



Evaluation of physiological responses and yield stability during long-term water deficiency memory of cotton (*Gossypium hirsutum* L.)

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Abstract

Crops are frequently subjected to simultaneous abiotic stresses, which negatively impact plant health and yield. A plant's "memory" of prior stress exposure, known as stress priming, can enhance its ability to cope with subsequent drought events. This memorial capacity triggers tolerant physiological, biochemical, and morphological mechanisms that help mitigate yield losses. Due to climate change and diminishing water resources, cotton cultivation is increasingly shifting to rainfed areas, which alters seed yield and fiber quality. This experiment evaluated the effects of drought stress memory on yield and fiber traits in cotton (*Gossypium hirsutum* L., cv. Golestan). The study employed a split-plot factorial design where plants were exposed to drought stress multiple times (simulating rainfed conditions and 33%, 66%, and 100% of field capacity (FC)). Results indicated that the activation of drought stress memory equipped plants with improved defenses against water deficit. Notably, this was observed through increased epicuticular wax accumulation on the seed coat, higher total phenol content, and elevated levels of secondary metabolites such as glycine betaine and protein in seeds. Furthermore, key agronomic traits like lint percentage, 30-boll weight, and total yield under the severe stress treatment (S33) were significantly higher in plants that had experienced prior stress compared to naïve plants. It can be concluded that subjecting cotton plants to 33% FC irrigation not only reduces their water requirements but also enhances seed tolerance, priming the next generation for improved performance under water-limited conditions. Therefore, stress memory serves as a crucial tolerance mechanism, enhancing the physiological preparedness of seeds for subsequent encounters with drought.

Keywords: Cottonseed, Drought stress, Memory stress, Phenol, Protein

1. Introduction

Drought is widely recognized as a major threat to ecosystems under global climate change (34). Approximately 45% of the world's cultivated area is subject to frequent and continuous drought, making it one of the most destructive abiotic stresses globally and a significant cause of crop production losses (26, 23, 33). Among environmental stresses, drought exerts some of the most severe negative effects on plant growth and development, often leading to substantial reductions in productivity (14). Cotton (*Gossypium hirsutum* L.) is a vital fiber and oilseed crop that provides 35% of the world's total fiber (1). Although it is considered a drought-tolerant, glycophytic species, its growth and productivity are adversely affected under severe stress conditions, which significantly impacts fiber quality and yield (32). To adapt to environmental fluctuations, plants have evolved a phenomenon known as stress memory (30). Plants pre-exposed to stress can develop a stable "memory" of the event, enabling a stronger and faster

activation of their defense systems upon subsequent stress challenges—a process also described as priming or acclimation (4, 13). This involves complex, multi-level responses, including altered physiological signaling, the accumulation of protective metabolites, and epigenetic modifications, which ultimately facilitate adaptation (3, 30).

In cotton, drought stress reduces leaf water potential, which is crucial for fiber development. Since fiber elongation depends on cell turgor, drought-induced loss of turgidity negatively affects fiber strength, length, and uniformity. Stress occurring at any growth stage can cause severe losses in boll formation; however, drought during the flowering and boll development stages is particularly detrimental, reducing seed number, fiber length, and overall yield (21).

Early-season drought before flowering decreases the production of photosynthetic products, limiting the resources available for developing bolls and ultimately reducing whole-plant yield, a response that varies among genotypes (22). In summary, drought severely impairs reproductive development by reducing photosynthetic activity, impairing pollen tube function, limiting carbohydrate availability, and altering metabolic processes. This leads to a smaller leaf area, further curtailing photosynthesis and the production of essential assimilates (20).

Plant drought tolerance strategies involve numerous biological mechanisms at the cellular, organ, and whole-plant levels. At the molecular level, drought stimulates the accumulation of stress-related proteins and metabolites that help adjust physiological processes (5, 18, 24). For instance, drought stress can increase respiration intensity while reducing nutrient uptake, leading to a decrease in stored carbohydrates and an increase in protein content (29). Plants also protect their cellular structures from reactive oxygen species by producing antioxidant compounds, such as phenolics and carotenoids (10). The response of these compounds to stress can vary; for example, total phenolic content in leaves has been shown to decrease with increasing drought stress in sweet potato (25), while it increased in *Hypericum brasiliense* under water-deficit conditions (15). Given this context, the present investigation aimed to evaluate the physiological responses and yield stability of cotton resulting from long-term water deficiency stress memory.

2. Material and methods

An experiment was conducted to evaluate the effects of drought stress memory on yield and fiber quality in cotton (*Gossypium hirsutum* L., cv. Golestan). The study was carried out over three crop seasons (2016-2018) at the Hashemabad Research Station, located in the southeast corner of the Caspian Sea (3651' N latitude, 5416' E longitude, 13.3 m above sea level).

A split-plot factorial design with three replications was employed. The main plots consisted of four irrigation regimes: rain-fed (W0), 33% of field capacity (W1), 66% of field capacity (W2), and 100% of field capacity (W3). The sub-plots consisted of five seed sources, which were derived from plants subjected to stress in previous generations.

Seed Source Generation:

2016 Season (Primary Stress Exposure): Plants were subjected to the four irrigation treatments (W0, W1, W2, W3). Seeds harvested from these plants served as the primed sources for the following season.

2017 Season (Second Stress Exposure): Seeds from the 2016 season were sown and again subjected to the same irrigation treatments.

S20: Control seed from commercial sources (non-primed).

S21: Double-stressed seed (rain-fed treatment in both seasons).

S22: Double-stressed seed (33% FC treatment in both seasons).

S23: Double-stressed seed (66% FC treatment in both seasons).

S24: Double-stressed seed (100% FC treatment in both seasons).

2018 Season (Third Stress Exposure): Seeds from the 2017 season were sown and subjected to the irrigation treatments for a third time (fig 1).

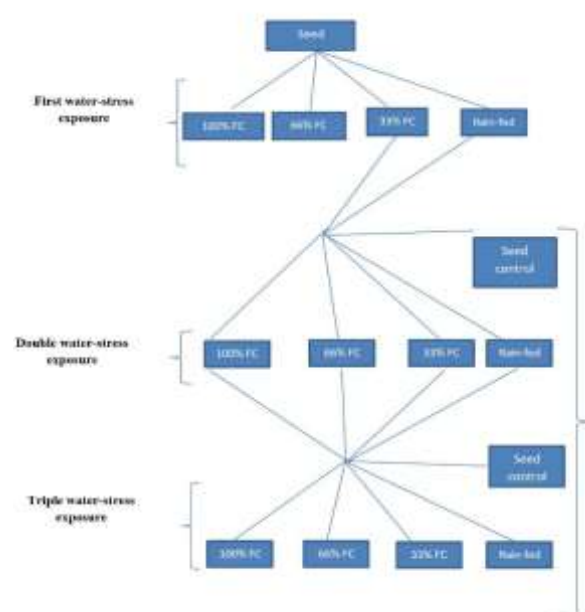


Figure 1. Research Plan-Seed production for experiment 2016 (crop season), Irrigation treatments and seed productions from single water-stress exposure for double water stress exposure (2017 crop season), Irrigation treatments and seed productions from the double water-stress exposure for the triple water-stress exposure (2018 crop season).

S30: Control seed (non-primed).

S31: Triple-stressed seed (rain-fed treatment for three seasons).

S32: Triple-stressed seed (33% FC treatment for three seasons).

S33: Triple-stressed seed (66% FC treatment for three seasons).

S34: Triple-stressed seed (100% FC treatment for three seasons).

To investigate the impact of drought on reproductive growth, the designated irrigation treatments were applied during the flowering stage using a drip system. Irrigation water was supplied from a deep well with an electrical

conductivity of less than 1 dS m^{-1} , confirming its suitable quality for agriculture.

Calculation of required water:

The amount of water required was calculated using weighted soil sampling before irrigation in the control treatment and with the help of formula (1) (6).

$$(1) \text{ } d_x = \text{RAW} = \text{MAD} * D_z * \rho_b * (\text{Fc} - \text{PWP}) * 10 / 100$$

d_x = Readily Available Moisture / Net Irrigation Application Depth (mm)

MAD = Management Allowable soil water Depletion (%)

D_z = Effective Root Depth (Cm)

ρ_b = Soil Bulk Density (g/Cm³)

FC = water content at Field capacity (FC) % weight

PWP = water content at Permanent Wilting Point (WP) % weight

After determining the required irrigation water depth, the irrigation system was started and the pump pressure was set to one atmosphere (10 meters). 10 minutes after the start of irrigation (ensuring that the pipes and irrigation strips/tips are full), small containers were placed under a number of drippers in different plots. After a period of three minutes, the volume of collected water was measured with the help of a graduated cylinder in the said containers. Finally, the average volume of falling water was calculated and the flow rate of the droplets was determined. According to the surface covered by each strip or dropper, the duration of irrigation was also calculated by formula (2):

$$(2) \text{ } t = d_x / q$$

t = duration of irrigation (h)

d_x = Readily Available Moisture / Net Irrigation Application Depth (mm) (from formula 1)

q = the intensity of water falling from the dripper (mm/h)

Determining the quantity (concentration) Protein Content of Seed

In this experiment, the amount of protein in the resulting samples was determined using the method of Bradford (1976).

For this purpose, the kit purchased from Bio-Rad was used. In this method, serum albumin (BSA) was used as a standard in different concentrations. To prepare the standard, first, the protein concentration determination kit A 20% diluted amount was added in separate tubes to albumin concentration (BSA) of 0, 2, 4, 8, 12, 16, and 18 microliters of the mother solution, 1 microgram per microliter of albumin for every 1 milliliter of Bradford solution. The final volume was considered 1014 μl . After at least 5 minutes of protein addition, the absorbance value of the standard samples was read at a wavelength of 595 nm using a spectrophotometer (Cary Varian-300). After drawing the standard curve and calculating the regression equation with R², 99% of

those tested plant samples were dissolved in a similar concentration of Bradford solution.

And the amount of protein concentration was determined. In order to determine the deviation from the standard and the error caused by the spectrophotometric method, the average of the data obtained from 2 steps was considered as the final concentration of the samples.

Determination of the Total Phenolic Content

The measurement of phenolic substances was done by the of Follen-Ciocalto method (10).

In this method, 100 μl of Folin-Ciocalto 0.2 normal, 15 μl of extract solution dissolved in 80% ethanol (final concentration: 50 $\mu\text{g/ml}$) and 585 microliters of distilled water were mixed together. After one minute, 300 μl of 5.7% sodium carbonate was added to this mixture and after the sample was placed in a dark environment for 30 minutes and the absorbance of the samples was read at a wavelength of 725 nm.

Determining Glycine Betaine Content of Seed

To measure the amount of glycine betaine by the method of Arakawa et al (1990) by using 1 grams of dry plant matter and sulfuric acid 2 normal dichloroethane and the potassium iodide reagent was used in the standard curve and absorbed at 365 nm wavelength. sulfuric acid was used as a blank.

Determining Epicuticular Wax of Seed

The concentration of wax in seed was determined based on the gradient of color change when potassium dichromate acid reacts with cuticular wax. Finally, its absorbance was read using a spectrophotometer at a wavelength of 590 nm (16).

lint percentage

After harvesting the entire crop of each plot, the fibers are separated from the cotton seeds and after weighing the weight of the fibers and cotton seeds with a sensitive scale, lint percentage is calculated from the following formula (Institute of Record Research and Seed and Seedling Certificate, 2009):

lint percentage

$$= \frac{\text{fiber weight}}{\text{Cotton seed weight} + \text{fiber weight}} \times 100$$

Calculations and statistical analysis:

At the end, the obtained data were analyzed using SAS software, and comparison of means was done with Duncan's multi-range test at the 5% level. EXCEL program was used to draw graphs and tables.

Results

Effect of different irrigation levels and seed sources on the 30- bolls weight

Analysis of variance indicated that for plants derived from double-stressed seeds (second exposure), the 30-boll weight was significantly affected ($p < 0.05$) by the interaction between irrigation level and seed source (Table 1). Mean comparison revealed that the highest 30-

boll weight was achieved by the S21 seed source under 66% field capacity (FC) irrigation, while the lowest was observed in the control seed source (S20) under rain-fed conditions (Fig2). For plants from triple-stressed seeds (third exposure), 30-boll weight was significantly influenced ($p < 0.01$) by the main effect of irrigation level alone (Table 1).

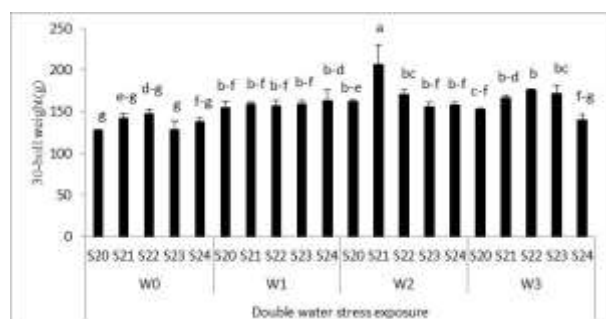


Figure 2. 30- boll weight (g) in double water stress in exposure

Effect of Irrigation Levels and Seed Sources on Total Yield

The interaction between irrigation level and seed source was not significant for total yield (Table 1). However, yield was significantly affected ($p < 0.01$) by the main effect of irrigation level. Furthermore, a significant interaction ($p < 0.05$) was found between irrigation level and triple-stressed seed sources. The highest total yield was recorded for the S32 seed source under 33% FC irrigation, whereas the lowest yield was observed for S31 under full irrigation (100% FC) (Figure 3). Under rain-fed conditions, the S32 source also yielded the highest, while S31 yielded the lowest.

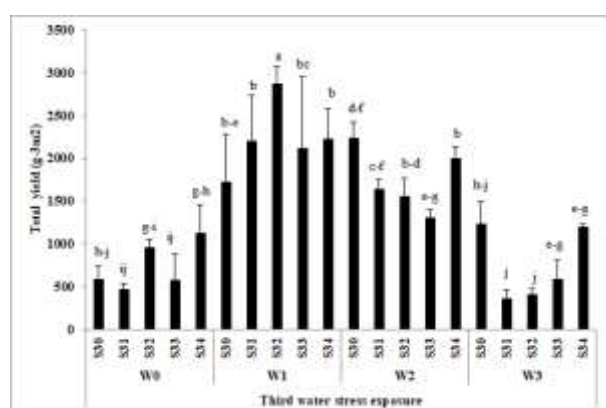


Figure 3. Total yield ($\text{g} \cdot \text{m}^{-2}$) in third water stress in exposure

Effect of Irrigation Levels and Seed Sources on Lint Percentage

The interaction between irrigation level and seed source had a significant effect ($p < 0.01$) on lint percentage in

plants from double-stressed seeds, but not in those from triple-stressed seeds (Table 1). Lint percentage was also significantly influenced ($p < 0.01$) by the main effect of irrigation level for both stress groups. For triple-stressed plants, a significant difference ($p < 0.05$) was observed among seed sources. The mean comparison showed that under double stress, the highest lint percentage was associated with the S22 source under 33% FC irrigation, while the lowest was found under 100% FC irrigation (Fig4).

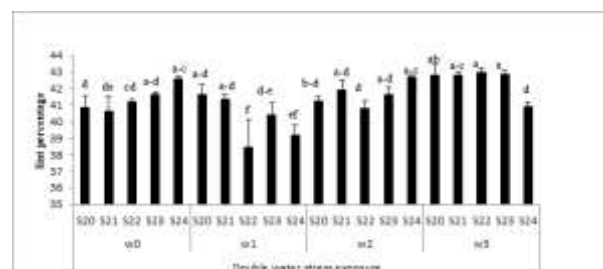


Figure 4. lint percentage in Double water stress in exposure

Effect on Glycine Betaine Content of Leaf

Second Year (Double Stress): glycine betaine content of Leaf was significantly affected by both the interaction of irrigation and seed source ($p < 0.01$) and the main effect of irrigation level ($p < 0.05$) (Table 2). The highest content was found in the S24 source under 66% FC irrigation, and the lowest in S22 under rain-fed conditions (Fig 5).

Third Year (Triple Stress): Content was significantly affected by the interaction between irrigation level and seed source ($p < 0.01$) (Table 2). The highest concentration was in the control source (S30) under 33% FC, and the lowest was also in S30 under rain-fed conditions (Fig 5).

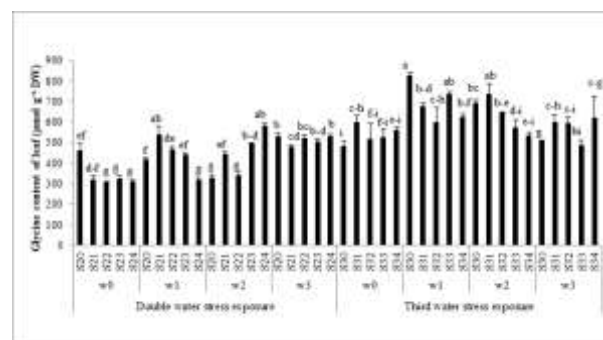


Figure 5. Glycine content of leaf ($\mu\text{mol g}^{-1} \text{DW}$) in double and third water stress in exposure

Effect Glycine Betaine Content on Seed

The interaction between irrigation level and seed source significantly affected ($p < 0.01$) glycine betaine content on Seed in both double- and triple-stressed plants (Table 2). The main effect of irrigation was also significant ($p < 0.01$). For double-stressed plants, the highest content was in the S21 source under 100% FC irrigation, while the lowest was in the control (S20) under the same irrigation (Fig 6). For triple-stressed plants, the highest concentration was in S33 under 66% FC irrigation (statistically similar to S30 under rain-fed conditions), and the lowest was in S31 under 100% FC irrigation (Fig 6).

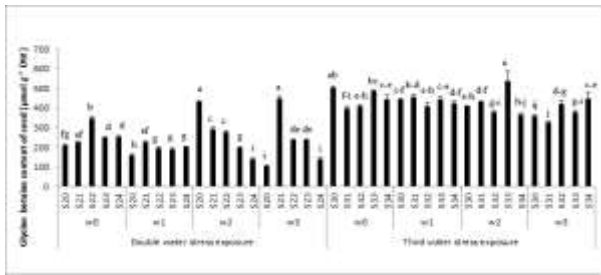


Figure 6. Glycine betaine content of seed ($\mu\text{mol g}^{-1}$ DW) in double and third water stress in exposure

Effect on Total Phenolic Content

Total phenolic content was significantly influenced by the interaction between irrigation level and seed source ($p < 0.01$) for both stress groups (Table 2). The main effects of irrigation and seed source were also significant ($p < 0.01$). In double-stressed plants, the highest phenol concentration was in S22 under rain-fed conditions (statistically similar to S24 under 100% FC), and the lowest was in S24 under rain-fed conditions (Figure 7). In triple-stressed plants, the highest concentration was in S33 under 33% FC irrigation, and the lowest was in S31 under 100% FC irrigation (Fig 7).

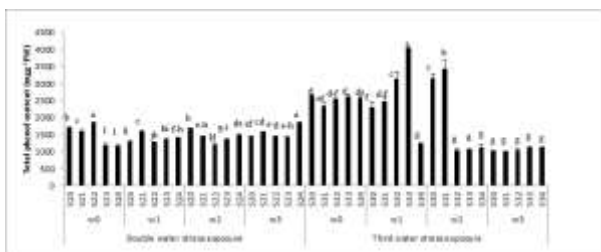


Figure 7. Total phenol content (mg g^{-1} FW) in double and third water stress in exposure

Effect on Epicuticular Wax Accumulation of Seed

A significant interaction ($p < 0.01$) between irrigation level and seed source was found for wax accumulation in

double-stressed plants, but not in triple-stressed plants (Table 2). Wax content was significantly affected by irrigation level for both groups ($p < 0.01$). For double-stressed plants, it was also significantly affected by seed source ($p < 0.01$). The highest wax content was in the control source (S20) under 66% FC irrigation, while the lowest was in S21 under full irrigation (not significantly different from S24 under 33% FC) (Fig8).

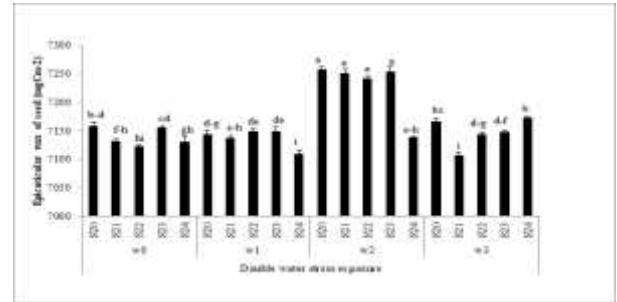


Figure 8. Epicuticular wax of seed (mg cm^{-2}) in double water stress in exposure

Effect on Protein Content of Seed

Seed protein content in double-stressed plants was significantly affected ($p < 0.01$) by the interaction between irrigation level and seed source (Table 2). Mean comparison showed that the highest protein content was in the S23 source under full irrigation (100% FC), while the lowest was in the control source (S20) under 66% FC irrigation (Fig 9).

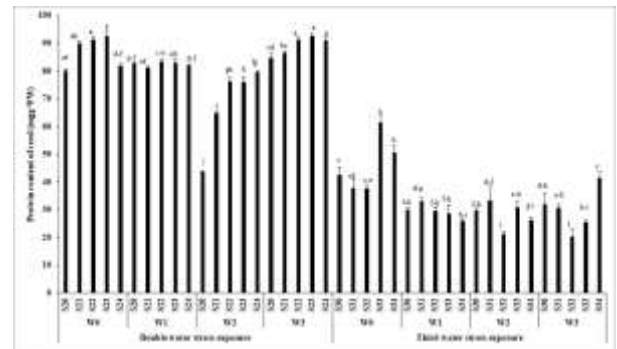


Figure 9. Protein content of seed (mg g^{-1} FW) in double and third water stress exposure

4. Discussion

In the present study, the observed reduction in boll weight under severe water deficit can be attributed to the detrimental impact of water stress on plant metabolism. This stress likely disrupts the translocation of photosynthetic compounds, including cellulose, to developing fibers, which is essential for their elongation and thickening (28). Conversely, under full irrigation conditions, excessive vegetative growth appears to occur

at the expense of reproductive growth. This shift in resource allocation can lead to a reduction in boll retention and an increase in boll shedding, ultimately resulting in a lower number of 30 bolls weight, as was observed in seeds from fully irrigated plants. This finding is consistent with previous reports of reduced boll load under drought conditions (27).

The significant yield reduction in the S31 seed treatment under full irrigation in the third year suggests that prior stress exposure can condition plants in a way that makes excessive water availability detrimental. In this case, it may have promoted vigorous but inefficient vegetative growth, negatively impacting boll number, boll weight, and final yield. In contrast, the yield increase in the S32 treatment under 33% field capacity (FC) irrigation demonstrates that a moderate water deficit can be beneficial. It appears to curb excessive vegetative growth, stimulate and sustain flower production, and enhance boll retention and weight, leading to a higher overall yield (9). This aligns with the findings of Sezner et al. (2015), who noted that drought stress applied during early growth stages can limit photosynthetic product availability for bolls, ultimately reducing yield in a genotype-dependent manner.

The lowest lint percentage was recorded for the S22 seeds under 33% FC irrigation, while the highest was found in seeds produced under 100% FC. This indicates that seeds developed under specific stress conditions may carry a physiological "memory" that influences their subsequent performance. When these seeds were cultivated again, they maintained a higher lint percentage under moderate to full irrigation (66% or 100% FC) but showed a reduction under more severe stress (rain-fed or 33% FC).

A higher concentration of the osmoprotectant glycine betaine was observed in plants that had undergone three stress exposures compared to those with two. This accumulation is a classic adaptive response, wherein glycine betaine acts to increase the osmotic pressure in aerial organs, enhancing water uptake and helping to maintain leaf cell turgor under drought conditions (17, 7). Glycine betaine is a highly effective compatible solute that safeguards plants from abiotic stress injury. Its accumulation enhances growth, survival, and tolerance by regulating various metabolic processes (19). The variation in glycine betaine concentration among seed treatments in this study likely reflects their differing inherited levels of drought tolerance.

The increase in phenolic compounds in triple-stressed plants represents a key antioxidant defense mechanism. Under drought stress, phenolic compounds act as potent scavengers of reactive oxygen species (ROS), thereby

stabilizing cell membranes and preventing the peroxidation of lipids, which would otherwise cause severe cellular damage (12).

Epicuticular wax accumulation on seeds was also found to be highly responsive to irrigation treatments, accumulating differently under stress compared to normal conditions. This wax layer is a critical barrier that reduces non-stomatal water loss.

The positive effect of water-deficit stress on protein content of seed observed in this research agrees with the findings of Foroud et al. (1993) in soybean. This increase may be an essential adaptive response to mitigate stress, playing a positive role in osmotic adjustment (2, 21). This phenomenon can be explained by the fact that drought stress often reduces the overall translocation of photosynthetic materials, disproportionately affecting starch synthesis in the endosperm. As the protein-rich bran and embryo constitute a larger proportion of the smaller grain, the percentage of protein increases. Furthermore, as noted by Noormohammadi et al. (2000), drought stress can increase respiration rates and reduce nutrient uptake, leading to a decline in stored carbohydrates and a consequent relative increase in protein content.

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Table 1. Results of analysis of variance of different irrigation treatments and seed source treatments on the studied traits							
Source	DF	30- bolls weight		Total yield		Lint percentage	
		Double water stress	Third water stress	Double water stress	Third water stress	Double water stress	Third water stress
rep	2	72.46	699.23	120.78	178.31	0.46	11.03
w	3	3131.74**	4296.89**	2135.96**	1195.76**	13.08**	222.47**
rep*w	6	294.21	469.51	42.28	96.46	0.81	5.92
g	4	880.68*	489.70 ^{ns}	142.44 ^{ns}	105.62 ^{ns}	1.48 ^{ns}	14.42*
w*g	12	455.73*	331.86 ^{ns}	82.52 ^{ns}	102.88*	3.28**	2.76 ^{ns}
error	32	149.42	290.70	58.72	45.18	31.63	164.87
Cv(%)		7.76	13.18	18.32	19.68	2.40	6.12

Table 2. Continued results of analysis of variance of different irrigation treatments and seed source treatments on the studied traits

Source	DF	Glycine betaine content of leaf		Glycine betaine content of seed		Total Phenol		Epicuticular wax of seed		Protein content of seed	
		Double water stress	Third water stress	Double water stress	Third water stress	Double water stress	Third water stress	Double water stress	Third water stress	Double water stress	Third water stress
rep	2	696.14	1705.35	183.80	118.73	3724.42	15213.44	37.15	1633.85	2.46	9.92
w	3	71003.14**	76287.68**	15394.12**	10419.55**	75962.54**	7868921.29**	28616.07**	11994.27**	1376.84**	1069.14**
rep*w	6	215.08 ^{ns}	4165.83 ^{ns}	142.36 ^{ns}	585.59 ^{ns}	2227.11 ^{ns}	21932.52 ^{ns}	83.97 ^{ns}	545.98 ^{ns}	2.52	22.86
g	4	2366.12*	11805.88 ^{ns}	23383.40**	6904.01**	83787.17**	1347471.45**	3651.12**	132.98 ^{ns}	351.40**	168.13**
w*g	12	21683.14**	16887.42**	25717.90**	7201.82**	144536.05**	2128787.87**	2577.18**	939.69 ^{ns}	144.37**	136.04**
error	32	28139.38	164075.88	5386.26	27628.72	57392.49	879727.45	3029.95	13635.65	4.52	16.87
Cv(%)		6.83	11.78	5.45	6.94	2.88	8.08	0.14	0.29	2.60	12.31

**Significance at the 1% level, *Significance at the 5% level ns Non-significance

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