



Biochemical and Physiological Traits of Pinto Bean (*Phaseolus vulgaris*) Lines Affected by Drought Stress: A Comprehensive Analysis

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Abstract

Determining the influential physiological and biochemical traits affecting seed yield under drought stress conditions is crucial for optimizing crop performance. A study conducted during 2022 and 2023 at the Bean Research Center in Markazi, Iran, utilized a split-plot design within a randomized complete block design with three replications to assess these traits in various *Phaseolus vulgaris* pinto bean lines (13 lines plus the 'Kusha' variety as a check) under drought stress. The results demonstrated that drought stress significantly reduced chlorophyll content, relative water content, and seed yield while increasing the activities of antioxidant enzymes, as well as proline and flavonoid content. Under non-stress conditions, lines 7 and 10 exhibited notably high total chlorophyll content of 3.55 and 3.46 mg. g⁻¹ FW, respectively, surpassing the control cultivar by 1.40 and 1.37 times. Line 13 displayed the highest activities of catalase, peroxidase, polyphenol oxidase, and ascorbate peroxidase under drought conditions. Regarding seed yield under drought stress, lines 3, 7, and 11 performed the best, being 1.79, 1.73, and 1.74 times higher than the control cultivar, respectively. Overall, the study highlighted that total chlorophyll content, proline content, polyphenol oxidase activity, and relative water content collectively explained 95.85% of the variations in seed yield under drought stress. Antioxidant enzyme activities, proline levels, and relative water content are key traits influencing seed yield under drought stress. Lines 3, 11, 12, and 13 are promising candidates for cultivation in water-limited regions based on these traits.

Keywords: Antioxidant activity, Proline content, Seed yield, Total chlorophyll, Water-limited.

Abbreviations: Maximum Allowable Depletion (MAD); catalase (CAT); Peroxidase (POX); polyphenol oxidase (PPO); ascorbate peroxidase (APX); superoxide dismutase (SOD); relative water content (RWC)

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Introduction

The pinto bean (*Phaseolus vulgaris*) is an annual, herbaceous, and self-pollinating plant of the

Fabaceae family, which is considered one of the main sources of human food supply due to its high protein, phosphorus, iron, vitamin C, vitamin B1, and fiber content, and it is cholesterol-free (Geleta et al., 2024; 2023). *Phaseolus vulgaris* is one of the world's most essential legumes, containing 20–25% protein and 50–56% carbohydrates (Islam and Dhaubhadel, 2023). According to the FAO report, the global cultivated area of this crop is around 29 million ha, and its production is 23.9 million tons with an average yield of 500 kg ha⁻¹ (Goharivahid and Yousefirad, 2024).

Drought stress is a significant challenge for farmers in arid and semi-arid regions like Iran. Beans, however, are known to be resistant to drought, heat, and cold and can be cultivated in various soil types (Geleta et al., 2024). Nevertheless, the reproductive stages of beans, including flower formation, full flowering, pod formation, and seed filling, are susceptible to drought stress, which can negatively impact seed yield, seed quality, and market value (Farzamisepehr et al., 2021; Mutari et al., 2023; Pierre et al., 2023). Approximately 60% of beans cultivated globally are at risk of experiencing terminal or intermittent drought stress (Mutari et al., 2023). The bean plant thrives best in environments with high altitudes ranging from 1,400 to 2,000 meters above sea level (MSAL). Due to its short cropping period and adaptability to different cropping systems, the bean plant has the potential to be a food security crop (Savita, 2023). However, this crop is at risk of experiencing moisture deficits, diseases, and pests resulting from climate change, which can negatively impact its yield (Geleta et al., 2024).

Research has shown that the number of stomata (plant pores) in leaves, which are necessary for taking in carbon dioxide, can be reduced due to a lack of water, ultimately leading to lower rates of photosynthesis and decreased seed production (Franks et al., 2015; Geleta et al., 2024)). Moreover, changes in plant structure and root system development can also adversely affect the plant's ability to take up water and nutrients, thus impacting its yield ((Kleine and Müller, 2014; Mehrasa et al., 2022). Water is essential for optimal plant growth and functioning, and its

physical properties and quantity significantly impact gas exchange. If water completely covers a plant, gases cannot be exchanged, leading to no growth and, ultimately, the plant's death (Kirkham, 2023). Therefore, balancing water is crucial in achieving the desired functions and maintaining good health in these plants.

Physiological and biochemical characteristics, including photosynthetic pigment content, antioxidant enzyme activity, amino acid content, and plant water relations, can significantly impact pinto bean performance. Previous studies have focused on changes in these traits. Still, the current research aims to identify the physiological and biochemical characteristics that affect pinto bean yield in different lines under both non-stress and drought stress conditions. Previous research has reported that drought stress significantly reduces seed yield in pinto beans (Desoky et al., 2021). Mehrasa et al. (2022) found that under drought stress conditions, photosynthetic pigments such as chlorophyll a, chlorophyll b, and total chlorophyll, as well as protein content, decreased, while the activity of antioxidant enzymes like catalase and superoxide dismutase increased.

When local varieties are not appropriately adapted, food production becomes challenging because they do not grow well, necessitating an import-based approach (Mladenov et al., 2023). Sometimes, during periods of extended dry spells, such as during hot weather or drought, the pressure exerted by drought leads to reduced cytoplasm and increased plasma membrane solute concentration, ultimately contributing to the inhibition of cell division and other issues like nutritional depletion (Desoky et al., 2021; Geleta et al., 2024)). This situation has various disadvantages, including stunted plant growth due to inadequate water supply and malnutrition resulting from poor absorption capacity in plants whose functions are typically impeded when water is limited. "The adverse outcomes are decreased crop yield and survival" (Mladenov et al., 2023). Because of this, focusing on the physiological indicators that mark enhanced plant characteristics expressed in times of drought is crucial for ongoing selection programs.

For instance, yield analysis from 24 commercial common bean cultivars was statistically conducted across various locations in Chile and Bolivia, with two cultivars studied exhaustively (Lizana et al., 2006). According to the findings, drought-tolerant genotypes exhibit better adaptive capacity for maintaining stomatal conductance, photosynthetic rate, abscisic acid synthesis, and resistance to photoinhibition. An additional study on drought-adapted bean varieties showed that increased yields in beans after periods of low rainfall are mainly due to the accumulation of several substances that provide osmotic protection and help the plant fight off cell damage caused by oxidation (Rosales et al., 2012). Evaluating the performance of different pinto bean genotypes under drought stress conditions is highly important. This research can help identify drought-tolerant lines, enhance understanding of the physiological and biochemical mechanisms of drought tolerance, and assess yield and seed quality parameters to select suitable cultivars for water-scarce regions (Raza et al., 2023). Identifying appropriate drought stress indices will assist breeding programs in accelerating the development of drought-resistant lines (Yahaya and Shimelis, 2022). Furthermore, comparing lines' performance under drought and optimal irrigation conditions will determine the extent of yield and quality reduction and help identify resistant lines. Given the significance of pinto beans as a food source and their role in food security, it is crucial to evaluate different lines and develop drought-resistant cultivars to improve overall crop quality and nutritional value, thereby playing a critical role in ensuring food security in arid and semi-arid regions. This experiment aims to evaluate the performance of various pinto bean lines under drought stress conditions by identifying the physiological and biochemical traits that contribute to drought tolerance and assessing their impact on yield and seed quality.

Materials and Methods

Plant Material and Experimental Design

To evaluate drought tolerance in 14 *Pinto bean* lines/genotypes (with the 'Kusha' genotype as the control), a study was conducted using a split-plot

design based on a randomized complete block design (RCBD) with three replications over two years (2022-2023) at the Khomein Bean Research and Education Complex in Markazi, Iran. The information on the lines and their coding is provided in Table 1. The Khomein Bean Research and Education Complex is situated in the Khorram Dasht region, 8 km from the city of Khomein, at coordinates 49°57' longitude and 33°39' latitude, with an elevation of 1930 m above sea level (MSAL). According to meteorological data, this area has an average annual rainfall of 300 mm. Khomein's climate is moderately mountainous, bordering on semi-arid, with freezing winters and moderately warm summers. The average variations in climatic parameters during the two growing seasons are presented in Table 2.

In this experiment, drought stress levels (control and drought stress) were evaluated in the main plots, while the 14 lines/genotypes of *Pinto beans* were assessed in the subplots. The experimental plot covered an area of 1200 m², with each plot measuring 10 m² (2 × 5 m). Seeds of each line were sown in eight rows, each 5 m long, with plant spacing within rows set at 5 cm. A non-planted row was left between each experimental plot (treatment). Land preparation included deep plowing in the fall, followed by shallow plowing in the spring, discing, and leveling. Macro- and micronutrients were applied to the soil based on soil test results. The two-year average soil analysis results are presented in Table 3. Before planting, urea fertilizer was applied at 25 kg nitrogen ha⁻¹, potassium fertilizer at 50 kg K₂O ha⁻¹, and phosphorus fertilizer from ammonium phosphate at 70 kg P₂O₅ ha⁻¹. Planting took place in early June in both years, with replanting conducted after germination at the V2 stage (emergence of initial leaves).

Method of Applying Drought Stress Treatments (Irrigation)

The water requirements for optimal irrigation, based on 40% Maximum Allowable Depletion (MAD), and for drought stress, based on 80% MAD, were calculated using methods described by Kandel et al. (2013). Readily Available Water (RAW) represents the amount of water required

Table 1
Name and characteristics of bean lines/genotype evaluated in this experiment

Lines/genotype	Code	Lines/genotype	Code
Genotype (check)	'Kusha'	L7	KS21606
L1	KS21538	L8	KS21600
L2	KS21565	L9	KS21500
L3	KS21318	L10	KS21597
L4	KS21607	L11	KS21563
L5	KS21331	L12	KS21488
L6	KS21601	L13	KS21373

*All lines have been received from CIAT

Table 2
The average climatic parameters during the plant growth season over two years of experimentation

		Monthly average minimum temperature (°C)	Monthly average maximum temperature (°C)	Monthly average steady wind (km/h)	Monthly sum precipitation (mm)	Pressure (mb)
First-year	June	16.05	34.33	27.67	0	1000.74
	July	18.13	36.13	30.03	0	995.45
	August	18.19	36.39	25.77	1.2	997.22
	September	11.58	32.9	27.09	0	1004.52
	October	9.6	28.87	23.47	0	1011.08
	November	7	30.34	26.75	0	1014.02
Second-year	June	15.67	32.86	20.93	7.2	1000.41
	July	19.23	36.55	28.37	0	996.47
	August	17.84	35.93	25.68	1.2	1000.38
	September	14.32	33.9	25.92	0	1002.83
	October	10	26.43	26.13	0.5	1012.12
	November	7.11	23.11	23	1.5	1015.99

Table 3
The physicochemical properties of the soil from 0 to 30 cm depth (average of two years)

Parameter	Method	Unit	Result
pH	Saturated extract	Units	7.24
EC	Saturated extract	dS m ⁻¹	0.78
P (ava)	Olsen	ppm	3.78
K (ava)	Flame photometer	ppm	254
Total N	Kjeldahl	%	0.14
Organic matter	Walkley-Black	%	3.44
TNV	Titration	%	12.85
Organic carbon	Walkley-Black	%	2
CLAY	Hydrometer	%	28
SILT	Hydrometer	%	18
SAND	Hydrometer	%	54
Soil texture	Hydrometer		Sandy Clay
Fe	Atomic	ppm	8.25
Zn	Atomic	ppm	1.12
Mn	Atomic	ppm	3.55
Cu	Atomic	ppm	0.98

for irrigation; Maximum Allowable Depletion (MAD) is the fraction of water depletion from the soil; D_r refers to root depth in meters; TAM is the Total Available Moisture in mm; and D_{rz} indicates the average depth of water stored in the root zone. For optimal irrigation at 40% MAD, the volumetric moisture content at field capacity (θ_{vFC}) and the permanent wilting point (θ_{vPWP}) was calculated.

The Total Available Moisture (TAM) is given by (Eq. 1):

$$TAM = \theta_{vFC} - \theta_{vPWP}$$

RAW is then calculated using (Eq.2):

$$RAW = (MAD \times TAM \times D_r)$$

The volumetric moisture content at 40% MAD is found in (Eq. 3):

$$MAD = \theta_{vFC} - RAW$$

These calculations were repeated for drought stress at 80% MAD. All plots were irrigated uniformly until drought stress was applied, maintaining soil moisture at 40% MAD. After plant establishment, drought stress was imposed at 80% MAD from the third trifoliate stage until maturity. Soil sampling was conducted regularly to monitor soil moisture levels, with measurements compared to the moisture curve to determine irrigation timing and amounts. Small valves were installed in each plot to control water flow, ensuring precise irrigation according to the experimental conditions for both optimal irrigation and drought stress treatments (da Silva et al., 2011; Pejić et al., 2010).

Measurement of Physiological and Biochemical Traits

During the reproductive stage, leaf samples were randomly collected (ten middle leaves from three plants) and immediately transferred to the laboratory in liquid nitrogen for physiological and biochemical analyses.

Measurement of Photosynthetic Pigments (Chlorophylls)

To measure chlorophyll content, 0.2 g of fresh plant material was homogenized with 5 ml of 80% acetone in a porcelain mortar. After centrifugation at 6,000 rpm for 10 minutes, the absorbance of the samples was measured at 663 and 645 nm using a spectrophotometer (Model Spectronic 20; Milton Roy Co., USA). Chlorophyll a, b, and total chlorophyll contents were calculated using the equations from Lichtenthaler and Buschmann (2001):

$$\begin{aligned} \text{Chlorophyll a} &= (19.3 \times A_{663} - 0.86 \times A_{645}) V / 100 W \\ \text{Chlorophyll b} &= (19.3 \times A_{645} - 3.6 \times A_{663}) V / 100 W \\ \text{Total chlorophyll} &= \text{Chl a} + \text{Chl b} \end{aligned}$$

Where V is the volume of the filtered solution, A is the absorbance at 663 and 645 nm, and W is the fresh sample weight in grams.

Measurement of Proline Content

The method proposed by Bates et al. (1973) was used to measure the proline amino acid content in the leaf. For this purpose, 0.2 g of the leaf sample was ground in 10 ml of 3% sulfosalicylic acid solution using a mortar and pestle, and the resulting extract was centrifuged at 13,000 rpm for 10 minutes at 4°C. Then, 2 ml of the filtered extract was transferred to capped tubes, and 2 ml of ninhydrin reagent and 2 ml of glacial acetic acid were added to each tube. After capping the tubes, they were placed in a water bath at 100°C for one hour. Following cooling, 4 ml of toluene was added to each tube. The proline concentration was calculated using a spectrophotometer at a wavelength of 520 nm, based on the standard curve.

Measurement of Antioxidant Enzyme Activities

To measure the activity of antioxidant enzymes, enzyme extraction was first conducted. For this purpose, 0.1 g of plant sample from each treatment was weighed and homogenized with 1.5 ml of extraction buffer (containing 2.72 g

Table 4

Analysis of variance of drought stress effects on some biochemical and physiological traits of different lines of pinto beans (*Phaseolus vulgaris*)

		Mean square (MS)				
SOV	df	Chl-a	Chl-b	Total-Chl	Proline	Flav
Year (Y)	1	0.125ns	0.098ns	0.528ns	0.561ns	0.0002ns
rep (Y)	4	0.017	0.013	0.007	0.043	0.01
Drought (D)	1	12.27*	5.61*	33.79*	3.95*	33.3**
Y x D	1	5.831**	0.666**	10.047**	0.002ns	0.004ns
Error D	4	0.014	0.02	0.007	0.017	0.039
Lines (L)	13	0.191ns	0.212ns	0.657ns	0.168*	0.74**
Y x L	13	0.146**	0.129**	0.399**	0.046**	0.029ns
D x L	13	0.236**	0.734*	1.829*	0.187*	0.663**
Y x D x L	13	0.052*	0.161**	0.339**	0.079**	0.014ns
Error	167	0.174	0.115	0.463	0.069	0.357
CV (%)		10.93	20.17	8.54	9.57	10.11

Table 4

Continue:

		Mean square (MS)				
SOV	df	CAT	POX	PPO	APX	SOD
Year (Y)	1	0.334ns	0.773ns	0.024ns	43.52ns	1.58ns
rep (Y)	4	0.043	0.399	0.179	1.24	2.95
Drought (D)	1	73.1*	148.5**	35.9**	575.3ns	4359.8**
Y x D	1	0.038ns	0.002ns	0.005ns	32.13**	0.01ns
Error D	4	0.009	0.307	0.06	0.14	0.39
Lines (L)	13	0.72**	7.858**	2.599**	4.27**	24.61**
Y x L	13	0.004ns	0.075ns	0.035ns	0.27ns	0.41ns
D x L	13	0.363**	0.574**	0.147**	0.91ns	24.21**
Y x D x L	13	0.004ns	0.032ns	0.02ns	0.37ns	0.25ns
Error	167	0.542	1.731	0.571	5.22	32.18
CV (%)		8.53	10.62	6.18	10.6	8.18

ns, *, and ** denote non-significant, significant at the 5%, and significant at the 1% levels, respectively.

Chl-a (Chlorophyll a), Chl-b (Chlorophyll b), Total-Chl (Total Chlorophyll), Proline, Flav (Flavonoids), CAT (Catalase), POX (Peroxidase), PPO (Polyphenol oxidase), APX (Ascorbate peroxidase), SOD (Superoxide dismutase), RWC (relative water content)

potassium phosphate with pH = 7.8, 0.029 g EDTA, and 5.64 g polyvinylpyrrolidone dissolved in 200 ml of distilled water) on ice in a porcelain mortar. The resulting extracts from each treatment were then transferred to 1.5 ml micro tubes, and the homogenates were centrifuged at 13,000 rpm for 30 minutes at 4°C. This extract was used to measure the activity of antioxidant enzymes.

Catalase (CAT) Enzyme Activity

The catalase (CAT) enzyme activity was measured using the protocol proposed by Chance and Maehly (1995). To 2.8 ml of 50 mM phosphate buffer (pH 7.0) containing 30 mM hydrogen peroxide, 100 µl of the enzyme extract was added, and the absorbance was measured at 240 nm

using a spectrophotometer at 0, 30, and 60 seconds. Adding H₂O₂ initiates its decomposition, causing a decrease in optical absorbance. The extinction coefficient for CAT is 0.0394 mM⁻¹ cm⁻¹.

Peroxidase (POX) Enzyme Activity

Peroxidase (POX) enzyme activity was measured using the method proposed by Kar and Mishra (1976). The reaction mixture contained 2.3 ml of 25 mM monosodium phosphate buffer (pH 6.8), 100 µl of 10 mM pyrogallol, 500 µl of the enzyme extract, and 100 µl of 40 mM hydrogen peroxide, bringing the final volume to 3 ml. Enzymatic activity was initiated by adding hydrogen peroxide to the reaction mixture. The enzyme extract was replaced with 25 mM monosodium phosphate

buffer in the blank sample. Changes in optical absorbance due to the formation of purpurogallin from pyrogallol were measured at 420 nm for 180 seconds using a spectrophotometer. POX enzyme activity was calculated based on the extinction coefficient of $2.47 \text{ mM}^{-1} \text{ cm}^{-1}$.

Polyphenol Oxidase (PPO) Activity

Polyphenol oxidase (PPO) activity was determined using a spectrophotometric method (Chance and Machely, 1995). The reaction mixture consisted of 2.4 ml of 50 mM phosphate buffer (pH 6.5) and 100 μl of enzyme extract. The reaction was initiated by adding 500 μl of 50 mM catechol as the substrate. The increase in absorbance due to the formation of the colored quinone product was monitored at 420 nm for 180 seconds. A blank sample was prepared by replacing the enzyme extract with phosphate buffer. The PPO activity

was calculated using the quinone product's molar extinction coefficient of $3.45 \text{ mM}^{-1} \text{ cm}^{-1}$.

Ascorbate Peroxidase (APX) Enzyme Activity

The ascorbate peroxidase (APX) enzyme activity was measured using the method proposed by Nakano and Asada (1981). The reaction mixture consisted of 2550 μl of 0.5 mM ascorbate solution in 100 mM potassium phosphate buffer (pH 7.0), 450 μl of 2 mM hydrogen peroxide solution in double-distilled sterile water, and 30 μl of the enzyme extract in a 3 ml quartz cuvette. The enzyme activity of APX was measured at a wavelength of 290 nm for 180 seconds using a spectrophotometer.

Superoxide Dismutase (SOD) Enzyme Activity

The measurement of superoxide dismutase (SOD) enzyme activity was performed using the method proposed by Giannopolitis and Ries (1977). The samples were exposed to light for 15 minutes, after which their absorbance was read at 560 nm using a spectrophotometer. A test tube containing the reaction mixture without the enzyme extract was used as a blank. The reaction mixture consisted of 50 mM phosphate buffer (pH 7.8)

containing 0.1 mM EDTA, 50 mM sodium carbonate (pH 10.2), 12 mM L-methionine, 75 μM nitro blue tetrazolium, 1 μM riboflavin, and 300 μl of the enzyme extract.

Measurement of Relative Water Content (RWC)

The method proposed by Smart and Bingham (1974) was used to determine the RWC of the leaf samples. The leaves were divided into 1 cm pieces, and their fresh weight was determined. Then, the leaf pieces were placed in distilled water for 16–18 hours at room temperature (approximately 25°C) and under low light conditions. After this period, the weight of the turgid leaves was quickly and accurately measured. The leaf pieces were then placed in a 70°C oven for 48 hours, and their dry weight was measured. Finally, the relative water content was calculated using the following equation:

$$\text{RWC (\%)} = [(W_f - W_d) / (W_t - W_d)] \times 100 \text{ (Eq. 7)}$$

Where W_f is the fresh weight of the leaf, W_t is the turgid weight, and W_d is the dry weight of the leaf.

Measurement of Seed Yield

Three middle rows of each plot were sampled to measure seed yield, following the principles of area-based sampling. An area of 1 m^2 was harvested from the center of each plot, and the samples were transported to the laboratory for evaluation. The measured values were then converted to kg ha^{-1} for statistical analysis.

Statistical Analysis

After data collection, Bartlett's and Levene's tests were first performed. Given the non-significant results, a combined analysis of variance (ANOVA) was conducted across years for all traits using the statistical software SAS version 9.2. Mean comparisons were made using Duncan's multiple range test at the 5% probability level. Simple correlations, cluster analysis, and stepwise regression were conducted under stress and non-stress conditions using Microsoft Excel 2021 and Minitab 2018. In the correlation analysis, positive correlation coefficients are presented in blue, and negative correlation coefficients are presented in

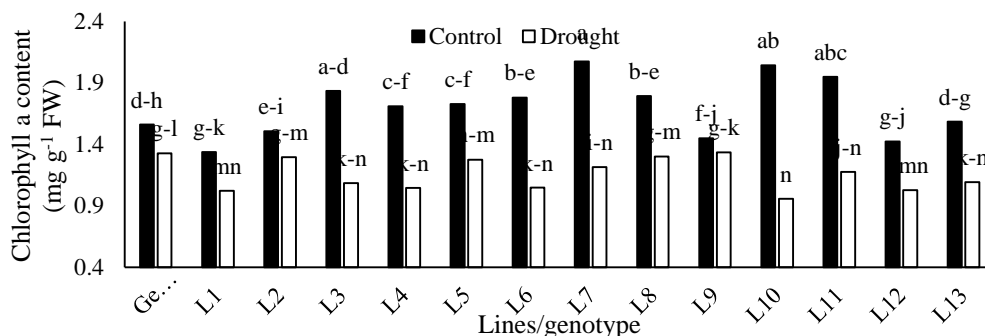


Fig. I. The effect of drought stress on the chlorophyll-a content of different pinto bean lines/genotypes. Means with similar letters are not significantly different according to the DMRT test at the 5% probability level.

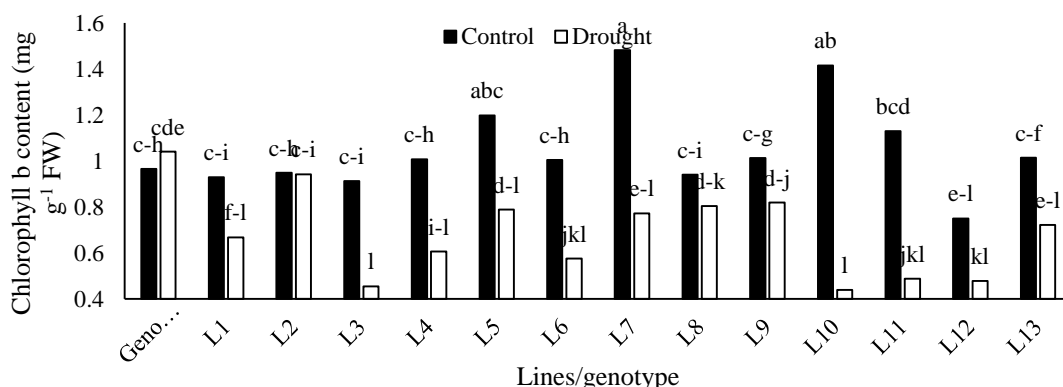


Fig. II. The effect of drought stress on the chlorophyll b content of different pinto bean lines/genotypes. Means with similar letters are not significantly different according to the DMRT test at the 5% probability level.

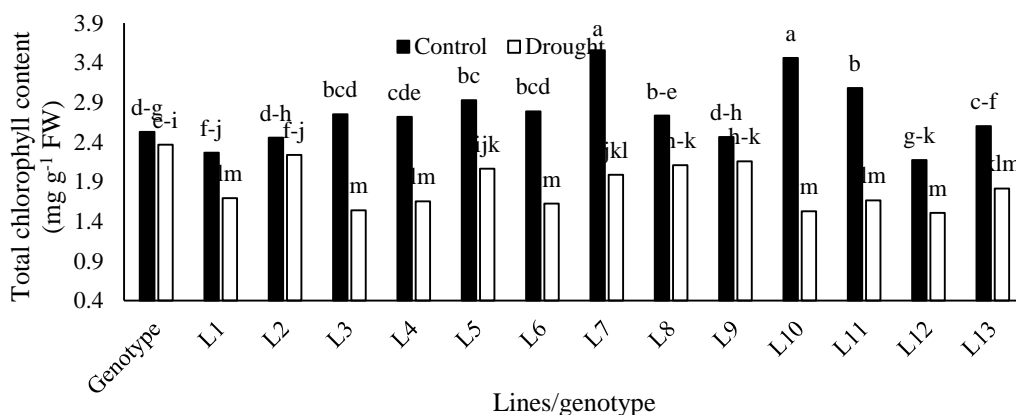


Fig. III. The effect of drought stress on the total chlorophyll content of different pinto bean lines/genotypes. Means with similar letters are not significantly different according to the DMRT test at the 5% probability level.

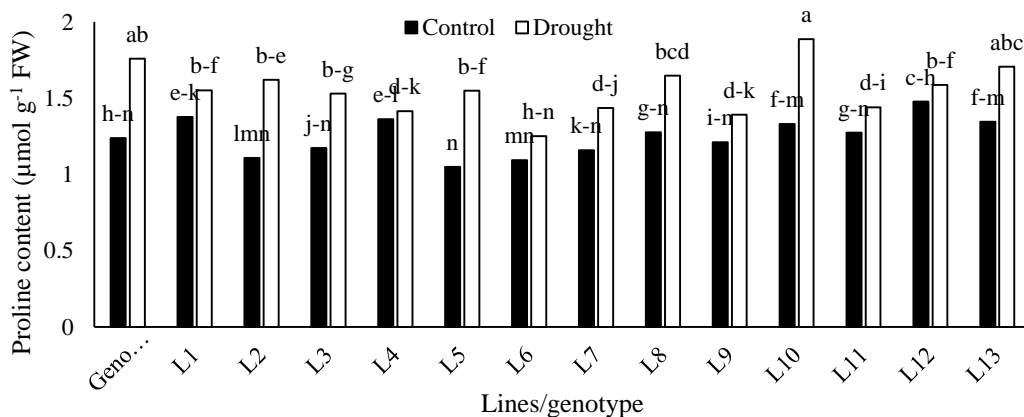


Fig. IV. The effect of drought stress on the proline content of different pinto bean lines/genotypes. Means with similar letters are not significantly different according to the DMRT test at the 5% probability level.

red, where the increasing intensity of the color indicates a stronger coefficient.

Results

Chlorophyll Content

The photosynthetic pigment content, including chlorophyll a, chlorophyll b, and total chlorophyll, was significantly influenced by drought stress and the interaction effects of drought stress on bean lines (Table 4). Line 7, under non-stress conditions, exhibited the highest chlorophyll a ($2.07 \text{ mg g}^{-1} \text{ FW}$), 1.32 times greater than the control cultivar. Under drought stress conditions, line 9 had the highest chlorophyll a ($1.33 \text{ mg g}^{-1} \text{ FW}$), but it did not demonstrate significant superiority over the control genotype under drought stress. Meanwhile, line 10, under drought stress conditions, had the lowest chlorophyll a (Fig. I).

The findings revealed that, in terms of chlorophyll b content, line 7 exhibited the highest mean for this trait under non-stress conditions ($1.48 \text{ mg g}^{-1} \text{ FW}$), while under drought stress, lines 3 and 10 had the lowest mean values (0.45 and $0.43 \text{ mg g}^{-1} \text{ FW}$, respectively). For chlorophyll b content, lines 5, 7, 10, and 11 were significantly superior to the control cultivar under non-stress conditions. In contrast, the tested lines did not demonstrate any significant superiority over the control genotype in terms of this trait under drought stress (Fig. II).

Under non-stress conditions, lines 7 and 10 had the highest total chlorophyll contents, at 3.55 and $3.46 \text{ mg g}^{-1} \text{ FW}$, respectively. These values were 1.40 and 1.37 times higher than the control cultivar. In contrast, under drought stress, all the tested lines exhibited lower total chlorophyll levels than the check genotype. The only exception was line 2, which had a mean value roughly on par with the control. The lowest total chlorophyll contents under drought stress were observed in lines 3, 6, 10, and 12 (1.54 , 1.62 , 1.52 , and $1.50 \text{ mg g}^{-1} \text{ FW}$, respectively) (Fig. III).

Proline Content

The ANOVA results indicated that drought stress, bean lines, and their interaction significantly

affected proline content at the 5% probability level (Table 4). Proline content increased dramatically in all lines under drought stress conditions. The highest proline content was observed in line 10 under drought stress ($1.88 \text{ } \mu\text{mol g}^{-1} \text{ FW}$), though it did not show a statistically significant advantage over the control genotype. The lowest proline content was observed in line 5 under non-stress conditions ($1.05 \text{ } \mu\text{mol g}^{-1} \text{ FW}$). In comparing drought and non-stress treatments, lines 4 and 12 showed only a minor difference in proline content, similar to what might be expected in native conditions. In contrast, lines 2 and 5 exhibited the most significant difference (Fig. IV).

Flavonoid Content

The results showed that drought stress, bean lines, and their interaction significantly affected flavonoid content at the 1% probability level (Table 4). Under drought stress conditions, line 11 had the highest flavonoid content ($3.53 \text{ mg g}^{-1} \text{ FW}$), which showed a 1.29-fold increase compared to the control genotype. The lowest flavonoid content was observed in lines 10 and 11 and the control genotype under non-stress conditions (1.62 , 1.55 , and $1.59 \text{ mg g}^{-1} \text{ FW}$, respectively) (Fig. V).

CAT Activity

The CAT enzyme activity was significantly impacted by drought stress, the different bean lines, and the interaction between these factors (Table 4). Generally, drought stress triggered an increase in CAT activity. The highest CAT activity was observed in line 13 under drought conditions, averaging $3.15 \text{ U mg protein}^{-1} \cdot \text{min}$. When subjected to drought stress, the control genotype and line 10 also exhibited excellent CAT activity. However, when grown under non-stress conditions, the tested bean cultivars and lines showed no statistically significant differences in their CAT enzyme activity levels (Fig. VI).

POX Activity

According to the results, the effect of drought stress on the activity of the POX enzyme was significantly different among the various bean

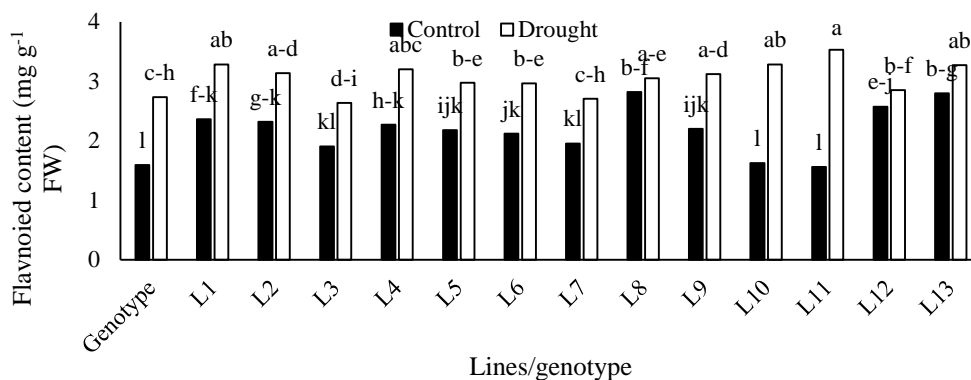


Fig. V. The effect of drought stress on the flavonoid content of different pinto bean lines/genotypes. Means with similar letters are not significantly different according to the DMRT test at the 5% probability level.

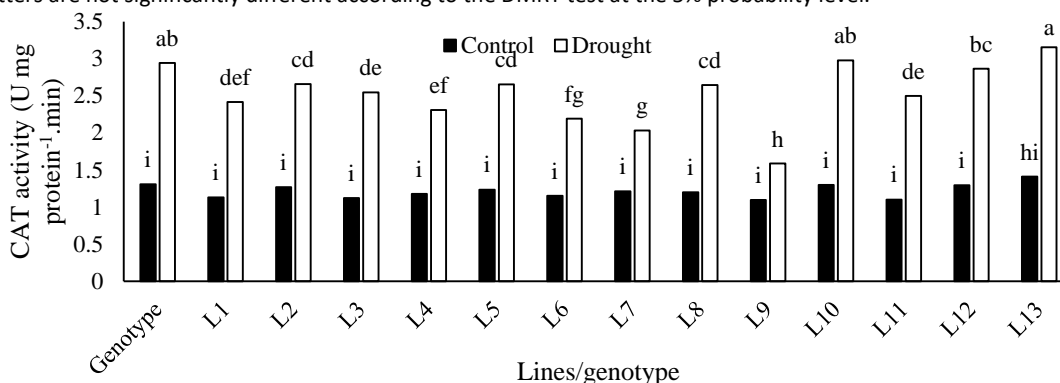


Fig. VI. The effect of drought stress on the CAT activity of different pinto bean lines/genotypes. Means with similar letters are not significantly different according to the DMRT test at the 5% probability level.

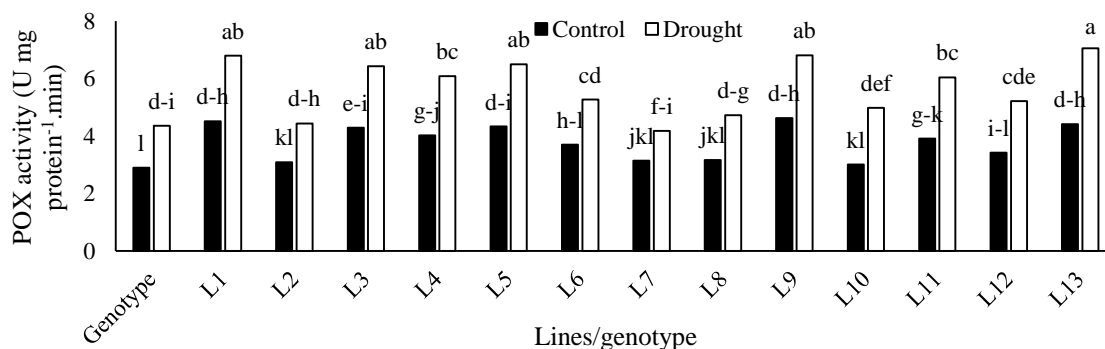


Fig. VII. The effect of drought stress on the POX activity of different pinto bean lines/genotypes. Means with similar letters are not significantly different according to the DMRT test at the 5% probability level.

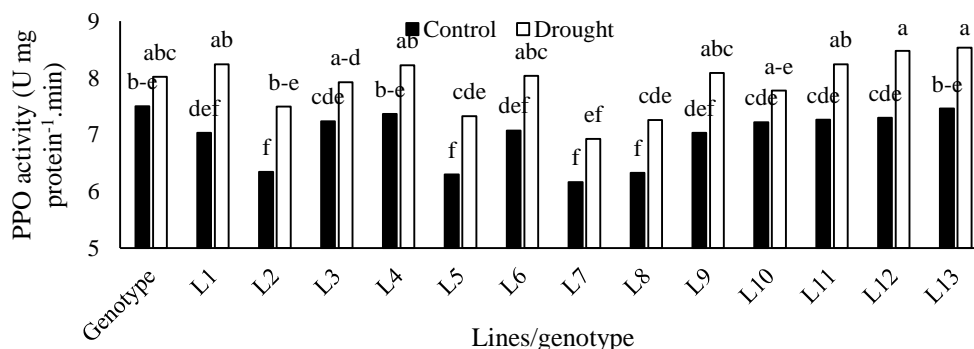


Fig. VIII. The effect of drought stress on the PPO activity of different pinto bean lines/genotypes. Means with similar letters are not significantly different according to the DMRT test at the 5% probability level.

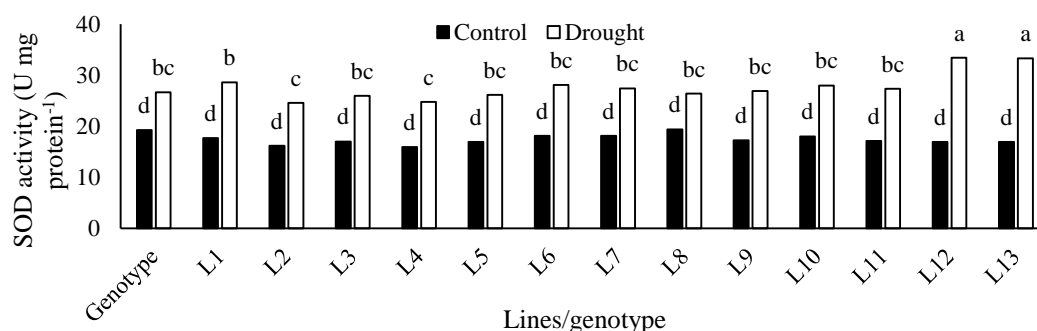


Fig. IX. The effect of drought stress on the SOD activity of different pinto bean lines/genotypes. Means with similar letters are not significantly different according to the DMRT test at the 5% probability level.

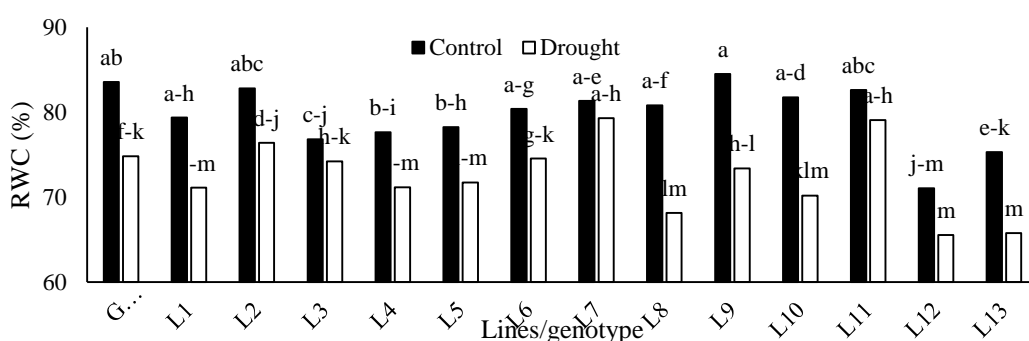


Fig. X. The effect of drought stress on the RWC of different pinto bean lines/genotypes. Means with similar letters are not significantly different according to the DMRT test at the 5% probability level.

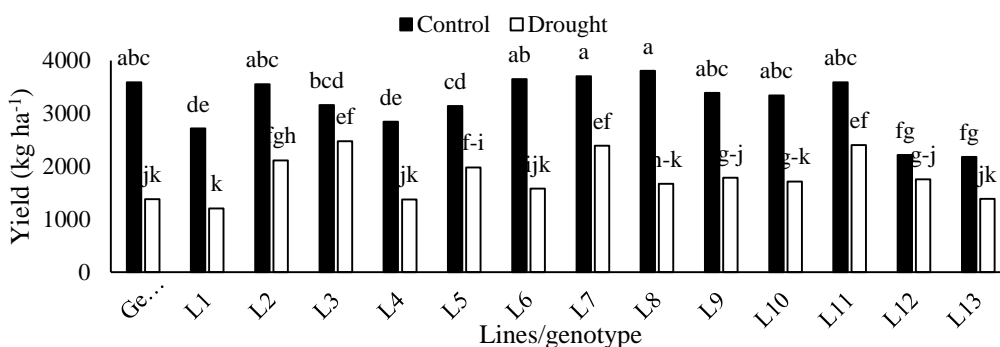


Fig. XI. The effect of drought stress on the seed yield of different pinto bean lines/genotypes. Means with similar letters are not significantly different according to the DMRT test at the 5% probability level.

lines at the 1% probability level (Table 4). The activity of this enzyme, similar to CAT, increased under drought stress conditions, such that line 13 under stress conditions exhibited the highest POX activity (7.05 U mg protein⁻¹.min), which was 1.62 times greater than the control. Based on the results, bean lines 1, 3, 4, 5, 9, and 11 showed higher POX activity compared to the control cultivar under drought stress conditions. The lowest activity of this enzyme was observed in the

control genotype under non-stress conditions (2.90 U mg protein⁻¹.min). The lowest activity was observed among lines 2 and 10 under non-stress conditions (Fig. VII).

PPO Activity

The activity of PPO was significantly affected by drought stress among the different bean lines at the 1% probability level (Table 4). The results

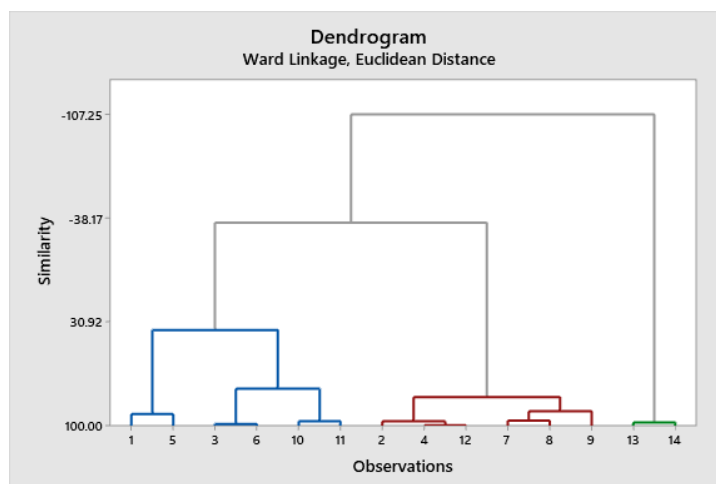


Fig. XII. Dendrogram of cluster analysis of pinto bean lines under non-stress conditions.

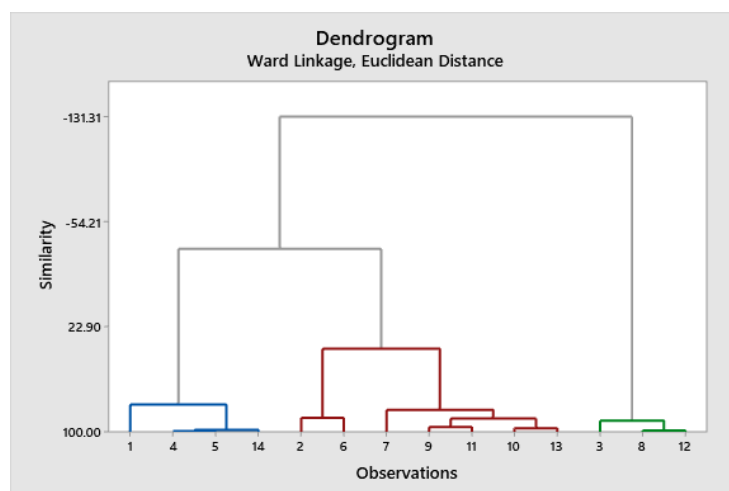


Fig. XIII. Dendrogram of cluster analysis of pinto bean lines under drought stress conditions.

showed that lines 12 and 13 under drought stress exhibited the highest PPO activity (8.47 and 8.53 U mg protein⁻¹.min, respectively). In all the tested lines/genotypes, drought stress led to an increase in the activity of the PPO enzyme. The lowest PPO activity was observed in lines 2, 5, 7, and 8 under non-stress conditions (6.34, 6.29, 6.16, and 6.32 U mg protein⁻¹.min, respectively) (Fig. VIII).

APX Activity

The results showed that the activity of the APX enzyme differed significantly among the tested bean lines. However, contrary to other antioxidant enzymes, drought stress had a non-significant

effect on the activity of this enzyme (Table 4). The highest APX activity was observed in lines 2, 12, and 13, with means of 11.71, 12.05, and 11.72 U mg protein⁻¹.min, respectively. The APX activity in the control genotype was 10.78 U mg protein⁻¹.min. The lowest activity was obtained in line 11, with a mean of 9.88 U mg protein⁻¹.min (data not shown).

SOD Activity

The effects of drought stress, line, and their interaction on the activity of the SOD enzyme were significant (Table 4). The experimental findings showed that drought stress led to a

Table 5
Simple correlations between physiological and biochemical traits with yield of different bean lines under drought stress conditions

	Chl-a	Chl-b	Total-Chl	Proline	Flav	CAT	POX	PPO	APX	SOD	RWC
Chl-b	0.76**										
Total-Chl	0.95**	0.93**									
Proline	-0.29ns	-0.31*	-0.32*								
Flav	-0.49*	-0.49*	-0.52**	0.27ns							
CAT	-0.09ns	0.06ns	-0.02ns	0.23ns	0.25ns						
POX	-0.34*	-0.23ns	-0.31*	0.02ns	0.24ns	-0.39*					
PPO	-0.22ns	-0.35*	-0.30*	0.57**	-0.22ns	0.11ns	0.23ns				
APX	-0.29ns	0.01ns	-0.17ns	0.45*	0.41*	0.29ns	-0.02ns	-0.04ns			
SOD	-0.05ns	0.01ns	-0.12ns	-0.05ns	0.02ns	0.05ns	0.06ns	-0.12ns	-0.06ns		
RWC	0.22ns	0.40*	0.32*	-0.48*	-0.51**	-0.33*	-0.24ns	-0.26ns	-0.38*	-0.30*	
Yield	0.61**	0.41*	0.69**	-0.64**	-0.48*	-0.40*	-0.46*	-0.48*	-0.50**	-0.44*	0.70**

ns, * and **: non-significant, significant at a 5% probability level, and significant at a 1% probability level, respectively.

Table 6
Simple correlations between physiological and biochemical traits with yield of different bean lines under non-stress conditions

	Chl-a	Chl-b	Total-Chl	Proline	Flav	CAT	POX	PPO	APX	SOD	RWC
Chl-b	0.82**										
Total-Chl	0.92**	0.97**									
Proline	-0.04ns	0.14ns	0.14ns								
Flav	-0.21ns	-0.21ns	-0.20ns	0.06ns							
CAT	-0.25ns	-0.06ns	-0.11ns	0.79**	0.07ns						
POX	-0.24ns	-0.30*	-0.32*	-0.22ns	0.39*	-0.13ns					
PPO	-0.49*	-0.38*	-0.47*	-0.03ns	0.36*	0.20ns	0.55**				
APX	-0.45*	-0.13ns	-0.28ns	0.37*	-0.25ns	0.51**	-0.04ns	0.30*			
SOD	-0.44*	-0.31*	-0.40*	0.20ns	0.08ns	0.39*	0.22ns	0.54**	0.79**		
RWC	0.36*	0.18ns	0.26ns	-0.42*	-0.11ns	-0.49*	-0.31*	-0.40*	-0.66**	-0.61**	
Yield	0.24ns	0.21ns	0.45*	-0.22ns	-0.24ns	-0.22ns	-0.18ns	-0.46*	-0.34*	-0.29ns	0.88**

ns, * and **: non-significant, significant at a 5% probability level, and significant at a 1% probability level, respectively

considerable increase in the mean activity of this enzyme, such that the highest SOD activity was observed in lines 12 and 13 under drought stress conditions (33.43 and 33.29 U mg protein⁻¹, respectively), which were 1.25 and 1.24 times higher than the control genotype. All the lines, as well as the control cultivar under non-stress conditions, exhibited the lowest SOD enzyme activity, similar to the pattern observed for CAT (Fig. IX).

RWC

The RWC of the leaves was significantly affected by drought stress in the tested bean lines (Table 4). The highest RWC was observed in line 9 under

non-stress conditions (84.5%), which did not show a significant increase compared to the control genotype. The lowest RWC was obtained in lines 12 and 13 under drought stress conditions (65.5% and 65.7%, respectively) (Fig. X).

Seed Yield

Seed yield was significantly affected by drought stress, bean line, and their interaction (Table 4). Based on the results, the highest yield was related to lines 7 and 8 with means of 3703.3 and 3807.4 kg ha⁻¹, respectively, under non-stress conditions, which were 3.19% and 5.83% higher than the control cultivar under non-stress conditions. Under drought stress conditions, the highest yield

Table 7

Cluster analysis of biochemical and physiological traits in pinto bean lines under non-stress conditions.

Variable	Cluster1	Cluster2	Cluster3
Chl-a	1.68	1.78	1.50
Chl-b	1.08	1.08	0.88
Total-Chl	2.76	2.86	2.39
Proline	1.25	1.19	1.41
Flav	2.09	2.06	2.68
CAT	1.18	1.21	1.35
POX	4.13	3.32	3.92
PPO	7.03	6.78	7.38
APX	9.07	8.96	9.54
SOD	17.15	18.05	16.96
RWC	79.72	81.92	73.16
Yield	3095.67	3646.50	2193.33
Number of lines	6	6	2

Table 8

Cluster analysis of biochemical and physiological traits in pinto bean lines under drought stress conditions.

Variable	Cluster1	Cluster2	Cluster3
Chl-a	1.12	1.18	1.16
Chl-b	0.76	0.69	0.57
Total-Chl	1.88	1.89	1.73
Proline	1.61	1.56	1.47
Flav	3.12	3.05	2.96
CAT	2.71	2.51	2.36
POX	6.07	5.42	5.55
PPO	8.25	7.78	7.70
APX	13.09	12.81	12.37
SOD	28.34	27.66	26.92
RWC	70.71	71.42	77.53
Yield	1334.37	1798.19	2422.55
Number of lines	4	7	3

Table 9

Stepwise regression analysis for yield as the dependent variable in different bean lines under drought stress conditions

Term	Coef	SE Coef	T-Value	P-Value
Constant	-2526	1602	-1.58	0.011
Total Chl (X1)	2843	440	6.46	0.000
Proline (X2)	-1538	266	-5.78	0.001
PPO (X3)	-464.8	84.9	-5.47	0.001
RWC (X4)	96.2	12.1	7.98	0.000

R-sq (adj) =95.85%

Y = -2526 +2843 (X1) - 1538 (X2) - 464.8 (X3) + 96.2 (X4)

Table 10

Stepwise regression analysis for yield as the dependent variable in different bean lines under non-stress conditions.

Term	Coef	SE Coef	T-Value	P-Value
Constant	4868	1012	4.81	0.015
Total Chl (X1)	-558	243	-2.30	0.024
RWC (X2)	58.4	22.4	2.61	0.023

R-sq (adj) =49.79%

Y = 4868 - 558 (X1) + 58.4 (X2)

was related to lines 3, 7, and 11, which were 1.79, 1.73, and 1.74 times higher than the control cultivar. The lowest yield was observed in line 1

under drought stress conditions, with a mean of 1204.2 kg ha⁻¹ (Fig. XI).

Correlation Analysis

The results of the simple correlation analysis between traits are presented in Tables 5 and 6 for drought stress and non-stress conditions, respectively. Under drought stress conditions, yield positively and significantly correlated with chlorophyll a, b, total contents, and RWC. In contrast, yield had a negative and significant correlation with proline content, flavonoid content, and the activities of CAT, POX, PPO, APX, and SOD enzymes. Under non-stress conditions, yield had a positive and significant correlation with total chlorophyll content and RWC, and a negative correlation with the activities of PPO and APX enzymes at the 5% probability level.

Cluster Analysis

The cluster analysis results of the tested lines under drought stress and non-stress conditions are presented in Tables 7 and 8 and Figures 12 and 13. Under non-stress conditions, six lines were grouped in the first cluster, six in the second cluster, and two in the third cluster. The second cluster was superior in photosynthetic pigment content, RWC, and yield. This cluster included the check genotype 'Kusha' and lines 3, 13, 8, 9, and 10.

Under drought stress conditions, the clustering of the lines was different, with the first, second, and third clusters containing 4, 7, and 3 lines, respectively. Regarding RWC and yield, the third cluster, which had the fewest lines (4, 9, 13), was superior. The first cluster, which included the check genotype, was exceptional in terms of the activities of the antioxidant enzymes CAT, POX, PPO, APX, SOD, as well as proline and flavonoid content.

Stepwise Regression Analysis

To identify the biochemical and physiological traits influencing seed yield under drought stress and non-stress conditions, stepwise regression analysis was performed, and the results are presented in Tables 9 and 10. The results showed that under drought stress conditions, the traits total chlorophyll, proline content, PPO activity,

and RWC were the influential factors, collectively explaining 95.85% of the variations in seed yield. In contrast, under non-stress conditions, the physiological and biochemical traits had a lesser effect, and only two traits, total chlorophyll content, and RWC, were included in the regression model, explaining 49.79% of the variations in yield.

Discussion

The objective of the current research was to assess the physiological and biochemical responses of various *Pinto bean* lines to different levels of drought stress and to identify the physiological and biochemical traits that influence seed yield under drought stress and non-stress conditions. The results revealed that drought stress significantly decreased the mean photosynthetic pigment content across all the tested bean lines. However, some lines/genotypes, such as 'Kusha' and lines 1, 2, 8, 9, and 12, exhibited less reduction in total chlorophyll content under drought stress conditions. Conversely, the total chlorophyll content of some lines, including lines 7, 10, and 11, was nearly halved under drought stress conditions. In other words, these lines were sensitive to drought stress in terms of their photosynthetic pigments. Reducing chlorophyll content under stress conditions may help plants mitigate photo-oxidative damage, as under these conditions, photosynthesis is inhibited, and the absorbed light energy exceeds the capacity for photosynthesis (Chauhan et al., 2023). The excess excitation energy absorbed by the photosynthetic pigments in Photosystem II can disrupt photosynthetic function and lead to the accumulation of reactive oxygen species (ROS), resulting in oxidative stress (Sachdev et al., 2023). Other studies have shown that plant photosynthesis is inhibited due to a decrease in chlorophyll concentration and the detrimental effects of drought stress on the Calvin cycle (Zahra et al., 2023). Various plant species have demonstrated similar results regarding reduced chlorophyll content under drought stress conditions (Desoky et al., 2021; Lizana et al., 2006; Mehrasa et al., 2022)).

Drought stress led to an increase in proline content and antioxidant enzyme activity in all the

tested lines. In selecting and breeding drought-tolerant bean lines, physiological traits such as high proline content and enhanced antioxidant enzyme activity under stress conditions are essential indicators of adaptive and stress tolerance capabilities (Azizi et al., 2024). The increase in proline content observed across all tested lines under drought stress is consistent with those findings reported by Sinha et al. (2018). The accumulation of proline is likely due to the increased activity of the enzymes involved in proline biosynthesis, namely ornithine aminotransferase and pyrroline-5-carboxylate reductase, as well as the reduced activity of proline oxidase and catabolic enzymes (Azizi et al., 2024). Previous research has shown that drought-tolerant genotypes accumulate higher proline levels than drought-sensitive genotypes (Singh et al., 2019).

Flavonoids act as antioxidants, scavenging reactive oxygen species and protecting cellular structures from oxidative damage. They also have signaling functions that activate plant stress response pathways (Dumanović et al., 2021). In the present study, an increase in the average content of this metabolite (flavonoids) was observed in the lines under drought-stress conditions. Research has demonstrated that drought-tolerant bean lines accumulate higher levels of total flavonoids than drought-sensitive genotypes when exposed to water stress (Soureshjani et al., 2019).

Under drought stress, the plant's induction of antioxidant enzyme activity is considered a defensive mechanism that helps counteract the cellular damage caused by oxidative stress (Shams Peykani and Farzamisepehr, 2018; Ullah et al., 2024). In this study, the activity of antioxidant enzymes such as CAT, POX, SOD, and PPO was higher in line 13 under stress conditions. The increased activity of these enzymes under stress conditions can reduce the damage caused by oxidative stress, and this target line can have desirable performance under stress conditions. In this study, the enzyme activity level differed among the lines, and the tolerant line, like 13, had the highest antioxidant enzyme activity. Similar results were reported in different lentil lines under drought stress conditions (Azizi et al., 2024).

Under drought stress conditions, the RWC significantly decreased. RWC is an essential physiological index that indicates the water status of the plant under stress conditions (Ghanem and Al-Farouk, 2024). In the case of bean lines, studies show that RWC varies among different genotypes under drought stress conditions (Geleta et al., 2024). Drought-tolerant lines typically have higher RWC compared to sensitive lines, which is due to better adaptive mechanisms, including more efficient water absorption and utilization, more effective osmotic adjustment, and reduced transpiration rates (Dumanović et al., 2021; Geleta et al., 2024; Ullah et al., 2024). Maintaining RWC in drought-tolerant lines is associated with better physiological performance, such as higher photosynthetic rate, plant growth, and yield stability, and can be helpful in the selection and breeding of cultivars with higher drought tolerance (Azizi et al., 2024; Kirkham, 2023).

The results showed that drought stress led to a decrease in yield in different lines. However, the extent of the reduction in lines 12 and 13 was lesser compared to the non-stress conditions. It seems that the efficacy of water use during this sensitive period caused a significant decrease in yield and yield components. In contrast, non-stress conditions increased photosynthetic products and their transfer to the seed, consequently increasing seed weight (Raza et al., 2023). Furthermore, insufficient moisture during flowering can significantly reduce seed formation and fertility. If water deficiency occurs during the seed-filling stage, it can considerably reduce the production of photosynthetic products and their transfer to the seed, resulting in smaller seeds and reduced seed weight (Azizi et al., 2024; Sinha et al., 2018). Other studies also indicate that seed yield and yield components are reduced under stress conditions (Azizi et al., 2024). Drought stress can lead to impaired photosynthesis and sterile pollen and facilitate reduced material transfer for seed storage, decreasing seed weight (Zahra et al., 2023).

One of the main objectives of this research was to determine the effective physiological and biochemical traits under drought stress and non-stress conditions on the yield of beans. The results

showed that the content of photosynthetic pigments (including chlorophyll a, b, and total), as well as the content of the amino acid proline and the activity of antioxidant enzymes CAT, POX, SOD, PPO, and APX, had a high correlation (positive and negative, respectively) with seed yield under drought stress conditions. The content of proline, the amount of chlorophyll a and b, and the activity of the antioxidant enzymes CAT and POX with seed yield under non-stress conditions were not significant, indicating a lesser effect of these traits under non-stress conditions. However, in the regression analysis for drought stress conditions, the traits of total chlorophyll, proline, PPO activity, and RWC were included in the regression model, which could explain more than 95% of the variations in seed yield with these traits. Based on these findings, it can be concluded that the total chlorophyll content, the activity of antioxidant enzymes, especially PPO, the proline amino acid content, and the high RWC are essential physiological and biochemical traits that significantly affect seed yield. In a similar experiment, it was reported that the activity levels of the SOD and PPO enzymes, as well as the RWC, were traits that significantly affected the yield of beans under salt-stress conditions (Anaya et al., 2017).

According to the cluster analysis results, lines 1, 4, and 13 exhibited superior activity levels of antioxidant enzymes, such as SOD and PPO, under drought stress conditions, indicating their improved capacity to mitigate oxidative stress. In contrast, lines 3, 7, and 11 demonstrated higher yield and maintained greater RWC under drought stress, suggesting their superior drought tolerance

mechanisms. Considering the overall evaluation of the assessed traits and the general conclusions, lines 11, 12, and 13 can be identified as the most promising *Pinto bean* genotypes for cultivation in drought-stressed regions, which can guide bean breeding programs to select the most suitable drought-tolerant lines for further evaluation and potential release in water-limited environments.

Conclusion

This study investigated the impact of drought stress on multiple physiological parameters in *Pinto bean* lines. Significant increases were observed in proline content, flavonoid content, and antioxidant enzyme activities (CAT, POX, PPO, APX, SOD) under stress conditions compared to non-stress conditions. In contrast, a decrease was observed in chlorophyll content, RWC, and seed yield. The results showed that the content of photosynthetic pigments (especially total chlorophyll), the activity level of antioxidant enzymes (especially PPO), as well as the amount of the amino acid proline and the RWC are considered the most critical physiological and biochemical traits affecting seed yield under drought stress conditions. The lines with higher mean values of these traits exhibit enhanced tolerance and adaptation for producing desirable yields under drought-stress conditions. Therefore, based on the overall assessment of traits and the analyses performed, lines 3, 11, 12, and 13 can be considered the most promising lines for further evaluation and potential cultivation in water-limited regions.

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