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Comparison of Pomological, Physiological and Molecular Responses of *Almond* Genotypes to Drought Stress under Field and Greenhouse Conditions

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ABSTRACT

Nowadays, drought stress is creating new challenges in agricultural production. The almond tree a crucial agricultural component with commercial importance and widespread cultivation, is considered a drought-tolerant species due to its pomological and physiological characteristics. To investigate the pomological, physiological and molecular responses in the field and greenhouse conditions and the effects of drought stress on new almond genotypes (11-10, D-11, B-6, D-7, A-33, 100-2-8, TT100, SU, 7-11 , 100-1-4, B-3, M-S-13, B-551, D-12, and D-5) grafted onto GF677 rootstock, an experiment was conducted with two irrigation period (every 5 (normal) and 10 (drought stress) days) in Karaj, Iran. In field conditions, pomological and physiological traits results showed that the TT100, SU, 7-11, 100-1-4, and D-12 genotypes exhibited the most appropriate responses to drought stress. In greenhouse conditions, 15 almond genotypes were studied under two irrigation levels with 100 ml of water applied to each pot. Screening based on the chlorophyll fluorescence index indicated that genotypes D-12, B551, and 11-10 were classified as resistant, semi-resistant, and sensitive, respectively, under drought stress conditions. Under applied stress, the leaf relative water content (RWC) (13.75%) and leaf chlorophyll content (3.80%) were decreased. Enzyme activity of catalase (108.3% in genotype 11-10) and superoxide dismutase (676.25% in genotype D-12) increased with the intensity of stress. Gene expression analysis of catalase under stress showed that only the D-12 cultivar exhibited the highest increase in gene expression, with a 206.8% increase. QRT-PCR analysis of miR159 expression revealed that in genotypes 11-10, a significant decrease in miR159 expression was observed under drought stress. The D-12 genotype was tolerant under applied drought stress conditions and could be useful in almond development projects in arid regions.

Keywords: Almond, Antioxidant enzymes, Irrigation intervals, miR159 expression, Phenol.

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INTRODUCTION

Climate change poses an important threat to worldwide agriculture, disrupting crop production, water resources, soil health, and regional food security (Prajapati et al. 2024). These changes could disrupt traditional farming practices and diminish crop yields, particularly significant in regions heavily dependent on agriculture like Iran. Based on the water crisis indicators, by 2025 Iran is one of the countries having a severe water crisis (National Research Council, 2005). In Iran, a country dominated by an arid and semi-arid climate, important climate anomalies have been observed (Alizadeh-Choobari and Najafi, 2018). Recently, Nouri et al. (2023) reported that around ninety percent of fresh renewable water is being used in the country, showing high water stress conditions across Iran. In brief, water shortages make drought stress inevitable.

Drought stress can decrease crop yields (Muhammad et al. 2024), making it a top concern for farmers globally. Drought stress is the most destructive and alarming among the abiotic stresses that affect agricultural production and nutritional security (Rajanna et al. 2023). As a result, a reduction in precipitation and its irregular distribution during the plant growth period can lead to drought stress (Haider et al. 2024).

The almond tree a crucial agricultural component with commercial importance and widespread cultivation, is considered a drought-tolerant species due to its morpho-physiological characteristics (Zokaee-Khosroshahi et al. 2014). The report of McClung et al. (2024) showed that reduced water availability strongly affected almond seedlings growth, biomass allocation, and leaf nutrition. In an in vitro experiment under drought stress, the number of developed leaves and plant height in different almond cultivars were significantly reduced, with significant differences observed between the cultivars (Akbarpour et al. 2017).

Identifying the physiological basis of drought resistance can provide valuable insights, allowing various morphological, biochemical, and physiological traits to be used as selection indicators in breeding programs (Kapoor et al. 2020). In addition to the physiological basis of plants under environmental stress, molecular studies have enabled the analysis of plant metabolic and physiological adaptations to drought conditions, leading to the identification of numerous genes involved in stress responses. By regulating the expression of stress-responsive genes and determining their expression patterns, plants can enhance their stress tolerance to environmental stress (Li et al. 2024).

DNA markers are used to identify cultivars, assess genetic diversity, characterize almond germplasm collections, and identify marker-trait associations (Sideli et al. 2023). On the

other hand, microRNAs and transcription factors are key regulators of gene expression. MiRNAs profiling is known to control gene expression in both plants and animals (Shi et al. 2024). The expression of miRNAs changes in response to stress, aiding plant adaptation by modulating gene expression (Islam et al. 2022). One of the main problems in Alborz province, Iran, as in other areas, is the decrease in groundwater level and even the drying up of many wells due to high water consumption. In this regard, selection of almond drought-tolerant genotypes is necessary to minimize irrigation water consumption. The aim of this study was to compare of pomological and physiological traits, antioxidant enzyme activity, and molecular responses of almond tree (*Prunus dulcis [Mill] D.A. Webb*) genotypes to drought stress under both field and greenhouse conditions. Pomological and physiological responses play a key role in almond adaptation to drought stress. Studies on morpho-physiological traits of almond cultivars have demonstrated that under water deficit conditions, relative water content and chlorophyll concentration decrease, while osmolyte accumulation and antioxidant enzyme activity increase. In addition, differences in stomatal regulation, photosynthetic efficiency, and leaf anatomical characteristics contribute to variable drought tolerance among cultivars (Safavi Bakhtiari et al. 2025; Oliveira et al. 2023; De Pascali et al. 2025).

The aim of the present study was to evaluate the effects of different irrigation regimes on some pomological and physiological traits of some almond genotypes under greenhouse conditions. This research will provide documentation to improve our understating of mechanisms involved in the response of almond genotypes under different irrigation regimes as well as breeding/selecting higher drought resistant genotypes.

MATERIALS AND METHODS

The experiments were carried out during the 2020 and 2021 growing seasons at the Horticultural Sciences Research Institute (Temperate Fruits Research Center), Karaj, Iran (35.8927° N, 50.8769° E; 1275 m a.s.l.). The soil and irrigation water characteristics were as follows: electrical conductivity of soil (EC_s) 0.7 dS m^{-1}, electrical conductivity of water (ECw) 0.5 dS m^{-1}, soil pH (pH_s) 6.8, water pH (pHw) 7.2, total neutralizing value (TNV) 7%, and sodium adsorption ratio of water (SARw) 1%. The experimental site is characterized by a mean annual rainfall of 251 mm, with absolute minimum and maximum temperatures of −20 and 42 °C, respectively, and an average relative humidity of 52%. In this research, 15 promising almond genotypes (11-10, D-11, B-6, D-7, A-33, 100-2-8, TT100, SU, 7-11, 100-

1-4, B-3, M-S-13, B-551, D-12, D-5) grafted on GF677 rootstock were cultivated and studied both in a pot experiment, Ten-kilogram pots (25 × 35 × 45 cm) were used, filled with loamy soil consisting of 45% sand, 30% silt, and 25% clay, under field conditions. The plant bases and soil were disinfected with benomyl fungicide at a concentration of 2:1000. Subsequently, commercial varieties were grafted onto the plant bases using wood bud grafting at a height of 15 cm above the soil surface in early June 2021 (20 Jun 2021). Following sufficient growth of the scions, drought stress treatments were imposed in September 2021 (two months post-grafting) and maintained for a duration of 15 days. Prior to the initiation of the experiment, a 2 kg soil sample was collected and subjected to laboratory analysis to determine its physical and chemical properties. Subsequently, experimental treatments and fertilization protocols were designed and implemented based on the analytical results. The field capacity (FC) of the potting soil was quantified prior to transplantation utilizing a pressure plate apparatus. Subsequently, irrigation scheduling was determined by monitoring the gravimetric variations of the pots and considering the necessity for soil leachate removal to prevent salt accumulation. Pots were initially saturated through water application, followed by a 48-hour drainage period, after which field capacity (FC) was quantified based on the volume of percolated water from each sample (Klute, 1986). Thereafter, irrigation regimes were administered gravimetrically at intervals of 5 and 10 days. Genotypic responses were assessed under two contrasting conditions: well-watered (control) and drought stress. Consequently, four distinct experimental treatments were designed and implemented:

Experiment 1

This experiment aimed to assess the genetic diversity of 15 almond genotypes grafted onto GF677 rootstock. The genotypes, sourced from the research orchard of the Iranian Institute of Horticultural Sciences, were evaluated over two consecutive years (1400 and 1401 SH; 2021–2022 AD) from mature trees. The study focused on characterizing genetic diversity through detailed pomological traits, including almond descriptors, various fruit and kernel attributes, and flowering phenology. These traits were meticulously measured and subjected to rigorous statistical analysis to provide a comprehensive understanding of the pomological variation of the genotypes under field conditions. The research was conducted across two growing seasons (2020–2021) utilizing pomological and physiological traits as outlined in established almond descriptors (Fruit weight, Fruit length, Fruit width, Fruit thickness, Kernel weight, Kernel length, Kernel thickness, Kernel width, Kernel twinning %, Kernel

percentage, and Yield). A randomized complete block design (RCBD) was implemented, and statistical analyses were performed on the collected dataset.

Experiment 2

In this part of the research, GF677 rootstocks were first cultivated in pots, and the studied almond genotypes were then grafted on GF677 rootstocks using bud grafting at a height of 15 cm from the soil surface delete it in early June 2020. After the scions had grown sufficiently, drought stress treatments were applied in August 2021 (two months after grafting) and too short time. The experiment was conducted as a two-factor factorial arrangement in a completely randomized design (CRD) under greenhouse conditions with three replications. The first factor consisted of 15 almond genotypes, while the second factor involved two irrigation regimes: normal irrigation and drought stress, the latter induced by doubling the irrigation period. Water stress was applied at tow levels: a) normal irrigation: irrigation based on the field capacity of the potting soil (100% field capacity with moderate irrigation every 5 days). b) Deficit irrigation: limited irrigation to induce stress, with irrigation every 10 days, maintaining 50% of the field capacity. Water stress was applied until the first signs of stress appeared in the plants. The field capacity of the potting soil was determined using a pressure plate device before the plants were transferred. The amount of irrigation was adjusted based on changes in the weight of the pots and the need for leaching. The pots were saturated with water, and after 48 h, excess water was collected to calculate the field capacity. Irrigation was applied to each pot according to the assigned treatment intervals: every 5 or 10 days. Two irrigation periods were defined: (A) Normal irrigation, corresponding to 100% of the potting soil's field capacity (FC), applied moderately every 5 days; and (B) Drought stress, applied for approximately one month—until the appearance of initial visible stress symptoms in the plants—corresponding to 50% of the soil's FC, with irrigation applied every 10 days. After applying the water treatments, the physiological behavior of all genotypes under stress conditions was assessed using chlorophyll fluorescence measurements (Fv/Fm). Based on the results, the genotypes were categorized into three groups: resistant, semi-resistant, and sensitive to drought stress (Maxwell and Johnson, 2000). Leaf samples from these genotypes were collected and transported to the laboratory in zip-lock bags under controlled conditions.

Experiment 3

Based on the results from the greenhouse study, after scion establishment in the greenhouse and the application of irrigation regimes, leaf chlorophyll content was assessed at 10:00 a.m.

using a chlorophyll fluorescence to determine plant responses to the imposed irrigation treatments. According to chlorophyll fluorescence index measurements, the genotypes were categorized into three groups, namely tolerant, moderately tolerant, and sensitive. Subsequently, one representative genotype from each group was selected for further molecular analyses. The genotypes were classified into three categories, therefore genotypes D-12, B551, and 11-10 were resistant, semi-resistant, and sensitive under drought stress conditions, receptively, and Leaf samples were collected (14 days after application deficit irrigation) from each group for further analysis. Various physiological traits were measured, including total phenol content (The amount of phenol was measured using the Folin-Ciocattoli reagent using a spectrophotometer), electrolyte leakage index (ELI), relative leaf water content (RWC) (based on fresh, dry, and swollen leaf weights) (RWC %=(fresh leaf weight – dry leaf weight) / (swollen leaf weight – dry leaf weight) ×100), and leaf chlorophyll content (Grant et al. 2010). To assess stress response more accurately, catalase enzyme (EC 1.11.1.6) and superoxide dismutase (SOD) enzyme activity were measured using the Aebi method (Aebi, 1974), and based on the inhibition of nitro blue tetrazolium photo reduction using enzymatic extraction (Winterbourn et al. 1976).

Experiment 4

Frozen leaf samples stored at −80 °C were used for molecular analyses. Total RNA was extracted using an RNA isolation kit (Vivantis, Taiwan) following a Trizol-based protocol with alcohol precipitation (GenAll, South Korea). The sequences of the target genes were retrieved from the National Center for Biotechnology Information database, and specific primers were designed using Geneious OligoV software. RNA concentration and purity were assessed spectrophotometrically at 230, 260, and 280 nm using a NanoDrop spectrophotometer (Thermo Scientific, USA) (Figure 1). To remove potential genomic DNA contamination, RNA samples were treated with DNase I (Fermentas, USA). Briefly, 1 µg of total RNA was incubated with 1 µL DNase I and 1 µL reaction buffer at 37 °C for 30 min. The reaction was terminated by adding EDTA followed by incubation at 65 °C for 10 min. RNA samples were subsequently re-quantified using the NanoDrop spectrophotometer. Gene expression analysis of target genes and regulatory microRNAs was performed using quantitative real-time PCR. Relative transcript levels were calculated using the 2-ΔΔCt method described by Livak and Schmittgen (2001), with an internal reference gene. All reactions were conducted in triplicate.

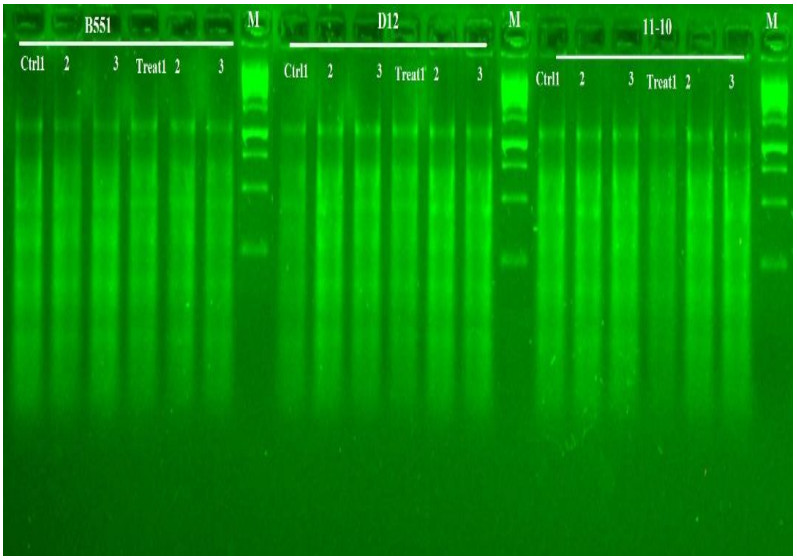

Figure 1. 1.5% agarose gel electrophoresis images showing the quality of RNA extracted from almond genotype leaves.

cDNA Synthesis from RNA

Stem-loop primers were mixed in equal volumes and used for microRNA reverse transcription. Briefly, 1 µL of the mixed primers was combined with 1 µg of total RNA in a final volume of 12 µL and incubated at 60 °C for 10 min, followed by immediate cooling on ice. Subsequently, 8 µL of reverse transcriptase reaction mixture containing the required reagents for cDNA synthesis was added. The reaction was incubated at 42 °C for 90 min and terminated by enzyme inactivation at 75 °C for 10 min.

Primer Design

To design specific primers for the target genes miR159, miR171, MYB33, SCL, WRKY, Catalase, Cu/Zn-SOD, and the 18S rRNA control gene, gene sequences obtained from the NCBI were used. Sequence alignment was performed in BioEdit software (Hall, 1999), and the conserved regions of these genes were identified. These conserved regions were then used for a primer design using Oligo7 software (Rychlik, 2007). Finally, the specificity of the designed primers was verified using the Primer-BLAST tool on the NCBI website (Table 1).

Table 1. List of primers designed for sequencing the studied genes.

Primer Name	Forward Primer (5'–3')	Length (bp)	Tm (°C)	Product Length (bp)
miR159-F	CGGCGGTGGAGTGACAGGA	21	58.7	
miR171-F	TTCCTTTTGAGCCGTGCCA	21	62	
miR	CCAGTGCAGGGTCCGAGGTA	20	63	
SOD-F	CAGAGGACATGGGTAGTGTGA	21	60	109
SOD-R	CCTTTTACTTGTGTAGGCCCG	21	59	
Cat-F	AGCCTGCACATGTTTACCTTC	21	64	123
Cat-R	TGTGCTTTCCCTGCCTTATTG	20	60	
SCL6-F	GGAATGGAAGACTGGGAGAG			163
SCL6-R	ACCCTGCACTGAATTCCAAAT			
MYB33-F	TGGAGCTCCCTTCACTCCAA			179
MYB33-R	ATGGAGCAAAGCATCCAGCA			
WRKY11-F	CGTCGTTTTTGTCCTCCATCA			120
WRKY11-R	ATTGGGAGAGAGGAGGTTTCC			

Real-Time PCR

Quantitative real-time PCR was performed in a 10 µL reaction mixture containing 1 µL cDNA, 0.5 µL gene-specific primers, 5 µL SYBR Green Master Mix, and DEPC-treated water to the final volume. The amplification program included an initial denaturation at 94 °C for 2 min, followed by 40 cycles of denaturation (94 °C, 30 s), annealing (58 °C, 30 s), and extension (72 °C, 20 s). A melting-curve analysis (60–95 °C) was conducted at the end of the run to verify amplification specificity.

Statistical analysis

An analysis of variance was made between the two farming systems (normal irrigation and drought stress) over two years. All data were analyzed using statistical software such as SAS or SPSS. Graphs were created using Excel and STATGRAPHICS software. Gene expression analysis was conducted using REST software. Expression data were processed using the delta- delta (ΔΔ) method, to calculate relative expression levels (Livak and Schmittgen, 2001), and entered into Excel software. Differences between means of different groups were carried out using one-way ANOVA followed by Duncan Multiple Range tests ($p<0.05$).

RESULTS

The analysis of variance in the field experiment revealed significant genotype effects for all studied traits at the 1% probability level (Table 2), prompting further investigation of these genotypes. The interaction between genotype and year was significant for fruit width, yield, and kernel double percentage ($p<0.01$), while kernel weight and kernel length were significant at the 5% level. Descriptive statistics for the 11 measured traits across all

genotypes indicated that the greatest variability was observed in kernel percentage (34.55%), followed by kernel length and fruit width (16). The highest coefficient of variation (158.1%) was observed for kernel twinning, while the lowest coefficient of variation (10.7%) was observed for fruit length.

Table 2. Descriptive statistics analysis of studied traits in 15 almond genotypes.

Traits	Range	Min	Max	Mean	Std. error	C.V (%)	Variance
Fruit weight (mm)	3.40	1	4.4	2.57	0.98	39.6	0.87
Fruit length (mm)	14	26	40	32	0.36	10.7	11.68
Fruit width (mm)	16	14	30	19.87	0.44	25.6	17.73
Fruit thickness (mm)	9	9	18	13.82	0.23	15.3	4.57
Kernel weight (mm)	1	0.6	1.6	1	0.023	22	0.048
Kernel length (mm)	16	18	34	25.78	0.37	13.7	12.49
Kernel thickness (mm)	3.51	5	8.51	6.94	0.079	10.8	0.57
Kernel width (mm)	8.51	8.49	17	11.94	0.24	19.1	5.15
Kernel twinning %	9	1	10	3.27	0.30	158.1	8.38
Kernel %	34.55	30.65	65.2	42.88	0.83	20.3	62.69
Yield (Dry nut, kg/tree)	7.69	4.96	12.65	8.23	0.22	29.7	4.44

Factor analysis based on principal component analysis (PCA) identified four main factors (Table 3). The factor coefficients highlighted the importance of traits related to yield and its components, which are crucial for selecting desirable genotypes. Based on the PCA, genotypes TT-100, SU, 7-11, and 100-1-4 showed positive results regarding the first and second factors and exhibited higher yields. However, genotypes 10-11, B-551, and D-12, which showed sensitive, semi-sensitive, and tolerant responses to drought stress under controlled conditions, were positioned near the axis of the second component in (Figure 2). Correlation analysis revealed a significant positive correlation between yield and traits such as fruit length, fruit width, kernel width, and kernel double percentage. Statistical analysis further indicated that fruit length, fruit width, kernel width, kernel length, and kernel twinning percentage were the most influential traits to affecting yield and could be used to identify superior genotypes for almond breeding programs.

Table 3. Principal component analysis between different traits studied in almond genotypes.

Traits	PCA1	PCA2	PCA3	PCA4
Fruit weight (mm)	0.78	-0.37	0.32	0.05
Fruit length (mm)	0.75	0.26	-0.20	-0.32
Fruit width (mm)	0.88	-0.10	-0.09	0.02
Fruit thickness (mm)	0.49	-0.32	0.20	-0.36
Kernel weight (mm)	0.56	-0.45	0.38	0.29
Kernel length (mm)	0.69	0.37	0.26	-0.09
Kernel thickness (mm)	0.22	0.59	0.52	-0.20
Kernel width (mm)	0.79	-0.25	-0.14	0.36
Kernel twinning %	0.67	0.56	-0.23	-0.09
Kernel %	0.14	0.53	0.001	0.77
Yield (Dry Nut (kg/ tree))	0.50	-0.12	-0.73	-0.05
Factor	25.63	22.44	17.11	11.07
Total	25.63	48.07	65.18	76.25

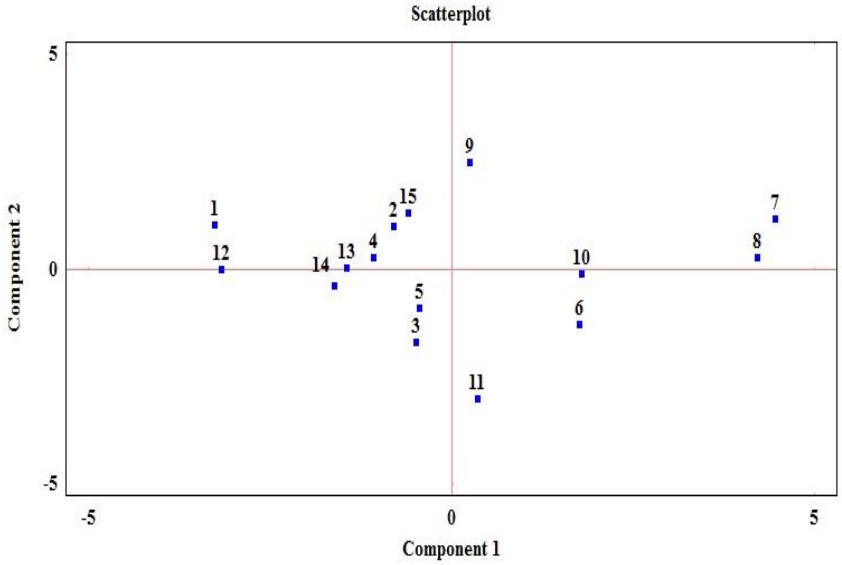

Figure 2. Scatterplot of 15 almond genotypes (1: 11-10, 2: D-11, 3: B-6, 4: D-7, 5: A-33, 6: 100-2-8, 7: TT100, 8: SU, 9: 7-11, 10: 100-1-4, 11: B-3, 12: M-S-13, 13: B-551, 14: D-12, and 15: D-5) based on the first and second components of the mean pomological traits from two-year data under field conditions.

In the second experiment, the results of the analysis of variance showed that the differences between genotypes were significant for all physiological traits except leaf chlorophyll content (Table 4). The results of the comparisons of the mean physiological traits revealed that genotypes D-12 and D-5 had the highest RWC levels, genotypes D-12 and D-5 had the lowest ELI, and most of the genotypes studied showed higher leaf chlorophyll levels under stress. Additionally, genotypes D-12 and D-5 exhibited higher total phenol levels. Based on the results, the highest reduction in the RWC index and leaf chlorophyll index under stress

were observed for genotype 11-10, while the lowest reduction in RWC and leaf chlorophyll index were recorded for genotype D-12. Regarding the total phenol index, the highest increase was noted for the B-551 genotype, which can be categorized as semi-sensitive to drought stress based on other indices. Furthermore, the D-12 genotype, which exhibited the least decrease in RWC and leaf chlorophyll, showed an intermediate behavior with a total phenol content increase of 297.7 %, compared to the other studied genotypes. As for the leaf ELI, cultivar D-12 exhibited the lowest ELI under stress conditions, while cultivar 11-10 showed the highest percentage of ELI.

Table 4. Average physiological traits and their percentage changes with the application of drought stress in almond genotypes.

	Genotype	RWC (Normal)	Rate of change (%)	ELI (Normal)	Rate of change (%)	Phenol (Normal)	Rate of change (%)	Leaf chlorophyll (Normal)	Rate of change (%)
normal	11-10	78.2	-21.1	15.2	250	0.35	228.6	0.785	-8.5
	D-11	78.6	-15.5	15.5	209.6	0.38	244.7	0.781	-3.5
	B-6	80.3	-17.7	14.7	229.2	0.35	262.8	0.801	-4.2
	D-7	78.6	-11.9	15.6	192.9	0.37	270.2	0.788	-2.2
	A-33	79.3	-14.4	15.0	196	0.39	251.3	0.795	-2.3
	100-2-8	76.9	-10.1	15.2	189.5	0.38	284.2	0.790	-0.25
	TT100	77.7	-12.5	14.4	202.7	0.40	252.5	0.791	-1.4
	SU	80.1	-18.2	13.6	267.6	0.37	237.8	0.799	-4.7
	7-11	71.0	-11.1	14.6	245.2	0.40	250	0.792	-5.5
	100-1-4	79.6	-16.9	14.5	235.8	0.41	260.9	0.795	-2.5
	B-3	78.3	-11.1	14.0	217.8	0.39	317.9	0.795	-1.8
	M-S-13	74.7	-15.5	14.3	249.6	0.40	255	0.803	-5.2
	B-551	80.1	-14.9	15.1	194	0.38	323.7	0.779	-0.89
	D-12	79.8	-7.4	14.5	178.6	0.44	297.7	0.785	-0.76
	D-5	79.7	-8.4	14.5	182.7	0.42	309.5	0.795	-1.25
drought Stress	Genotype	RWC (drought stress)		ELI (drought stress)		Phenol (drought stress)		Leaf chlorophyll (drought stress)	
	11-10	91.7		53.2		0.15		0.718	
	D-11	66.4		48.0		1.31		0.754	
	B-6	66.1		48.4		1.27		0.767	
	D-7	69.2		45.7		1.37		0.771	
	A-33	67.9		44.4		1.37		0.777	
	100-2-8	69.1		44.0		1.46		0.788	
	TT100	68.0		43.6		1.41		0.780	
	SU	65.5		50.0		1.25		0.761	
	7-11	63.1		50.4		1.40		0.748	
	100-1-4	66.1		48.7		1.48		0.775	
	B-3	69.6		44.5		1.63		0.781	
	M-S-13	63.1		50.0		1.42		0.761	
	B-551	68.1		44.4		1.61		0.772	
	D-12	73.9		40.4		1.75		0.791	
	D-5	73.0		41.0		1.72		0.785	

RWC: relative leaf water content; ELL: electrolyte leakage index.

Drought stress applied to the almond genotypes led to significant changes in the RWC, ELI, total phenol, and leaf chlorophyll indices (Table 5). In general, based on the results obtained under greenhouse conditions and by examining the behavior of these genotypes under field conditions, genotype D-12 was identified as tolerant, B-551 as semi-tolerant, and 11-10 as sensitive to water deficit stress compared to the other genotypes evaluated in this study.

Table 5. Mean and percentage changes of physiological indices in almond genotypes under water deficit stress conditions.

Different levels of water deficit stress	RWC	ELI	Total phenol	Leaf chlorophyll
Normal irrigation	78.13^{a}	14.79^{b}	0.39^{b}	0.79
Water deficit stress	67.39^{b}	46.47^{a}	1.44^{a}	0.76
Percentage of change	-13.75	214.20	269.23	-3.80

RWC: relative leaf water content; ELL: electrolyte leakage index; Different letters indicate a significant difference at the 5% probability level.

In the third experiment, the results of the analysis of variance for the three almond genotypes showed that the effects of genotype and drought stress were significant for both catalase and superoxide dismutase enzymes (Table 6). The interaction effect of genotype × water deficit stress was significant only for the superoxide dismutase enzyme. Comparison of the mean values for antioxidant enzyme genes activities among the three almond genotypes showed that genotypes 11-10 and D-12 exhibited higher catalase activity, while genotype 11-10 showed higher superoxide dismutase activity than the other genotypes. The average data and percentage changes for antioxidant enzyme genes activities revealed that enzyme activity increased with increasing stress intensity from normal to stressed conditions. The catalase enzyme activity showed the highest increase in activity under stress conditions compared to normal irrigation, with an increase of 108.3% in cultivars 11-10. The superoxide dismutase activity enzyme of genotype D-12 showed the highest increase with a 676.25% rise. On the other hand, genotype 11-10 showed the lowest increase in superoxide dismutase enzyme activity under stress conditions, with a 42.4% increase compared to normal irrigation.

Table 6. Mean and percentage changes in antioxidant enzyme activity in almond genotypes under water deficit stress conditions.

Drought stress	Genotypes	Catalase	superoxide dismutase
Normal irrigation	11-10	0.12^{b}	8.04^{a}
	B-551	0.11^{ab}	1.72^{b}
	D-12	0.12^{b}	1.60^{b}
Water deficit stress	11-10	0.25	11.45^{a}
	B-551	0.16	7.21^{b}
	D-12	0.22	12.42^{a}
Percentage of changes in enzyme expression under stress compared to normal irrigation	11-10	108.3	42.4
	B-551	45.5	319.2
	D-12	83.3	676.25

Different letters indicate a significant difference at the 5% probability level.

Based on the results of field studies, physiological indices (chlorophyll index) and yield under normal conditions and water deficit stress, three genotypes were identified: D-12 as tolerant, B-551 as semi-tolerant, and 11-10 as sensitive to drought stress. In the fourth experiment, the results of the analysis of variance for the relative expression data of superoxide dismutase, catalase, and the miR171, miR159, MYB-33, SCL, and WRKY genes in three almond genotypes (D-12, B-551, and 11-10) under water deficit stress showed a significant difference between stress levels for all genes except miR171 and CAT (Table 7). Additionally, the interaction effect of water deficit stress × almond genotypes was significant for all genes except WRKY, SCL, and superoxide dismutase at the 1% and 5% probability levels.

Table 7. Comparison of mean gene expression and percentage changes in gene expression in the three almond genotypes under two irrigation conditions.

Drought stress	Genotypes	MiR159	myb	wrky	MiR171	SCL	sod	cat
Normal irrigation	11-10	1.11^{a}	0.002^{b}	1.13^{b}	1^{a}	1.1^{b}	1.08^{b}	1.02^{a}
	B-551	1.12^{a}	0.002^{b}	1.01^{b}	1^{b}	1^{ab}	1^{b}	1.04^{a}
	D-12	1.05^{a}	0.001^{b}	1^{b}	1.04^{a}	1.01^{b}	1.04^{b}	1.03^{a}
Water deficit stress	11-10	0.049^{b}	1.75^{a}	2.27^{a}	0.18^{b}	2.93^{a}	3.32^{a}	0.29^{b}
	B-551	1.09^{a}	0.07^{a}	2.45^{a}	2.01^{a}	1.30^{a}	1.80^{a}	0.19^{b}
	D-12	0.91^{a}	1.79^{a}	1.54^{a}	0.24^{b}	3.15^{a}	2.94^{a}	3.16^{a}
Percentage of changes in gene expression under stress compared to normal irrigation	11-10	-95.58	87400	100	-82	166	207.4	-71.57
	B-551	-2.68	3400	142.6	101	30	80	-81.7
	D-12	-13.3	17800	54	-76.9	211.9	182.7	206.8

Under water deficit stress, the miR159 gene showed the highest percentage decrease in expression in genotype D-12 compared to normal irrigation. In contrast, the miR171 gene showed a 101% increase in expression in cultivar B-551. Under stress, the target gene MYB-3 exhibited the highest percentage increase in expression, with an increase of 87400% compared to normal irrigation conditions. Overall, with increasing water deficit stress intensity compared to normal irrigation, the expression levels of most of the studied genes increased (Figures 3 and 4). Comparisons of the mean expression of the studied genes showed that gene expression changed differently under water deficit stress treatment compared to normal irrigation for each of the seven studied genes. For example, for the superoxide dismutase gene, an increase in gene expression was observed in all three almond genotypes under water deficit stress, with the highest increase in expression in genotype 11-10 compared to normal irrigation. However, for the catalase gene, the results showed a decrease in expression in the two genotypes 11-10 and B-551 compared to normal irrigation, while the expression of the D-12 genotype increased by 206.8% compared to normal irrigation (Table 9, Figure 5).

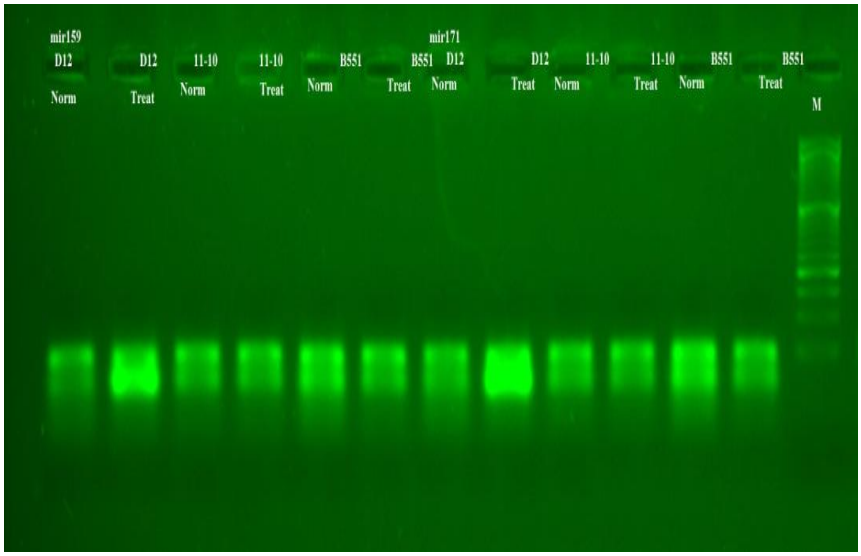

Figure 3. Expression of miR159 and miR171 microRNAs under normal and drought stress conditions in three almond genotypes.

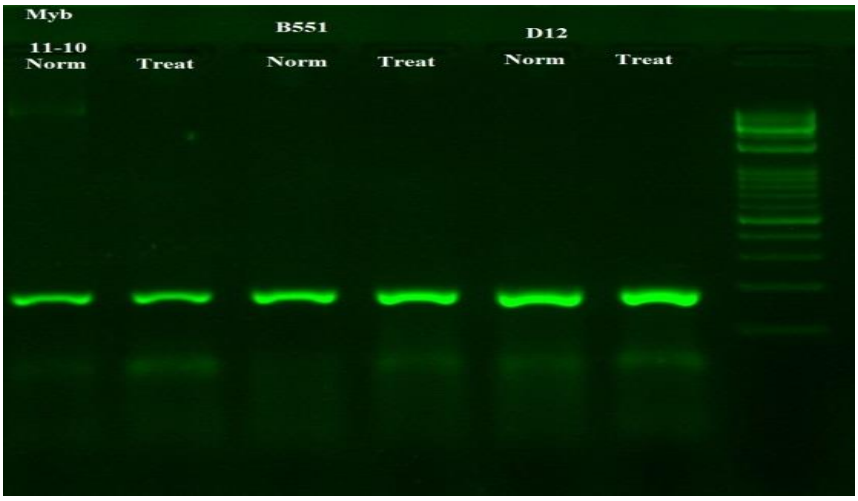

Figure 4. Image of MYB33 gene expression under normal and drought stress conditions in three almond genotypes.

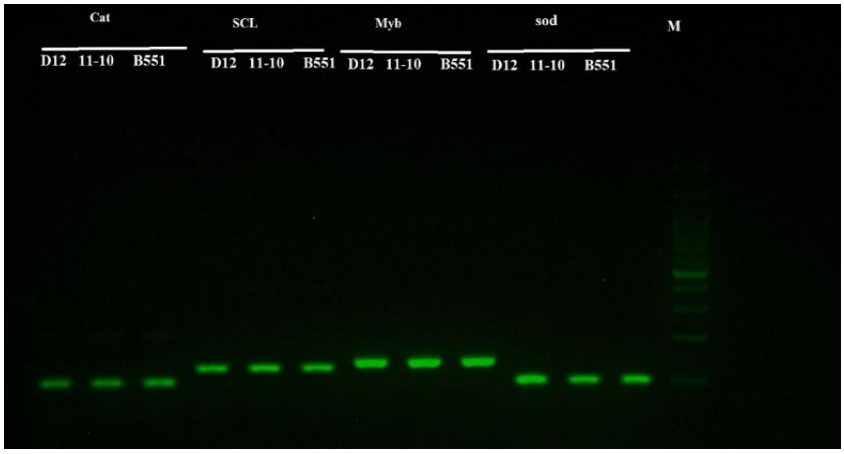

Figure 5. Image showing changes in the expression of catalase, superoxide dismutase, MYB, and SCL genes in the three almond genotypes studied

DISCUSSION

According to means comparisons, in both normal irrigation and drought stress conditions there are significant differences among the genotypes in all the pomological and physiological traits. Under field conditions, D-12 outperformed other genotypes, demonstrating superior yield, favorable physiological and molecular responses, and enhanced expression of antioxidant enzyme genes and their target genes. These findings explain D-12's high yield, resistance, and tolerance to water deficit stress. This aligns with previous research by Kim et al. (2020) which demonstrated the effectiveness of assessing genetic diversity based on growth and functional characteristics for identifying drought-resistant genotypes. Furthermore, the results of catalase gene expression showed that only the D-12 cultivar exhibited the highest increase in gene expression. These results are consistent with the findings of Fahim et al. (2023), who showed that catalase enzyme activity in grape cultivars significantly increased under different levels of drought stress. Similarly, the study of changes in the expression of the superoxide dismutase enzyme gene under stress conditions revealed that only the D-12 cultivar showed the highest percentage increase in expression. Based on these results, it can be concluded that water deficit stress-tolerant cultivars, by increasing the activity and expression of antioxidant enzyme genes (Yildrim et al. 2021), microRNAs, and their target genes, as well as enhancing physiological characteristics such as RWC, leaf chlorophyll, and phenols, help plants adapt to stress and prevent further reductions in almond yield.

To date, few local studies have focused on the molecular responses of almond genotypes to drought stress in Iran. Plant miRNAs play crucial roles in various aspects of plant growth and development, including organ morphogenesis, signal transduction, hormone function, pathogen invasion, signal transduction, and response to environmental stimuli (Luo et al. 2024). Therefore, identifying candidate genes associated with water deficit tolerance and sensitivity in almond cultivars may provide valuable insights into the molecular mechanisms of drought resistance in almond genotype. Drought induces an increase in abscisic acid levels in leaf mesophyll cells causing stomatal closure which leads to a decrease in CO_2 concentration in the leaf mesophyll tissue, thus closing stomata to reduce water loss (Luo et al. 2019). O_2 acts as an electron acceptor in the electron transport chain, generating superoxide free radicals or the primary form of reactive oxygen species (ROS) (Napolitano et al. 2022). These ROS play a significant role as messengers in the abiotic stress signal transduction pathway. In the early stages of stress, when the expression levels of transcription

403 factors are high, these factors likely enhance the expression of delayed genes related to
404 drought stress, including the superoxide dismutase enzyme gene, particularly Cu/ZnSOD,
405 which serves as the first line of defense against ROS caused by abiotic stresses (Hashemi et
406 al. 2013). Our results demonstrate that enzyme activity was influenced by water deficit
407 stress, responding to the severity and intensity of the stress. The superoxide dismutase and
408 catalyzes enzymes for dismutation of superoxide radicals (O_2^-) to hydrogen peroxide (H_2O_2)
409 and oxygen, making superoxide dismutase a primary defense mechanism against ROS
410 (Ashraf, 2009). Bian and Jiang (2009), in their study on the expression of the Cu/ZnSOD
411 gene in Kentucky bluegrass, found that Cu/ZnSOD gene expression decreased 48 h after
412 drought stress compared to the control, but increased during the recovery period. In our
413 study, the expression of antioxidant enzyme genes was elevated in the drought-tolerant
414 genotype (D-12) compared to the semi-tolerant (B-551) and sensitive (11-10) genotypes
415 under moisture stress. This suggests that, while enzyme production may not always directly
416 correlate with gene expression levels, D-12 showed increased antioxidant enzyme activity as
417 water deficit stress intensified. Overall, the response of superoxide dismutase to moisture
418 stress was found to be variable, in line with previous studies (Hashemi et al. 2013; Saed-
419 Moucheshi et al. 2021). In this study, all three almond genotypes (D-12, B-551, and 11-10)
420 showed an increase in the activity of catalase and superoxide dismutase enzymes under stress
421 conditions.
422 These findings highlight the significant role of antioxidant enzyme gene expression in
423 drought tolerance in almond cultivars. Antioxidant enzyme genes play a critical role in
424 abiotic stress tolerance across different species. Transcription factors emerge as pivotal
425 regulators of stress-responsive genes, positioned as promising candidates for enhancing crop
426 resilience (Liu et al. 2024). These factors regulate the expression of downstream genes and
427 play a key role in plant stress responses. Poor growth, a characteristic of plants under abiotic
428 stress, especially drought, reduces metabolism and reallocates resources to adapt to new
429 conditions.
430 In our study, the microRNAs miR159 and miR171, which are involved in plant regulation
431 and development, showed significant expression changes under stress conditions. QRT-PCR
432 results indicated that miR159 expression did not change significantly in genotypes D-12 and
433 B-551 under stress compared to normal irrigation ($p>0.05$). However, in genotype 11-10, a
434 significant decrease in miR159 expression was observed. The MYB33 gene, a target gene for
435 miR159, exhibited a significant increase in expression in both D-12 and 11-10 under stress.

In contrast, B-551 showed a modest 20% increase. miR159 furthermore plays an important regulatory role in abscisic acid signaling (Guo et al. 2021) and abscisic acid plays a key role in stress responses by regulating stomatal closure, root growth, and preventing shoot growth (Ge et al. 2019). The pattern of miR159 expression in this study was consistent with those observed in almond, peach, and GN rootstocks under stress (Eldem et al. 2012; Sunkar et al. 2012). Notably, miR159 expression increased in 11-10 and D-12 but was less pronounced in B-551, indicating that miR159 plays a lesser role in drought adaptation in this genotype. Increased miR159 expression during drought stress likely helps regulate plant growth by redirecting energy to stress adaptation (Esmaili et al. 2015). Although miR159 expression decreased under stress in almonds, its target gene (MYB33) showed increased expression, suggesting a complex regulatory mechanism. This could be due to a form of inconsistent regulation, where miR159 and its target gene are located in different cells (Saini et al. 2012), or another regulatory mechanism could be involved in the gene's upregulation. Additionally, since miR159 influences abscisic acid signaling, the decrease in miR159 expression in D-12 and B-551 (and the severe decrease in 11-10) may point to other mechanisms contributing to drought tolerance, possibly linked to the genotypes' overall drought tolerance. MiR159, which regulates plant growth and development, furthermore modulates the transition between growth phases and the initiation of flowering (Achard et al. 2004). In this study, miR171, another microRNA involved in plant growth and development, showed a significant decrease in expression in D-12 and 11-10 and an increase in B-551 compared to normal irrigation. Both miR171 target genes, SCL and WRKY, showed increased expression under stress, suggesting that miR171 regulates its target genes in a non-coordinated manner.

Conclusions

This study demonstrated significant differences among almond genotypes in pomological and physiological traits under both normal irrigation and drought stress conditions. The drought-tolerant genotype D-12 consistently outperformed others, showing superior yield, enhanced physiological responses, and elevated expression of key antioxidant enzyme genes, notably catalase and superoxide dismutase. These molecular and physiological adaptations contribute to D-12’s resilience and high productivity under water deficit stress. The differential expression patterns of microRNAs, particularly miR159 and miR171, alongside their target genes, further reveal complex regulatory mechanisms underpinning drought tolerance. These findings underscore the critical role of antioxidant enzymes, transcription factors, and

microRNAs in facilitating almond adaptation to drought, offering valuable insights for breeding programs aimed at improving stress resistance. Overall, this research highlights the importance of integrating molecular and physiological approaches to better understand and enhance drought tolerance in almond cultivars.

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