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Divergent Regulation of Trehalose Accumulation by Exogenous Proline in Drought-Stressed Sorghum and Maize

Tülay Turgut Genç[1,a,*], Timuçin Taş[2,b]

[1]*Çanakkale Onsekiz Mart University, Faculty of Science, Department of Biology, 17100, Çanakkale, Türkiye*
[2]*Balıkesir University, Kepsut Vocational School, Division of Veterinary Medicine, 10660, Balıkesir, Türkiye*
[*]*Corresponding author*

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ABSTRACT

This study investigated the effects of varying water availability and exogenous proline application on trehalose accumulation in two economically important crops, sorghum (*Sorghum bicolor* L.) and maize (*Zea mays* L.), which exhibit distinct drought tolerance strategies. The experiment was conducted under controlled conditions with four water availability levels (100%, 75%, 50% and 25%) and three proline concentrations (200, 400, and 600 mg/L). Leaf area and leaf curling were also measured to verify the successful establishment of drought stress prior to biochemical analyses. Trehalose content was determined at the V8 growth stage using a modified enzymatic assay. Results showed a reduction in leaf area with decreasing water availability and a concurrent rise in leaf curling, confirming effective induction of drought stress. Maize displayed stronger curling responses, whereas sorghum largely maintained leaf structural integrity. Regarding trehalose, sorghum naturally accumulated higher levels under severe drought stress without proline, indicating a robust innate stress response. In contrast, maize exhibited lower trehalose levels under the same conditions but showed a marked increase with proline application, suggesting a greater reliance on external osmoprotectants. Under moderate and mild stress, proline enhanced trehalose accumulation in both crops, though the response was more consistent in sorghum. Interestingly, under optimal water conditions, proline application increased trehalose levels in sorghum but had minimal effect on maize, highlighting differences in the metabolic utilization of proline. Overall, these findings underscore the importance of tailoring proline application strategies based on crop-specific stress responses and environmental conditions to optimize drought resilience. This study provides valuable insights into the complex interplay between water availability, proline, and trehalose metabolism, offering potential pathways for improving crop stress tolerance in drought-prone regions.

[a] tturgutgenc@comu.edu.tr https://orcid.org/0000-0001-5074-3572 | [b] timucin.tas@balikesir.edu.tr https://orcid.org/0000-0002-2144-9064

Introduction

Plants are exposed to various abiotic stresses, such as drought, salinity, and extreme temperatures, which negatively impact their growth and productivity. To counteract these adverse conditions, plants have developed intricate mechanisms involving osmolytes, stress-related proteins, and signaling pathways to enhance their survival. Among these osmolytes, trehalose and proline play crucial roles in plant stress tolerance and metabolic regulation (Hayat et al., 2012; Eh et al., 2024).

Trehalose is a non-reducing disaccharide composed of two glucose units linked by an α,α-1,1-glycosidic bond. Trehalose serves multiple functions, such as energy storage, stress protection, signaling and metabolism regulation across various organisms such as bacteria, fungi, insects and plants, except vertebrates. (Elbein et al., 2003; Perfect et al., 2017; Lunn et al., 2014; Becker et al., 1996; Kosar et al., 2018; Jagdale & Grewal, 2003). Its protective attributes are often associated with membrane stabilization, protein protection, and regulation of the osmotic balance (Fernandez et al., 2010; Abdallah et al., 2016; Ali & Ashraf, 2011). It also serves as a signaling molecule that modulates stress-responsive gene expression (Liu et al., 2020; Paul et al., 2018; Eh et al., 2024). Increased trehalose accumulation is commonly observed in plants subjected to drought stress, suggesting its essential role in osmoprotection and desiccation tolerance (Iturriaga et al., 2006). Studies have demonstrated that trehalose biosynthesis genes, such as TPS (Trehalose-6-phosphate synthase) and TPP (Trehalose-6-phosphate phosphatase), are upregulated under drought conditions, leading to enhanced stress resilience (Acosta-Perez et al., 2020; Habibur Rahman Pramanik and Imai 2005; El-Bashiti et al., 2005; Morabito et al., 2021).

Proline, an amino acid, is similarly known to accumulate under stress conditions in plants (Hare et al., 1998; Munns, 2005; Hayat et al., 2012). It functions as an osmoprotectant, radical scavenger, and a regulator of redox status (Ashraf & Foolad, 2007; Tripathi & Gaur, 2004). Exogenous application of proline, commonly through foliar spraying, accelerate its build up in leaf tissues and also enhance stress tolerance (Wang et al., 2009; Roy et al., 1993; Li et al., 2011). However, the relationship between externally applied proline under stress and trehalose accumulation remains insufficiently explored, especially in key cereals such as maize and sorghum.

Maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* L.) have significant economic importance as staple crops and as livestock feed worldwide. Both can grow in similar regions but exhibit distinct responses to drought stress, making them ideal models for studying the effects of water availability and osmoprotectants like proline. Maize generally has higher yields under optimal conditions but is highly sensitive to drought, especially during flowering. In contrast, sorghum is more drought-tolerant due to its deep root system, osmotic adjustment and delayed reproductive development, making it well-suited arid and semi-arid environments (Baloch et al., 2023).

In drought-prone areas, water availability remains a critical factor limiting crop productivity. Understanding how plants respond to combined water and proline treatments is essential for developing sustainable management practices. Based on the known differences in drought response between the two crops, we hypothesize that (i) decreasing water availability would enhance trehalose accumulation in both species as a drought-induced protective response, and (ii) exogenous proline would differentially modulate trehalose biosynthesis in sorghum and maize depending on drought severity. Therefore, this study aimed to investigate how trehalose accumulation in maize and sorghum is influenced by varying water availability and exogenous proline applications.

Materials and Methods

Plant Materials and Growth Conditions

The experiment was conducted in a controlled plant growth chamber with adjustable light (12 000 lux, 16-hour photoperiod), temperature (20/25 °C night/day), and relative humidity (65%). The 'Kale' (FAO-650-700) maize variety and the 'Erdurmuş' sweet sorghum variety were used in the study. After surface sterilization in 5% sodium hypochlorite for 10 minutes, they were rinsed thoroughly in distilled water and dried on filter paper (Naeem et al., 2017). The soil used was fertile garden soil, sieved and mixed after removing plant residues and stones. Five seeds were planted in pots filled with 9.5 kg of soil, and after germination three seedlings per pot were retained. The experiment was set up in a completely randomized design (CRD) with three replications. Each treatment combination consisted of three pots, and each pot contained three plants. Measurements from the three plants within the same pot were averaged and treated as one independent biological replicate, ensuring that the pot served as the experimental unit and avoiding pseudoreplication. All pots were randomly positioned at the start of the experiment and rotated weekly in a clockwise manner among corner positions within the growth chamber to minimize microsite effects such as light, airflow, and temperature variation. The main pots consisted of drought stress treatments categorized as follows: as optimal condition (100% water availability), mild stress (75% water availability), moderate stress (50% water availability) and severe stress (25% water availability). Subplots included proline treatments (P0: control no proline, P200: 200 mg/L, P400: 400 mg/L and P600: 600 mg/L). Before sowing, 800 mg P_2O_5/kg and 1000 K_2O/kg were mixed into the soil, and 1600 mg N/kg was applied in three stages (V2, V4 and V6 growth stages). The experiment was conducted between April 15 and June 15, 2024.

Application of Drought Stress and Proline

Drought stress was applied using gravimetric method. Before sowing, three pots were filled with 9.5 kg of soil, watered until saturation, and allowed to drain for four hours before recording their weight. The difference between wet and dry weight determined the 100% water holding capacity (WHC) (Li et al., 2024). Pots were weighted every three days, and water levels were maintained at 100%, 75%, 50%, and 25% WHC according to the respective drought treatments.

Proline (purity≥98.5%) was applied as a foliar spray to plant seedlings at the V2, V4, V6 and V8 growth stages in concentrations of 200 mg/L, 400 mg/L and 600 mg/L with a control receiving only water. To enhance adhesion, 0.1% Tween-20 was added to the solution (Kibria et al., 2016). Plants were fully sprayed, and measurements were taken at the V8 stage, three days after the final proline application, with averages calculated from three plants per pot. Leaf samples were collected at the V8 growth stage and weighed as 1gram of samples (Noein & Soleymani, 2022). The samples were first flash-frozen in liquid nitrogen and stored at -80°C for trehalose measurements.

Determination of Leaf-based Stress Indicators

Leaf area (LA) and leaf curling degree (LCD) were measured to evaluate drought-induced morphological changes and the potential mitigating effects of proline application. Leaf area was calculated by measuring the length and maximum width of fully developed leaves using the formula LA= leaf length (cm) X width (cm) X 0.75 (Plénet et al., 2000). Leaf curling was assessed visually from four directions and scored on a standardized 1-5 scale based on the UPOV guidelines, where 1 indicates fully open leaves and 5 represents fully curled, onion-shaped leaves (Bänziger et al., 2002). Together, these parameters served as morphological indicators of plant stress severity under varying water availability and proline treatments.

Trehalose Assay

Trehalose content of leaf samples was determined with the protocol described previously with the following modifications (Parrou & François, 1997). The weighted leaf samples (1 gr) were resuspended in 1 ml of 0.25 M Na_2CO_3 and 500 mg glass beads were added, then samples were incubated in boiling water to inactivate enzymes and proteins for 2 hours. During incubation the samples were vortexed every 30 min for 30 seconds. After boiling, the samples were centrifuged, and 250 µL supernatant was transferred to new tube including 150 µL 1M acetic acid and 600 µL 0.2 M sodium acetate buffer (pH: 5.2). The

mixture was incubated at 37 °C for 18 hours with 3mU of trehalase enzyme (Sigma T8778) to hydrolyze trehalose into glucose. As a control, an enzyme-free supernatant was included to measure residual glucose from other sources in leaf cells. The released glucose was quantified enzymatically using the glucose oxidase-peroxidase (GOD-POD) assay with a commercial kit (Fluitest®-GLU, Analyticon, Germany). Trehalose content was expressed as milligrams of trehalose equivalent per gram of fresh weight of plant sample (mg/g FW).

Statistical Analysis

Statistical analyses were performed using both one-way and two-way analysis of variance (ANOVA) depending on the objective of each comparison. One-way ANOVA was used to evaluated the effects of proline treatments with each water availability, particularly for trehalose measurements. When one-way ANOVA indicated significant differences ($p<0.05$), means were separated using Tukey's HSD post-hoc test. In addition, a two-way ANOVA (Water × proline) was conducted to assess the overall effects of water availability, proline concentration, and their interaction on leaf area, leaf curling, and trehalose accumulation. All data are presented as mean ± standard deviation (SD), and statistical significance was set at $p<0.05$.

Results

Leaf Area and Leaf Curling Responses Under Drought Stress

To verify whether drought stress was effectively induced in both plant species, changes in leaf area and leaf curling were evaluated under different irrigation levels and proline treatments. These morphological parameters were used as primary indicators of stress formation.

Decreasing water availability led to a marked reduction in leaf area in both maize and sorghum plants (Figure 1 and Figure 2).

In both species, leaf area progressively decreased with the severity of water limitation. Two-way ANOVA revealed that water availability had a highly significant main effect on leaf area in both species ($p<0.0001$), confirming that reduced irrigation strongly restricted leaf expansion and the development of photosynthetic surface area. No significant difference was observed between the two species in terms of the magnitude of reduction, suggesting that maize and sorghum exhibited similar responses to drought in leaf area. In contrast to the initial assumption, proline exhibited a small but statistically significant main effect on leaf area (sorghum $p<0.005$; maize $p<0.001$), although no water × proline interaction was detected, and proline did not lead to visually noticeable differences within individual irrigation levels.

Leaf curling results followed a similar trend both in maize and sorghum (Figure 3 and Figure 4). As water availability decreased, leaf curling values increased progressively. Two-way ANOVA similarly showed that water availability had a highly significant effect on leaf curling in both species ($p<0.0001$). In sorghum, curling also increased under limited irrigation but this pattern was not statistically significant within individual water levels, aligning the nonsignificant water × proline interaction. Interestingly, in sorghum, despite a noticeable reduction in leaf area, leaf curling did not increase markedly under 50% and 25% irrigation levels, suggesting that the species mitigated drought-induced water loss through structural or physiological adaptations rather than extensive morphological leaf rolling. However, a significant main effect of proline was detected for leaf curling, with proline reducing curling more strongly in maize ($p<0.001$) and more modestly in sorghum ($p<0.01$).

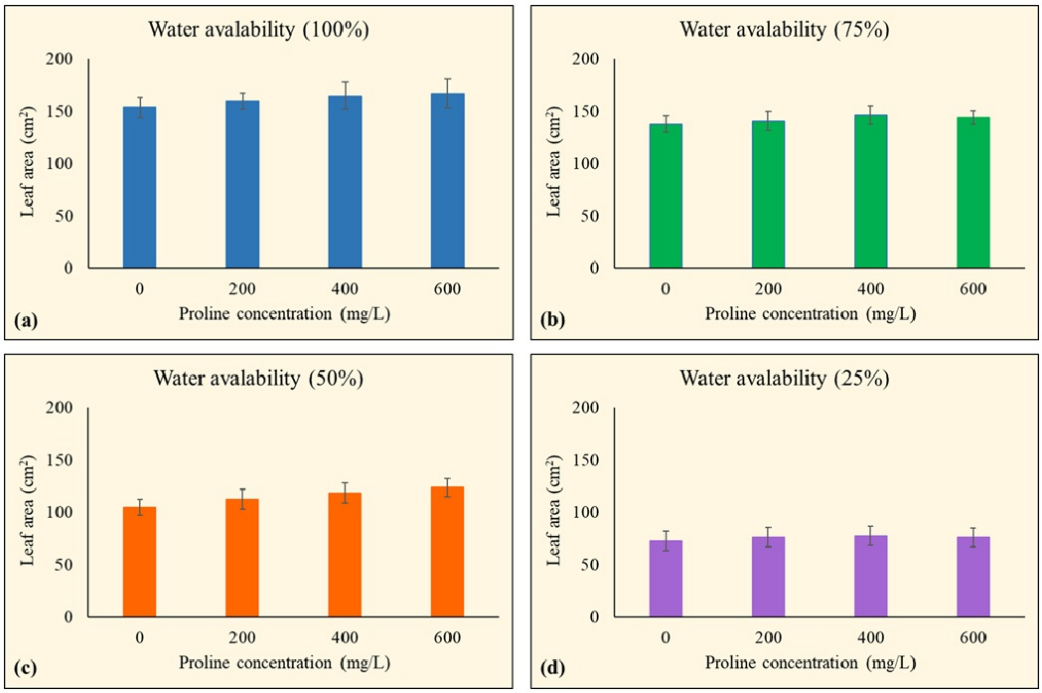

Figure 1. Leaf area of sorghum under different water availability levels combined with proline concentrations. Two-way ANOVA (Water × Proline) results are reported in the text.

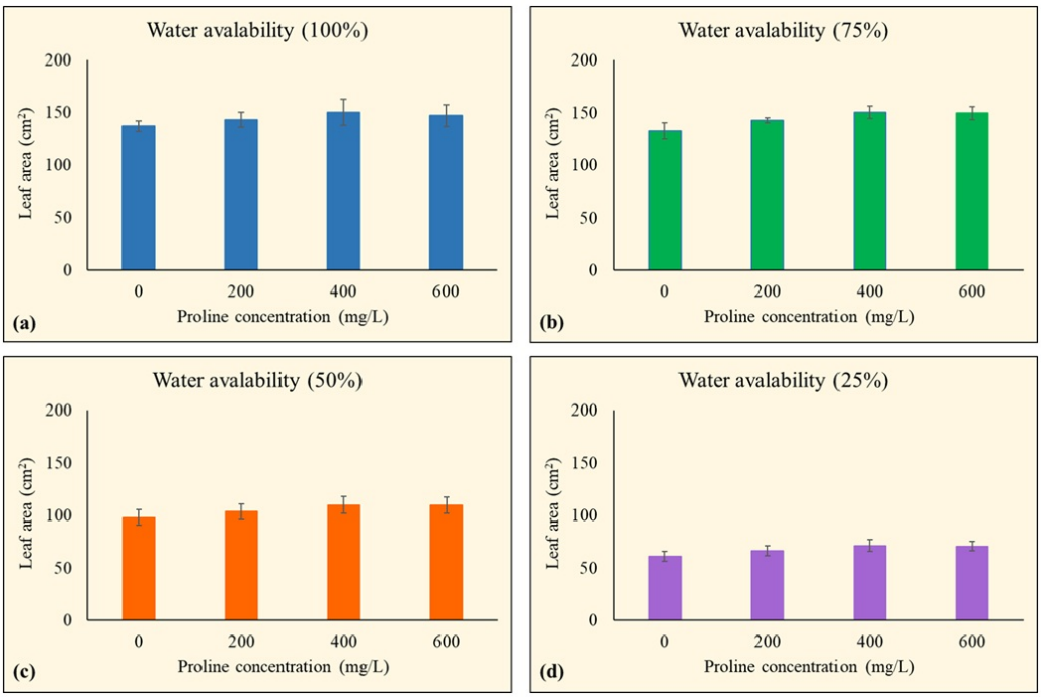

Figure 2. Leaf area of maize under different water availability levels combined with proline concentrations. Two-way ANOVA (Water × Proline) results are reported in the text.

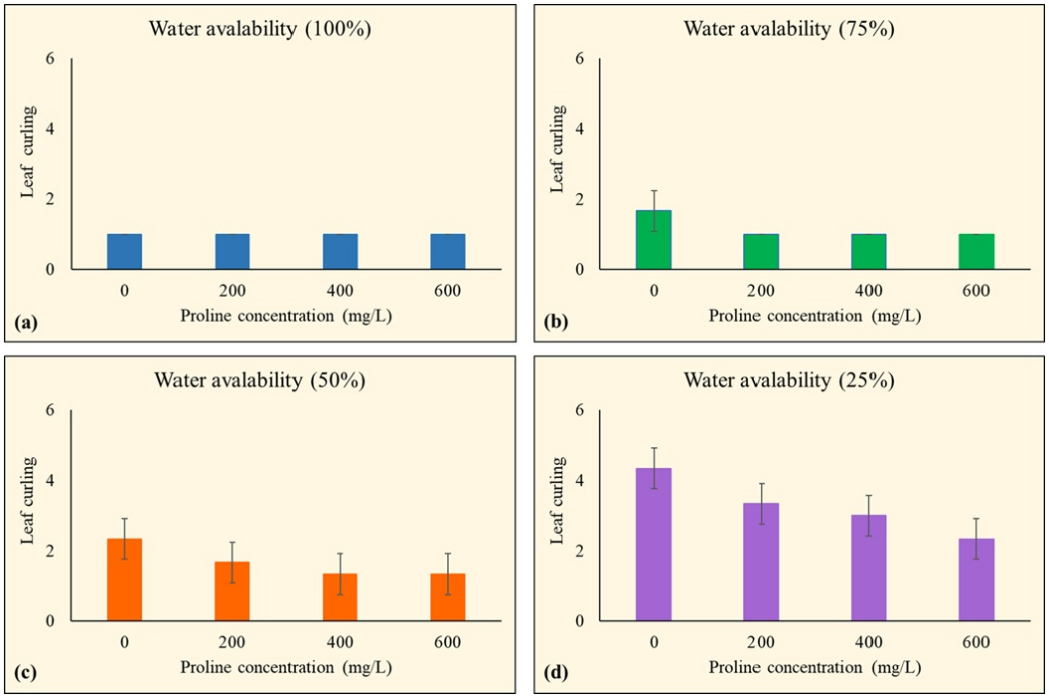

Figure 3. Leaf curling of sorghum under different water availability levels combined with proline concentrations. Two-way ANOVA (Water × Proline) results are reported in the text.

Overall, leaf area tended to decrease as leaf curling increased, under drought conditions, consistent with expected stress response, confirming that drought stress was successfully induced. When evaluated together, these two morphological parameters indicate that water limitation effectively established stress conditions in both maize and sorghum, providing a reliable physiological basis for subsequent trehalose accumulation analysis.

Trehalose accumulation in sorghum plant

Trehalose accumulation in sorghum exhibited significant variation in response to both water availability and exogenous proline application, and this response was strongly shaped by a significant water × proline interaction (Figure 5). Under well-watered conditions (100% water availability), trehalose content in control plants was 2.09 mg/g FW. Foliar proline application at 200, 400, 600 mg/L progressively enhanced trehalose accumulation, with levels rising to 3.89, 5.65 and 4.75 mg/g FW, respectively. Under mild drought stress (75% water availability), a similar promotive effect was observed. The trehalose concentration increased from 2.44 mg/g FW in control to 4.70 mg/g FW at the highest proline concentration.

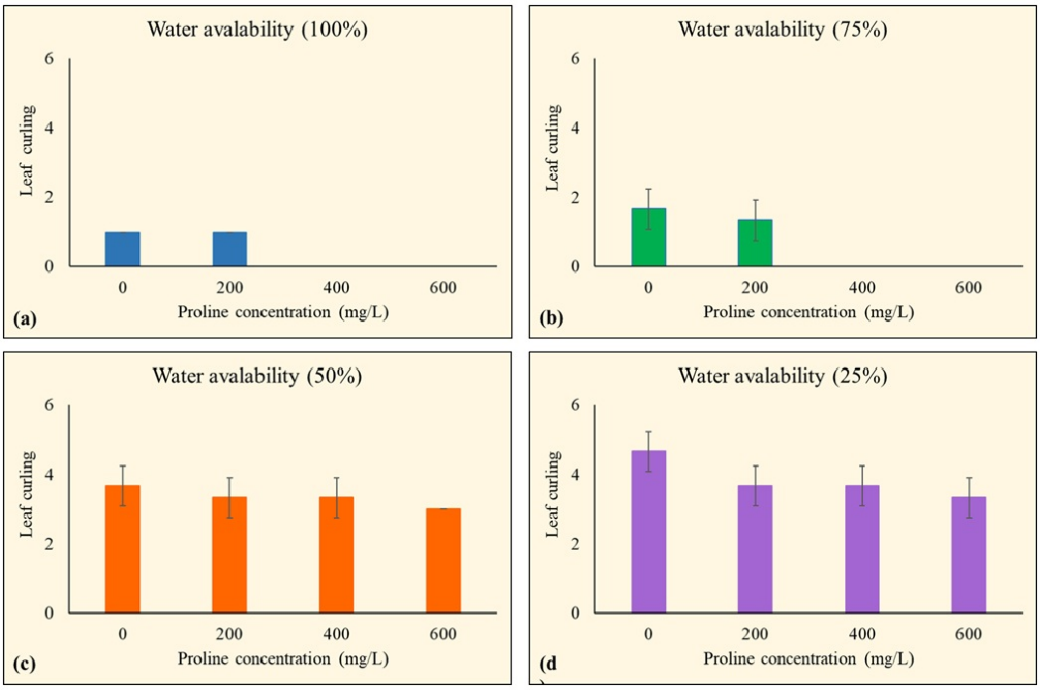

Figure 4. Leaf curling of maize under different water availability levels combined with proline concentrations. Two-way ANOVA (Water × Proline) results are reported in the text.

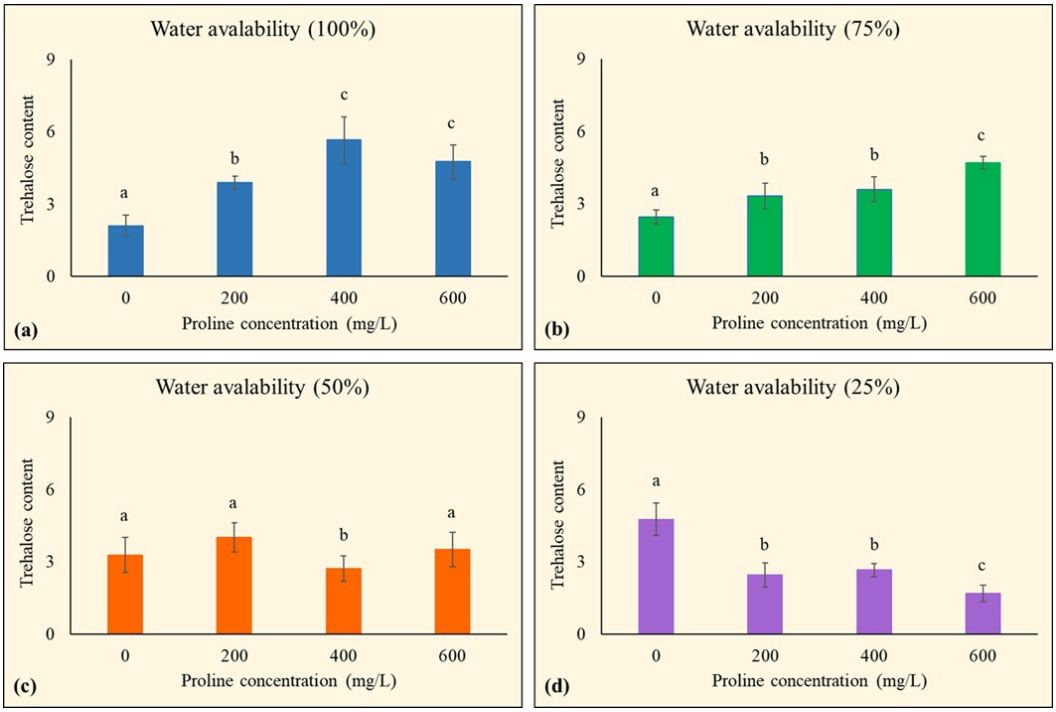

Figure 5. Trehalose content in sorghum under different water availability levels combined with proline concentrations.
Trehalose content was expressed as mg/g fresh weight (FW). Different letters indicate significant differences among proline treatments within each water level (one-way ANOVA, Tukey test, $p<0.05$). Two-way ANOVA results: Water effect: $F=20.76$, $p<0.000001$; Proline effect: $F=4.99$, $p=0.0059$; Water × Proline interaction: $F=34.51$, $p<0.000001$.

This trend shifted under moderate drought (50% water availability). Low proline concentration slightly increased trehalose (4.01 mg/g FW compared to the control at 3.28 mg/g FW), whereas higher proline doses caused a reduction, most notably at 400 mg/L (2.72 mg/g FW, $p<0.05$). Notably, a contrasting pattern emerged under severe drought stress (25% water availability). The highest trehalose accumulation was recorded in the prolin-untreated control plants (4.76 mg/g FW). In contrast, all proline treatments lowered trehalose content, with the greatest reduction observed at 600 mg/L (1.68 mg/g FW, $p<0.001$), indicating a strong suppressive effect of proline under extreme water deficit.

Trehalose accumulation in maize plant

Trehalose accumulation in maize was significantly influenced by water availability and exogenous proline application (Figure 6).

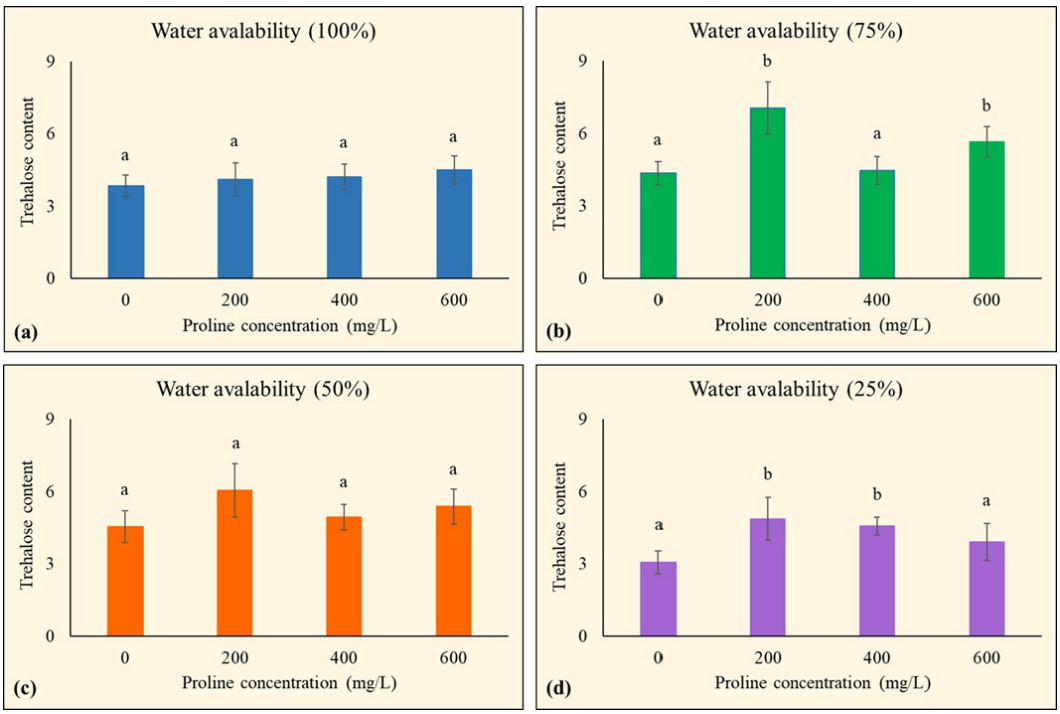

Figure 6. Trehalose content in maize under different water availability levels combined with proline concentrations.
Trehalose content was expressed as mg/g fresh weight (FW). Different letters indicate significant differences among proline treatments within each water level (one-way ANOVA, Tukey test, $p<0.05$). Two-way ANOVA results: Water effect: $F=19.66$, $p<0.0001$; Proline effect: $F=18.10$, $p<0.0001$; Water × Proline interaction: $F=3.60$, $p<0.002$.

Under optimal watering conditions, trehalose content in control plants was 3.83 mg/g FW and proline application induced a modest, concentration-dependent increase (reaching 4.50 mg/g FW at the highest proline dose), although the magnitude of this response varied significantly with water availability. As mild water deficit was imposed a pronounced increase in trehalose accumulation was triggered by proline. Relative to the control (4.35 mg/g FW), the 200 mg/L proline treatment caused a substantial rise in trehalose accumulation (7.06 mg/g FW, $p<0.0019$). The 600 mg/L treatment also elevated trehalose levels (5.65 mg/g FW), although this specific comparison was not statistically significant despite a significant overall proline effect. This enhancing effect of proline persisted under moderate drought stress. The highest trehalose accumulation was observed with 200 mg/L proline (6.04 mg/g FW, over the control at 4.55 mg/g FW), consistent with the significant main effect of proline and the significant water proline × interaction. Under severe drought stress, the lowest native trehalose level was recorded in the control plants (3.06 mg/g FW). Proline application markedly boosted trehalose accumulation, with 200 mg/L producing the strongest increase (4.87 mg/g FW, $p<0.001$), demonstrating a critical role for exogenous osmoprotectants in mitigating severe stress in maize.

Discussion

Morphological responses to drought

The observed reductions in leaf area and increases in leaf curling under stress are well-established morphological adaptations that serve to minimize water loss and preserve plant water status. In this study, both maize and sorghum exhibited a progressive decrease in leaf area with increasing drought severity, confirming the effective induction of stress conditions. Leaf curling responses were generally more pronounced in maize than in sorghum, particularly under moderate and severe drought, reflecting differences in the adaptive morphology of two species. Sorghum's ability to maintain leaf structural integrity even under severe drought likely stems from its inherent xerophytic traits, such as a thicker cuticle, reduced stomatal density, and efficient osmotic adjustment (Wang et al., 2022; Baloch et al., 2023). The absence of significant proline effects on these morphological indicators implies that foliar proline supplementation primarily functions at the metabolic and cellular level, rather than by altering visible structural adaptations, which are consistent with previous reports (Kibria et al., 2016; Li et al., 2024). Collectively, these contrasting morphological responses highlight the differential drought tolerance mechanisms between maize and sorghum, with the latter exhibiting superior morphological stability under water limitation.

Trehalose and proline interplay in sorghum

The results for sorghum revealed a complex, dose-dependent interaction between water availability and proline application in regulating trehalose accumulation. As expected, trehalose content increased under drought stress in the absence of proline, consistent with its role as a key osmoprotectant in abiotic stress tolerance, particularly in resilient species like sorghum (Garg et al., 2002; Elbein et al., 2003; Eh et al., 2024). This accumulation is a recognized protective mechanism to stabilize cellular structures and maintain osmotic balance under water deficit (Wang et al., 2022).

A key finding was that under severe drought stress, proline application significantly reduced trehalose accumulation compared to the proline-untreated control.

This pattern may indicate that under severe drought, plants possibly relied more on externally supplied proline as an osmoprotectant, which coincided with lower trehalose levels in proline-treated plants. However, the underlying biochemical mechanisms remain to be clarified in future studies. Under moderate water deficiency, proline application did not produce a consistent enhancing effect on trehalose levels, instead higher doses (particularly 400 mg/L) caused a significant reduction. Interestingly, under mild stress and well-watered conditions (≥75% water availability), proline application markedly enhanced trehalose accumulation. This suggests that proline not only mitigates stress but may also actively promote growth and metabolic activity when water is non-limiting, aligning with its reported involvement in various biochemical and physiological processes beyond osmoregulation (Kishor & Sreenivasulu, 2014; Funck et al., 2012; Szabados & Savoure, 2010). The response was concentration-dependent, with 600 mg/L performing best at 75% water availability and 400 mg/L being most effective at 100% water availability, indicating a non-linear relationship and a potential threshold for proline's efficacy. Overall, proline plays a critical role in modulating sorghum's metabolic response, but its effect -whether stimulatory or suppressive on trehalose- is contingent upon both its concentration and the prevailing water conditions.

Trehalose and proline interplay in maize

In maize, trehalose accumulation was shaped by a clear interaction between water availability and proline application, revealing a reliance on external osmoprotectant support. Under severe drought stress, innate trehalose levels in untreated plants were low, a finding consistent with maize's less efficient innate trehalose biosynthesis pathway compared to drought-tolerant cereals like sorghum (Ali & Ashraf, 2011; Acosta-Perez et al., 2020). This inherent limitation likely stems from differences in the expression or activity of key biosynthetic enzymes, such as trehalose-6-phosphate under acute stress. Critically, and in contrast to sorghum, proline supplementation under severe stress tended to increase trehalose accumulation in maize, although these increases were not statistically significant. This trend suggests that exogenous proline does not merely act as an alternative osmoprotectant but may help facilitate, or could be associated with increasing in trehalose accumulation in this stress-sensitive species, although the underlying biosynthetic mechanisms remain unclear. This is consistent with observations that exogenous proline can enhance osmoprotectant synthesis in stress-sensitive plants (Kishor et al., 2015; Semida et al., 2020). This suggests that proline plays a crucial role in augmenting the plant's limited innate capacity to cope with extreme water scarcity. This effect appeared to diminish at the highest proline concentration, indicating a potential threshold beyond which excessive proline may disrupt metabolic balance or trigger feedback inhibition.

Under moderate and mild water deficiency, the effect of proline was more nuanced. Trehalose accumulation was generally higher than under severe stress, yet, the responses were more variable and often not statistically significant compared to the control, particularly at the 400 mg/L concentration. This implies that a moderate water deficit alone may not induce sufficient stress to consistently potentiate a strong, proline-mediated trehalose response. The specific effectiveness of the 400 mg/L treatment highlights that the relationship is not purely dose-dependent but involves specific concentration thresholds that interact with the plant's stress perception and signaling networks. The fact that the 200 mg/L concentration was consistently the most effective under these conditions underscores the dose-dependent nature of proline's effect, where optimal, rather than maximal, concentration is key to bolstering stress tolerance. Furthermore, under optimal water conditions, proline application resulted in only a marginal increase in trehalose. This strongly supports the concept that trehalose biosynthesis in maize is primarily an inducible, adaptive response to water limitation rather than a constitutive metabolic process. The minimal response to proline in the absence of significant stress suggests that the signaling pathways which link proline to trehalose accumulation are predominantly activated under hydric stress.

Overall, the findings indicate that trehalose accumulation in maize is responsive to both water availability and proline concentration, but the relationship is complex and dependent on the degree of water availability and proline concentration. While proline appears to enhance trehalose accumulation under water-limited conditions, its effect diminishes under optimal conditions. Future studies should explore the underlying mechanisms regulating proline-induced trehalose synthesis and its interaction with other metabolic pathways involved in plant water stress responses.

Comparison analysis and implications

The results of this study clearly delineate the contrasting drought survival strategies of sorghum and maize, which are further modulated by exogenous proline. Sorghum exemplifies a strategy of inherent resilience, characterized by a robust, constitutive accumulation of trehalose under severe drought stress without proline assistance. The finding that proline application suppresses this innate trehalose accumulation under severe stress reveals a sophisticated metabolic trade-off, where the plant may preferentially utilize the externally provided osmoprotectant, thereby conserving energy.

In contrast, maize employs a more facilitated tolerance strategy. Its low innate trehalose levels under severe stress underscore a deficient constitutive response mechanisms. Its capacity to show an upward trend in trehalose accumulation in response to proline supplementation suggests a potential reliance on external osmolyte support. This pattern may indicate that maize relies more heavily on externally supplied osmoprotectants, and proline application was associated with higher trehalose levels under stress; however, whether proline influences biosynthetic signaling requires further molecular investigation.

The divergence in proline utilization extends to non-stress conditions. Sorghum's ability to leverage proline to significantly elevate trehalose even under optimal watering suggests a broader role for this amino acid in its general metabolic efficiency and growth potential. Maize, however, shows minimal response, confining the proline-trehalose relationship primarily to stress contexts.

Conclusion

This study elucidates the contrasting drought resilience strategies of sorghum and maize, governed by their distinct metabolic interplay between proline and trehalose. Sorghum demonstrates a constitutive tolerance mechanism, maintaining high innate trehalose levels under severe drought, while maize exhibits a facilitated response in which trehalose accumulation shows a positive trend following proline supplementation, particularly under water-limited conditions. These physiological insights dictate crop-specific proline management it serves as a crucial rescue treatment for drought-sensitive maize but requires precise dosage in inherently tolerant sorghum to avoid disrupting its native osmoprotective balance. Optimizing proline as a biostimulant therefore demands a species-specific approach. Future research should decipher the molecular signaling underlying this proline-trehalose cross-talk, particularly the synergistic gene activation in maize and the suppressive regulation in sorghum, to pave the way for next-generation drought mitigation strategies in cereals.

Declarations

Ethical Approval Certificate

This article does not contain any studies with human participants or animals performed by any of the authors.

Author Contribution Statement

Tülay Turgut Genç: Data collection, methodology, investigation, formal analysis, and writing the original draft, review and editing

Timuçin Taş: Project administration, conceptualization, methodology, data collection, review and editing

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Conflict of Interest

The authors declare no conflict of interest.

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