n in Persian walnut. The observed significant positive correlation between the P_n and g_s, confirms this suggestion.

Previous studies revealed that reduction in leaf RWC under drought stress leads to an imbalance of photon energy captured by PSII and its utilization in the process of CO_2 reduction. Consequently, the electrons transfer to molecular oxygen (O_2) generating reactive oxygen species (ROS) which damage the protein complexes in the thylakoid membrane (Flexas et al., 2009). This statement supported by Bano et al. (2021), who have reported the destruction of thylakoid pigment-protein complexes in photosynthetic reaction centers can cause a decrease in photosynthetic pigments, resulting in the disruption of the electron transport chain. Thus, the observed divergence in drought stress tolerance among walnut families could be linked to their better capability to protect the photosynthetic machinery, which has been reported in numerous crops (Sousaraei et al., 2021). We found differential drought tolerance in locally-adapted walnut families. Our finding further revealed that drought stress caused a greater detrimental effect in the drought-sensitive families on the PSII, which can be because of a lower RWC-induced decrease in membrane fluidity or to poor photoprotective mechanism. While, in the drought tolerant families, different photo-protective mechanisms may have been activated, such as photo-respiration and the Mehler reaction. Our results suggested that drought stress caused a significant inhibition of both PSII and beyond PSII electron transport activities in Persian walnut families.

The effects of drought stress on target sites, such as components of photosynthetic electron transport, were identified and analyzed using the OJIP-test and its parameters (Strasser et al., 2004). The results of chlorophyll-a fluorescence transient analysis demonstrated that fluorescence substantially changed at O, J, I, and P time-points under drought condition, which is indicative of PSII electron transport inhibition (Strasser et al., 2004). In the drought-sensitive group, the multiple primary fluorescence parameters and their ratios, which are indicative of the PSII's structural stability or the site of damage, exhibited significant changes. In contrast, these parameters remained unchanged in the drought-tolerant group (Fig. 3). For example, F_M, F_I, F_J, F_V, F_0, F_M/F_0, F_V/F_0, and F_V/F_M were considerably reduced in drought-sensitive group. However, all these fluorescence parameters either remained almost at the same level as detected in the control or only slightly altered in the drought-tolerant group (Fig. 3), indicating that drought stress had slight effect on the structural damage to PSII at both donor and acceptor end of PSII in this group. We observed reduced F_M and F_0 at the end of severe drought stress, especially in drought-sensitive families, indicating the increase in the number of inactive PSII reaction centers. These results are in contrast with some of the previous studies. For instance, Faseela et al. (2020) showed an increase in F_0 under drought stress. Nevertheless, consistent with prior studies on abiotic stress, the reduction of F_M and F_0 in drought sensitive plants under severe drought stress were probably because of the damage of chloroplast, antenna structure or inactivation of the PSII reaction center (Tsai et al., 2019). According to Guidi et al. (2019), a decline in both F_0 and F_M also suggests a potential occurrence of photoinhibition due to an increase in the rate of non-radiative energy dissipation.

Notably, at the end of severe drought stress, the F_0 and F_V/F_M of drought-sensitive group decreased which may be due to damage or reversible inactivation of PSII reaction centers. Conversely, the drought-tolerant group exhibited a slight increase in F_0, suggesting that the transport of energy absorbed by the PSII antenna pigments to the photochemical reactions were partially suppressed as reported by Li et al. (2013) in other crops. Therefore, these plants demonstrated the ability to protect PSII reaction centers from damage. The findings suggested that in the drought-sensitive group under severe drought stress, there was a restriction in the transfer of energy that was absorbed by the light-harvesting complex to the reaction center. Additionally, there was a decreased probability of electron transport occurring from the donor end of PSII to the acceptor side of PSII. These results supported by Mehta et al. (2010) who reported that the reduction in F_M, F_V, and F_V/F_M under stress conditions could prevent transfer of electrons from PSII reaction center to electron transporters. Re-watering the walnut families after severe drought stress shows substantially lower primary fluorescence parameters (F_0, F_M, F_I, F_J, and F_V) and ET_0/RC in the drought-sensitive group, implicating a partial restoration in PSII and electron transport.

Drought stress more significantly reduced F_V/F_0, F_M/F_0, and F_V/F_M in the drought-sensitive group as compared to drought-tolerant group (Fig. 3). The reduction in F_M/F_0 can be explained by the damage to the reaction centers (RCs) and an increase in the number of inactive RCs. This leads to an increase in fluorescence and a decrease in the transfer of excitation energy from the antenna complex to the RCs (Kalaji et al., 2016). On the other hand, decrease in F_V/F_0 indicates that the transfer of electrons from the OEC to the donor side of PSII was less efficient in the drought-sensitive families under drought stress. Based on our finding, a decrease in the maximal quantum yield of PSII (F_V/F_M), and decline of Ψ_0 and φ_{Eo} in the drought-sensitive group (Fig. 3) indicates that drought stress inhibits the redox reaction after Q_A and slowdown the electron transfer between Q_A^- and Q_B, which result in Q_A accumulation. The reduction in F_V/F_M also indicated the fact that PSII RCs were impaired or not functioning effectively in photosynthesis under severe drought stress. In addition, the drought-tolerant group showed no photo-inhibition, as indicated by the unchanged F_V/F_M, in contrast to the drought-sensitive group where the decrease in PSII efficiency was mainly caused by photoinhibition (Flexas et al., 2009; Lawlor and Tezara, 2009). On the other hand, when the walnut families were re-watered chlorophyll fluorescence ratios (F_V/F_0, F_M/F_0, F_V/F_M), PI_{ABS}, φ_{Eo}, and Ψ_0 returned to their initially normal status in drought-tolerant and intermediate groups, implicating fully restoration occurred in PSII and electron transport. But sensitive families could not fully restore.

Accordingly, significant enhancement in variable fluorescence at step J (V_J) and rate of accumulation of closed RCs (M_0) under drought stress, especially in drought-sensitive families, reflects the accumulation of Q_A, indicating blockage of electron transfer from Q_A to Q_B on the PSII acceptor side, as also reported by Kalaji et al. (2016). PSII electron

transport chain over-reduction may be a possible cause of the reduced proportion of opened PSII reaction centers which may be reflected by energy dissipated as heat. Photosynthetic performance (PI_{ABS}) as a key chlorophyll fluorescence parameter is a combination of three partial components including the energy absorption, trapping, and conversion into the electron transport (Strasser et al. 2004). Our results align with previous studies indicating that this parameter is highly responsive to various environmental stress situations and varies significantly across different crops (Banks, 2018; Sousaraei et al., 2021; Strasser et al., 2000). PI_{ABS} sensitivity can serve as a useful biomarker to screen genotypes for their susceptibility to drought stress. PI_{ABS} did not change much in the drought-tolerant group, while it greater decreased in the drought-sensitive group (Fig. 3), which is associated with system structure, potential activity of PSII, photoinhibition of PSII, and disturbed PSI function. Furthermore, the greater reduction of PI_{ABS} in sensitive families compared to tolerant ones is indicative of a greater capability of tolerant families to maintain more active reaction centers capable of trapping and transporting more energy per unit of excited leaf area, in line with the finding of Kalaji et al. (2016).

A reduction in the F_V/F_M and PI_{ABS} values is considered appropriate indicators of photoinhibition, which can occur due to two distinct processes: a decrease in the rate at which PSII photochemistry caused by damages to the PSII reaction centers and/or an increase in the rate at which excitation energy is dissipated non-radiatively (Guidi et al., 2019). Wang et al. (2018) also reported a reduction in the overall performance of photosynthesis (PI_{ABS}) under stress conditions linked to a decrease in electron transport capacity. Therefore, maintaining walnut plant electron transfer capacity appears to play a crucial role in increasing its tolerant to drought stress. In agreement with Liu et al. (2019), our results showed F_V/F_M and PI_{ABS} decreased slightly in the drought-tolerant group suggesting a slight impact of drought stress on the openness of PSII reaction centers, and thus the plants did not damage seriously. In contrast, the reduced F_V/F_M and PI_{ABS} values of drought-sensitive group indicated impaired photosynthetic capacity and reduced electron transport through PSII. These findings demonstrate that drought-induced photoinhibition directly affects the ability of plants to carry out photosynthesis efficiently.

Damage to PSII reaction centers under drought stress have been influenced through electron transport and energy transformation. Some OJIP-related parameters such as ABS/RC, TR_0/RC, ET_0/RC, and DI_0/RC have been calculated to identify the damage sites on the acceptor side of PSII, including energy absorption, energy trapping, electron transport, and energy dissipation per reaction center (Strasser et al. 2004). Under severe drought stress, ABS/RC and DI_0/RC dramatically increased while substantial and slight decreases were detected in ET_0/RC and TR_0/RC, respectively, implying the dissipation of most of the absorbed energy by the PSII reaction center (Guha et al., 2013) According to Strasser et al. (2004), increasing ABS/RC under drought stress might indicate a change in the number of light-harvesting complexes (HLC) per PSII reaction center or inactivation of some reaction centers, *i.e.*, a reduction in active Q_A reducing reaction centers. Additional reasons for increment in ABS/RC include the degradation of chlorophyll due to early leaf senescence caused by severe drought stress and the regrouping of antennae from inactive PSII RCs to active ones (Boureima et al., 2012; Guha et al., 2013). Moreover, ABS/RC increased significantly in drought-sensitive families by drought exposure, however, significant changes were not observed in tolerant families, indicating that these families are capable of better regulation of energy amount reaching the reaction centers. Hence, the increase of ABS/RC in drought-sensitive families might point out a decrease in the effective antenna size per active RC (Q_A-reducing) that could be result from PSII inactivation. In agreement with Çiçek et al. (2019) our finding showed that DI_0/RC increased under drought stress, especially in drought-sensitive families, implying the dissipation of absorbed excitation energy, fluorescence emission, and/or transfer of energy to other systems, rather than electron transport. Furthermore, an increase in the DI_0/RC may be due to the disruption of connectivity

between PSII units. The above findings support the reduction of F_0 in drought-sensitive families, which is often caused by nonphotochemical energy dissipation by PSII antenna pigments as reported by Li et al. (2013). Overall, the main causes of disruption of PSII performance in the drought-sensitive group are a contribution to the inactivation of reaction centers, impaired electron transfer on the acceptor side of PSII, and enhanced energy dissipation in walnut under water-withholding.

Additionally, re-watering reduced the energy flux parameters (ABS/RC, TR_0/RC, and DI_0/RC), as well as φ_{Do}, M_0, V_J, and V_I in the drought-tolerant families. This suggests that there is a relaxation process that responds to changes in water availability. As a result, more energy can be converted from heat dissipation into CO_2 fixation, which can enhance the efficiency of photosynthesis. We observed that re-watering only partly restored photosynthetic functions in terms of chlorophyll fluorescence parameters in the drought-sensitive group (Fig. 4). Whereas re-watering fully restored the PSII efficiency and electron transport in the drought-tolerant and intermediate groups (Fig. 4). Thus, both drought limitations and the subsequent re-watering recovery ability will be the crucial determinants of the photosynthetic efficiency in the breeding of drought-tolerant walnut cultivars. Our findings suggest that the Ma2 and Ya1 walnut families can be introduced as tolerant walnut families under drought and recovery conditions. These families, by reducing the rate of M_0 and φ_{Do} as well as maintaining the F_V/F_0, F_V/F_M and quantum yield of electron transport demonstrate high photosynthetic efficiency under drought stress and recovery conditions.

Previous studies suggested that the parameters of JIP-test have the potential to serve as indicators of structural disorders in PSII and the carriers of intersystem electrons (Banks, 2018; Kalaji et al., 2016; Sousaraei et al., 2021). Several studies have shown that F_V/F_M and PI_{ABS} are highly responsive indexes that can be utilized to determine the photosynthetic physiological status of plants and to assess their overall health status when facing stressful conditions (Kalaji et al., 2016). In addition, it has been reported that PI_{ABS} is more sensitive to abiotic stresses than F_V/F_M (Çiçek et al., 2019). Nonetheless, there is decisive proof that discourages relying on F_V/F_M and PI_{ABS} as the most significant parameters (Banks, 2018; Sousaraei et al., 2021). The data presented here strongly suggest that the F_V/F_M, φ_{Eo}, φ_{Do}, PI_{ABS}, ABS/RC, and DI_0/RC are valuable screening indicators under both drought stress and recovery conditions because they exhibit strong correlation with the drought tolerance score (DS). Our results add evidence against an over-reliance on the F_V/F_M index alone. These findings are in line with the results of the previous studies, which showed significant advantages over F_V/F_M were achieved by the application of the other OJIP parameters (Banks, 2018; Kalaji et al., 2016; Strasser et al., 2004; Sousaraei et al., 2021). To summarize, understanding physiological responses and identifying reliable biomarkers for drought tolerance is crucial for breeding Persian walnut trees. Our findings showed that indices such as leaf RWC and WUE, as well as biophysical parameters related to chlorophyll fluorescence analysis (F_V/F_M, φ_{Eo}, φ_{Do}, PI_{ABS}, ABS/RC, ET_0/RC, and DI_0/RC), can provide valuable insights into the plant's water status and photosynthetic performance under drought stress. These non-destructive biomarkers can aid in the rapid and efficient screening of genotypes, allowing breeders to select drought-tolerant individuals for targeted breeding efforts and speeding up the development of more resilient and productive cultivars, ensuring walnut production sustainability in the context of climate change conditions.

4.3. Correlations between photosynthetic traits and the influence of geography

Improving photosynthetic efficiency is considered as a key goal to improve crop biomass production and yield potential. Therefore, leaf photosynthetic properties play an important role in determining biomass accumulation (Qu et al., 2017). Two main approaches to improve the photosynthetic efficiency of a plant species under stress conditions are identifying the superior genotype and genetically

engineering photosynthetic apparatus if an engineering target is well defined (Long et al., 2006). Therefore, identifying genotypes with high photosynthetic efficiency in drought stress conditions plays a key role in genetic improvement programs. Iranian local walnut populations exhibited different photosynthetic responses to drought stress and recovery in most studied photosynthetic traits, suggesting genetic control of drought adaptation. Under the well-watered condition, P_n was higher in populations originating from semi-arid and cold regions such as Hamadan and West Azerbaijan but these populations (especially the sensitive families) showed a greater reduction in photosynthesis under drought stress than populations collected from hot and dry areas such as Yazd, suggesting populations originated from arid habitat are particularly well adapted to the drought stress. Our results agree with Adams et al. (2016), who demonstrated that habitat temperature and precipitation of *Arabidopsis thaliana* ecotypes influences their photosynthetic efficiency in a climate-controlled growth chamber. Higher photosynthetic capacities of populations originated from cold region with higher altitudes would be expected to feature a greater ratio of reaction centers, compared to the outer light-harvesting proteins (Adams et al., 2016). In contrast, these populations showed a further reduction in photosynthesis under drought stress condition compared to the populations collected from hot and dry areas such as Markazi and Yazd provinces, suggesting that populations originated from arid habitat are particularly well adapted to the drought-stressed condition. On the other hand, populations from Markazi, Kerman and Yazd, which have arid climates, showed higher WUE under drought stress, indicating proper control of water loss with slight decrease in photosynthesis for any given g_s. The slight decrease in photosynthesis along with the decrease in g_s, which results in high WUE in populations originating from hot and dry regions of Iran, confirms the appropriate stomatal behavior of these populations. These results are in line with those of Dittberner et al. (2018) reporting that stomata size co-varies with WUE and may contribute to local adaptation (Dittberner et al., 2018). Our finding is also supported by Cornic (2000), who indicated that stomatal closure prevented water loss under drought stress condition though it can subsequently lead to inhibition of photosynthesis.

Under severe drought stress, the Markazi and Yazd populations had greater F_V/F_M, PI_{ABS}, ET_0/RC, and TR_0/RC values than those from Ilam, Fars and Kerman (Figs. 7 and 8). Higher F_V/F_M and PI_{ABS} for the Markazi and Yazd populations indicate greater maximal quantum efficiency of PSII in the dark-adapted state, most likely reflecting an ability to maintain a higher level of active PSII centers and electron transport beyond PSII. Higher ET_0/RC in the Markazi and Yazd populations is indicative of a greater capacity to dissipate excess light energy as heat, as revealed by lower values of DI_0/RC as an adaptive strategy to protect photosynthesis apparatus under drought stress (Monneveux et al., 2003). On the other hand, the ABS/RC and DI_0/RC increased in all populations under drought stress but their lowest values were detected in the drought-tolerant families from arid habitat (Markazi and Yazd populations). Whereas the TR_0/RC decreased in most families, especially in sensitive families under drought stress, we observed a slight increase in TR_0/RC in some drought-tolerant families of the Markazi and Yazd populations. These results indicate that drought stress limited the transfer of absorbed energy from the antenna (LHC) to the reaction center, resulting in reduced or interrupted electron transport from the donor end of PSII to the acceptor side in the drought-sensitive families. In general, it can be concluded that the O-J, J-I, and/or I–P phases of the fluorescence curve were more strongly disturbed in drought-sensitive families from semi-arid and cold habitats than in drought-tolerant walnut families from arid habitat. This indicates that drought stress caused damage to acceptor end of PSII as well as resulted in a poor redox state of Q_B pool, cytb6f and acceptor end of PSI in the drought-sensitive families. These findings suggest the existence of an adaptive mechanism that provides families from drier habitats with more photosynthetic capacity under severe drought stress exposure than other families is highly likely. Prinzenberg et al. (2020) have reported such a mechanism

in *Arabidopsis thaliana* under cold stress (Prinzenberg et al., 2020). Xiao et al. (2008) also demonstrated that the Populus population originated from dry climate aquired higher drought tolerance than the populations originated from wet climate, but there is no information regarding fruit trees, particularly walnuts.

In our study, re-watering of walnut plants after severe drought exposure caused nearly full recovery of photosynthetic efficiency in most populations, especially those that originated from arid habitats (Yazd and Markazi provinces). This implies that the plants were not permanently damaged by the water withholding, and drought recovery is distinct from drought tolerance. Such a recovery is consistent with the ecology of Persian walnut across Iran, where it grows in temperate regions in which exposure to water deficit in the growing cycle is common. Previous studies have shown that walnut may have mechanisms to adapt to drought stress (Arab et al., 2020; Aletà et al., 2009).

The correlation network based on photosynthetic traits and climate and soil variables was largely consistent with previous studies in walnut and other tree species (Arab et al., 2020; Lind et al., 2017; Postma and Ågren, 2016; Aletà et al., 2009). In our study, variation in photosynthetic traits was more strongly associated with climate parameters (especially temperature and precipitation) rather than with soil characteristics at the location of origin. Our findings demonstrated negative correlations between WUE under stress and DSI of WUE with the BIO12, suggesting that the populations from arid and hot regions are efficient in water usage under drought stress. These results agree with Lind et al. (2017) who examined white bark pine (*Pinus albicaulis* Engelm.) adaptation. DSI of WUE also showed strong significant negative correlation with PC3 of climate variables, PC1 of soil variables, BIO1, minimum temperature of coldest month (BIO6), and BIO11 suggested that drought stress had more reduction effects on WUE of populations originating from cold regions. In addition, DSI of Ci showed strong positive correlation with BIO1, and BIO5 suggesting that drought stress has less effect on photosynthesis of populations originating from warm regions. On the other hand, DSI of chlorophyll fluorescence related traits such as F_M/F_0, F_V/F_0, and F_V/F_M were negatively correlated with BIO16, and BIO19. Also, ABS/RC was positively correlated with BIO6, BIO11 and BIO16. These results suggesting a more severe disruption of photosynthetic apparatus under drought stress, especially PSII performance, has occurred in populations originating from wet regions. Our results are consistent with those of Adams et al. (2016) in *Arabidopsis thaliana*. It can be concluded that temperature and precipitation, as the two main components determining WUE, have been key climatic variables in walnut adaptation and evolution across Iran. Correlations of photosynthetic traits with environment and soil variables in our study suggest that variation in adaptive traits in walnut is partially driven by geography and climate.

5. Conclusion and perspectives

Our study provides new insights into the physiological and photosynthetic responses of locally adapted populations of Persian walnut under different irrigation regimes. We characterized photosynthetic-related traits through powerful and non-destructive systems in diverse walnut families (n = 60) grown in a controlled greenhouse under well-watered, water-stressed, and re-watered conditions. Based on the obtained findings RWC, gas exchange (P_n, g_s, T_r, and C_i/C_a), chlorophyll fluorescence (F_M, F_V, F_V/F_0, F_M/F_0, and F_V/F_M), quantum yields and specific energy fluxes (PI_{ABS}, φ_{Eo}, Ψ_0, and ET_0/RC) parameters decreased, while, WUE, V_J, M_0, DI_0/RC, ABS/RC, and φ_{Do} indices increased in walnut families under water-withholding condition. Families also responded differently to drought stress and recovery conditions. Cluster analysis properly assigned the 60 locally-adapted walnut families into three main groups: drought-tolerant, moderately drought-tolerant, and drought-sensitive. The tolerant group displayed less change for most of the measured parameters compared to other groups. The families in the drought-sensitive group experienced damage to both

PSII and beyond PSII electron transport activities when water was withheld, while the drought-tolerant families maintained a high RWC without affecting the structural stability and functional activity of PSII. Moreover, most of the families except sensitive ones fully recovered after being rewatered. Among the examined families, four of them including Ke23, Ma2, Se2, and Ya1 originating from Kerman, Markazi, Semnan, and Yazd provinces, which are arid regions in Iran, exhibited the highest photosynthetic efficiency under drought stress. In addition, fourteen families, such as Ma2 and Ya1, originating from different parts of Iran showed a high recovery percentage from drought stress. Multivariate analyses on phenotypic data revealed that parameters obtained from chlorophyll fluorescence analysis (F_V/F_M, φ_{Eo}, φ_{Do}, PI_{ABS}, ABS/RC, ET_0/RC, and DI_0/RC) along with RWC can be used as biomarkers for the screening of drought-tolerant genotypes of Persian walnut. Furthermore, climate data revealed that precipitation and temperature of the regions of origin drive signatures of local adaptation in walnut across Iran. This study suggests genetic resources adapted to dry climates, such as Ma2 and Ya1 families, which had the highest drought tolerance and recovery potential can be utilized directly or indirectly as founder parents in walnut genetic improvement programs. Consequently, the introduction of drought-tolerant genetic resources can play an important role in dealing with water shortage and water management of walnut orchards in arid and semi-arid regions.

Author contributions

M.M. Arab, H. Askari and K. Vahdati: Conceptualisation. M.M. Arab, S. Aliniaeifard, A. Mokhtassi-Bidgoli and M. Sadat-Hosseini: Physiological Data collection and curation. M.B. Mesgaran: Environmental data collation and respective analysis. M.M. Arab, H. Askari, S.S. Sohrabi and M. Sadat-Hosseini: Formal analysis. M.M. Arab: Writing – original draft. S. Aliniaeifard, A. Mokhtassi-Bidgoli, A. Estaji, M.B. Mesgaran, C.A. Leslie, P.J. Brown and K. Vahdati: Writing – review and editing. All authors have read and approved the manuscript for publication.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2023.107859.

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