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Exogenous applied proline may enhance the tolerance of sweet sorghum (*Sorghum bicolor* (L.) Moench) under water deficit stress

La aplicación exógena de prolina puede mejorar la tolerancia del sorgo dulce (*Sorghum bicolor* (L.) Moench) bajo estrés por déficit hídrico

A aplicação exógena de prolina pode aumentar a tolerância do sorgo doce (*Sorghum bicolor* (L.) Moench) sob estresse por déficit hídrico

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Abstract

Drought is a major abiotic stress that threatens global food security by reducing crop yield and quality. Foliar application of osmoprotectants such as proline offers a promising means to mitigate drought-induced damage. This study examined the effects of exogenous proline (P0, P200, P400, and P600 mg.L^{-1}), sorghum genotype, and their interaction on morphological, physiological, biochemical, forage quality, and microbial traits under different drought levels (I100, I75, I50, and I25). Proline application increased dry matter by over 100 % under medium to severe deficits and enhanced root dry weight by 90 % at 75 % water reduction. The strongest response occurred in chlorophyll content (SPAD), reflecting improved photosynthetic stability. Exogenous proline reduced leaf drying by 25 % and alleviated drought-related declines in forage quality, as evidenced by improvements in NDF, ADF, and ADL. It also boosted peroxidase activity more than superoxide dismutase and catalase, minimizing hydrogen peroxide (H_2O_2) toxicity and oxidative stress. Even under extreme drought (I25), proline maintained plant vigor and improved water-use efficiency by 25 - 40 % at the seedling stage. Compared with the control, leaf chlorophyll content (SPAD values) decreased by 13.91 %, 24.28 %, and 31.85 % under the I75, I50, and I25 treatments, respectively, suggesting that SPAD measurements at the seedling stage may serve as a practical and cost-effective indicator for identifying drought-tolerant sorghum genotypes.

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Resumen

La sequía es un importante estrés abiótico que amenaza la seguridad alimentaria mundial al reducir el rendimiento y la calidad de los cultivos. La aplicación foliar de osmoprotectores como la prolina ofrece un medio prometedor para mitigar el daño inducido por la sequía. Este estudio examinó los efectos de la prolina exógena (P0, P200, P400 y P600 mg.L^{-1}), el genotipo de sorgo y su interacción sobre rasgos morfológicos, fisiológicos, bioquímicos, de calidad forrajera y microbianos bajo diferentes niveles de sequía (I100, I75, I50 e I25). La aplicación de prolina incrementó la materia seca en más del 100 % bajo déficits medios a severos y aumentó el peso seco de la raíz en un 90 % con una reducción del 75 % del agua. La respuesta más fuerte se observó en el contenido de clorofila (SPAD), lo que refleja una mayor estabilidad fotosintética. La prolina exógena redujo el secado foliar en un 25 % y alivió las disminuciones de la calidad forrajera relacionadas con la sequía, como lo evidencian las mejoras en NDF, ADF y ADL. También incrementó la actividad de la peroxidasa en mayor medida que la superóxido dismutasa y la catalasa, minimizando la toxicidad del peróxido de hidrógeno (H_2O_2) y el estrés oxidativo. Incluso bajo sequía extrema (I25), la prolina mantuvo el vigor de la planta y mejoró la eficiencia en el uso del agua en un 25 - 40 % en la etapa de plántula. En comparación con el control, el contenido de clorofila foliar (valores SPAD) disminuyó en un 13.91 %, 24.28 % y 31.85 % bajo los tratamientos I75, I50 e I25, respectivamente, lo que sugiere que las mediciones SPAD en la etapa de plántula pueden servir como un indicador práctico y rentable para identificar genotipos de sorgo tolerantes a la sequía.

Palabras clave: sorgo, prolina exógena, estrés hídrico, SPAD, peso seco de la raíz.

Resumo

A seca é um importante estresse abiótico que ameaça a segurança alimentar global ao reduzir o rendimento e a qualidade das culturas. A aplicação foliar de osmoprotetores, como a prolina, oferece um meio promissor para mitigar os danos induzidos pela seca. Este estudo examinou os efeitos da prolina exógena (P0, P200, P400 e P600 mg.L^{-1}), do genótipo de sorgo e de sua interação sobre características morfológicas, fisiológicas, bioquímicas, de qualidade forrageira e microbianas sob diferentes níveis de seca (I100, I75, I50 e I25). A aplicação de prolina aumentou a matéria seca em mais de 100 % sob déficits médios a severos e elevou o peso seco da raiz em 90 % com uma redução de 75 % da água. A resposta mais intensa ocorreu no conteúdo de clorofila (SPAD), refletindo maior estabilidade fotossintética. A prolina exógena reduziu o ressecamento foliar em 25 % e atenuou os declínios da qualidade forrageira relacionados à seca, conforme evidenciado pelas melhorias em NDF, ADF e ADL. Também aumentou a atividade da peroxidase em maior grau do que a superóxido dismutase e a catalase, minimizando a toxicidade do peróxido de hidrogênio (H_2O_2) e o estresse oxidativo. Mesmo sob seca extrema (I25), a prolina manteve o vigor das plantas e melhorou a eficiência do uso da água em 25 - 40 % na fase de plântula. Em comparação com o controle, o conteúdo de clorofila foliar (valores SPAD) diminuiu em 13.91 %, 24.28 % e 31.85 % sob os tratamentos I75, I50 e I25, respectivamente, sugerindo que as medições SPAD na fase de plântula podem servir como um indicador prático e de baixo custo para identificar genótipos de sorgo tolerantes à seca.

Palavras-chave: sorgo, prolina exógena, estresse hídrico, SPAD, peso seco das raízes.

Introduction

Drought, intensified by climate change, poses a major threat to global agriculture by impairing plant growth, water status, and photosynthesis (Marček *et al*., 2019). It disrupts key physiological and biochemical processes, reducing carbon assimilation and chlorophyll content. Exogenous application of proline can mitigate these effects (Zahra *et al*., 2023). Water deficit triggers oxidative stress, causing electrolyte leakage, enhanced respiration, and overproduction of reactive oxygen species (ROS) including superoxide (O_2^-), hydroxyl radicals (OH), and hydrogen peroxide (H_2O_2). Plants activate a coordinated antioxidant defense system, comprising enzymatic (SOD, POD, CAT, APX) and non-enzymatic antioxidants, to maintain cellular redox homeostasis (Mittler *et al*., 2022). The efficiency of this system varies by species and drought intensity (Sher *et al*., 2023).

Rising drought frequency has renewed interest in tolerant crops. Maize (Zea *mays* L.), though the world's second most cultivated cereal, is highly water-sensitive and unsuitable for marginal lands. Consequently, attention has turned to alternative crops with potential for both forage and bioethanol production. Among these, Sorghum (*Sorghum bicolor* (L.) Moench) is notable for its drought tolerance and adaptability to semi-arid regions (George *et al*., 2022).

In order to alleviate drought impacts, strategies such as breeding, genetic modification, and the use of osmoprotectants like proline have been explored (Nguyen *et al*., 2018). Proline supports osmotic adjustment, water uptake, and turgor maintenance (Trovato *et al*., 2019), enhancing physiological traits such as relative water content and chlorophyll stability (Hayat *et al*., 2012). Although sorghum is considered drought-tolerant, the role of exogenous proline remains underexplored. Drought increases Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF), and Acid Detergent Lignin (ADL), reducing forage quality, whereas exogenous proline may enhance digestibility and dry matter yield (Yahaya *et al*., 2021).

The following aims are investigated herein: i) To evaluate the extent to which exogenously applied proline improves drought tolerance in sorghum. ii) To determine water savings during the seedling stage of the sorghum. iii) To detect the morphological, physiological, biochemical, feed quality, and enterobacteria responses of sorghum under drought stress treatments and foliar application of proline.

Materials and Methods

Plant material and growth conditions

The experiment was conducted using the Erdurmuş sweet sorghum genotype (*Sorghum bicolor* (L.) Moench). Plants were grown in a controlled growth chamber with temperatures of 20/25 °C (night/day), and 65 % relative humidity. The physicochemical properties of the potting soil are presented in Table 1. Before sowing, basal fertilization consisting of 800 mg.kg^{-1} of P_2O_5 and 1000 mg.kg^{-1} of K_2O was homogenously incorporated into soil. Additionally, 1,600 mg.kg^{-1} of N was applied in split doses at the V2, V4, and V6 growth stages which correspond to the appearance of two, four, and six leaves with visible collars, respectively (Kordas *et al*., 2024). After germination, three seedlings were retained per pot. The experiment followed a completely randomized design with three replications.

Drought stress treatments

Drought stress was applied using the gravimetric method. Before sowing, 9 L pots were filled with 9.5 kg of soil mixture and gradually irrigated until drainage occurred. After a 4 h drainage period, pot weights were recorded to determine the soil water-holding capacity (WHC). Pots were weighed every two days, and irrigation was adjusted by replenishing the lost water to maintain the target irrigation levels corresponding to 100 %, 75 %, 50 %, and 25 % of WHC. Four irrigation levels were applied: I100 (full, Irrigation100), I75 (upper medium stress), I50 (medium stress), and I25 (severe stress), corresponding to 100 %, 75 %, 50 %, and 25 % of WHC (Water Holding Capacity) (Li *et al*., 2024).

Foliar application of prolin

Proline (purity ≥ 98.5%) was applied as a foliar spray using a calibrated sprayer, with solutions prepared in distilled water and applied until uniform leaf wetting was achieved. To enhance leaf wetting and adhesion, 0.1% Tween-20 was used as a surfactant. Proline was applied at concentrations of 0 (control), 200, 400, and 600 $mg.L^{-1}$ at the V2, V4, V6, and V8 growth stages. Control plants received distilled water only (Noein & Soleymani, 2022).

Plant growth parameters

The experiment was concluded 50 days after seed germination. Measurements were then taken for Root length (RL, cm) and Root dry weight (RDW, $g.plant^{-1}$) (Kalhoro *et al*., 2018). Dry matter (DM, %) was determined according to Mi *et al*., (2018).

Physiological measurements

Leaf chlorophyll content (SPAD) was measured at the V8 stage using a portable chlorophyll meter on six points of a fully expanded leaf (Zhang *et al*., 2022). Leaf drying degree (LDD, 1 - 10) was evaluated from four directions using the UPOV scale, 0 = no drying and 10 = completely dry (Bänziger *et al*., 2000). Relative Water Content (RWC, %) was calculated using the formula described by Smart & Bingham (1974).

Forage quality determination

Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) were determined using an ANKOM fiber analyzer following the method of Van Soest *et al*. (1991).

Determination of oxidant and antioxidant activities

At the V8 stage, healthy sorghum leaves below the topmost leaf were collected under control and stress conditions, frozen in liquid nitrogen, and stored at -86 °C until analysis. Antioxidant enzyme (Superoxide Dismutase (SOD, $U.g^{-1}$ FW), Peroxidase (POD, $U.g^{-1}$ FW), Catalase (CAT, $U.g^{-1}$ FW) and Hydrogen peroxide (H_2O_2, $nmol.g^{-1}$ FW) were determined following the methods of Velikova *et al*. (2000), and Jack *et al*. (2019).

Detection of enterobacteriaceae in feed

Enterobacteriaceae in feed samples was analyzed according to ISO 21528-2 (ISO, 2018).

Statistical analysis

Statistical analysis was performed using JMP software (JMP Version 13.2.0; SAS Institute, Cary, NC, USA). A two-way factorial analysis of variance (ANOVA) was applied to evaluate the main effects of irrigation and proline treatments, as well as their interaction, on all measured parameters.

Variance analysis was conducted to determine the degrees of freedom and F-values. Treatment means were compared using the least significant difference (LSD) test at a probability level of $p \leq 0.05$.

Results and discussion

Analysis of variance

According to variance analysis, drought and proline treatments had significant effects ($p \leq 0.01$) on agronomic, physiological, feed quality, antioxidant, oxidative stress, and Enterobacteriaceae traits in sorghum. The drought × proline interaction was also significant ($p \leq 0.01$) for RL, RWC, LCC, LDD, SOD, CAT, and H_2O_2, while DM, RDW, NDF, ADF, ADL, and POD showed moderate significance ($p \leq 0.05$). Enterobacteriaceae counts (EBC) was not significantly affected. Degrees of freedom and F-values for these traits are presented in Table 2.

Plant growth traits

Compared to full irrigation, biomass decreased by 20.29 %, 26.20 %, and 47.64 % under I75, I50, and I25 drought levels, respectively. Exogenous proline markedly enhanced dry matter (DM), increasing by 23.72 %, 66.67 %, and 115.07 % with P200, P400, and P600 compared to P0. Under combined treatments, DM rose by 90.66 % - 139.84 % with P600 across irrigation levels, indicating greater drought tolerance at higher proline doses. Root length (RL) increased under moderate drought (I75: +9.15 %, I50: +20.89 %) but decreased under severe drought (I25: -35.81 %). Proline applications raised RL up to 9.20%, and under combined treatments, by 8.18 % - 10.58 %. Root dry weight (RDW) declined by 15.16 % - 45.30 % under drought but rose by 21.38 % - 94.78 % with proline, reaching 83.65 % - 115.28 % increases in combined treatments (Table 3).

Drought stress significantly reduced sorghum dry matter (DM) due to limited water uptake and inhibited photosynthesis. Foliar-applied proline improved DM by enhancing osmotic regulation, reducing water loss, and sustaining metabolism under stress, consistent with findings in other crops (Ibrahim *et al*., 2022). Root growth declined under severe drought but was restored by proline, which stimulated elongation and metabolic activity (Khan *et al*., 2025). Proline may also regulate abscisic acid and antioxidant responses, maintaining osmotic balance. Increased root dry weight (RDW) under proline treatments, especially in moderate drought, aligns with previous studies (Shah *et al*., 2020; Cheng *et al*., 2021), likely due to improved water retention and chlorophyll stability.

Physiological traits

Relative water content (RWC) decreased by 10.49 %, 20.13 %, and 37.91 % under mild (I75), moderate (I50), and severe (I25) drought treatments, respectively, compared to full irrigation (I100). Increasing doses of exogenous proline significantly improved RWC. Compared to the no-proline treatment (P0), RWC increased by 15.42 %, 20.29 %, and 27.56 % with P200, P400, and P600, respectively. Compared to the control combinations (I100×P0, I75×P0, I50×P0, I25×P0), RWC increased by 29.24 %, 31.77 %, 21.22 %, and

Table 1. Physical and chemical properties of the pot soil.

EC	pH	Organic matter	Sand	Clay	Silt	P	K	Cu	Mn	Fe	Zn
($dS.m^{-1}$)				(%)					($mg.kg^{-1}$)		
0.94	7.65	1.02	35.82	18.96	45.22	55.40	2110	1.31	3.55	4.18	1.22

Table 2. F values and degrees of freedom of the investigated traits in experiment.

SV	DF	F_{values}						
		DM	RL	RDW	RWC	LCC	LDD	NDF
R	2	1.07	61.12	0.10	8.02	1.06	0.23	2.58
I	3	163.11**	1038.82**	55.82**	977.61**	75.91**	140.21**	528.60**
P	3	264.75**	150.15**	222.50**	187.54**	282.60**	23.28**	274.05**
I*P	9	3.11*	5.55**	2.86*	6.16**	2.84**	3.57**	3.17*
Error	6							
CV (%)		7.11	1.07	6.70	2.54	5.29	18.91	4.65
SV	DF	F_{values}						
		ADF	ADL	SOD	POD	CAT	H2O2	EBC
R	2	3.79	45.25	2.58	0.82	0.99	0.37	1.24
I	3	142.00**	69.75**	206.34**	148.17**	1946.15**	720.53**	323.53**
P	3	336.75**	170.94**	69.94**	948.23**	1829.94**	658.10**	71.40**
I*P	9	2.81*	2.87*	4.10**	2.73*	33.52**	35.01**	21.86^{ns}
Error	6							
CV (%)		6.33	1.57	1.28	3.83	1.84	4.08	28.94

*,** significant at 0.05 and 0.01 level, ns = non-significant, SV: source of variation, DF: degree of freedom, CV: coefficient of variation, R: replication, I: Irrigation, P: proline

DM: dry matter (%), RL: root length (cm), RDW: root dry weight (g.plant^{-1}), RWC: relative water content (%), LCC: leaf chlorophyll content (SPAD), LDD: leaf drying degree (1-10), NDF: neutral detergent fiber (%), ADF: acid detergent fiber (%), ADL: acid detergent lignin (%), SOD: superoxide dismutase (U.g^{-1} FW), POD: Peroxidase (U.g^{-1} FW), CAT: catalase (U.g^{-1} FW), H2O2: hydrogen peroxide (nmol.g^{-1} FW), EBC: Enterobacteriaceae counts (cfu.m^{-1})

27.64 % under I100×P600, I75×P600, I50×P600, and I25×P600, respectively. Leaf chlorophyll content (LCC, SPAD value) decreased by 13.91 %, 24.28 %, and 31.85 % under I75, I50, and I25 treatments, respectively, compared to the control. Proline application increased LCC significantly by 46.81 %, 67.16 %, and 92.27 % with P200, P400, and P600, respectively, compared to P0. The protective effect of proline against drought was clearly visible. Compared to the control combinations, LCC increased by 78.02 %, 95.46 %, 90.51 %, and 112.58 % under I100×P600, I75×P600, I50×P600, and I25×P600, respectively. Under control and mild drought (I75) conditions, leaf desiccation was minimal and statistically similar. However, under I50 and I25, about 25 % and 40 % of the leaves showed drying symptoms. Proline application reduced leaf desiccation by 23.33 %, 33.33 %, and 46.67 % with P200, P400, and P600, respectively, compared to P0 (Table 3). Severe drought led to the greatest water loss and reduced relative water content (RWC), while proline application improved RWC by enhancing osmotic adjustment. Increased antioxidant activity (SOD, CAT) further supported drought tolerance. Drought decreased leaf chlorophyll content (LCC), but proline maintained higher levels, likely by alleviating oxidative stress and sustaining photosynthesis (Ibrahim *et al*., 2022). Leaf drying remained below 20 % across treatments, indicating high tolerance. Proline preserved turgor, cell structure, and green leaf area, consistent with previous studies (Ali *et al*., 2022).

Feed quality variations

Compared to full irrigation, NDF, ADF, and ADL increased by up to 70.59 %, 63.35 %, and 56.93 % under severe drought, respectively. Proline application (P200, P400, P600) reduced NDF by 9.92 - 41.30 %, ADF by 22.74 - 54.83 %, and ADL by 4.61 - 12.85 % compared to the control (P0). In interactions, compared to respective controls (I100 × P0, I75 × P0, I50 × P0, I25 × P0), P600 (I100 × P600, I75 × P600, I50 × P600, I25 × P600) decreased NDF by 45.49 - 38.28 %, ADF by 59.93 - 52.18 %, and ADL by 11.23 - 16.46 %. These results indicate that proline consistently alleviated drought-induced increases in cell wall components across all irrigation levels (Table 4). As drought intensified, NDF and ADF rose due to greater lignification, as sorghum strengthened cell walls to limit water loss. Proline likely supported this by enhancing antioxidant defense and maintaining water balance. The I × P interaction showed proline improved forage quality by regulating cell wall composition, especially under moderate drought. These findings agree with Kale *et al.* (2018) and Ferreira *et al*. (2021).

Enzymatic antioxidants and oxidant activity

Compared to full irrigation, SOD activity increased by 4.68 %, 7.77 %, and 13.77 % under mild, moderate, and severe drought, respectively. Proline treatments (P200, P400, P600) further enhanced SOD by 2.64 %, 4.55 %, and 7.67 % relative to P0. Combined treatments increased SOD by 5 - 10 % depending on drought intensity. POD activity rose sharply by 19.31 %, 33.30 %, and 50.44 % with increasing drought severity, while proline application enhanced POD by 33.69 %, 78.05 %, and 125.73 %. Under combined treatments, POD increased by 97 - 152 %, indicating strong synergy between drought stress and proline response. CAT activity increased by 20.45 %, 51.15 %, and 75.59 % under mild to severe drought and was further stimulated by 22.82 %, 46.69 %, and 71.66 % with increasing proline doses. Combined treatments (I×P600) enhanced CAT by 68 - 75 % across all stress levels. H_2O_2 content rose by 17.52 %, 56.92 %, and 80.93 % with drought but decreased by 14.46 %, 39.65 %, and 48.78 % following proline treatment.

Combined applications reduced H_2O_2 by up to 54 %, confirming the ROS-scavenging role of exogenous proline. Drought stress activated antioxidant enzymes, while exogenous proline amplified their activity, reducing oxidative damage. POD showed the greatest increase, supporting H_2O_2 detoxification and lignin synthesis for cell wall stability. CAT and SOD together maintained redox equilibrium and membrane integrity. Overall, foliar-applied proline effectively mitigated oxidative stress by enhancing the enzymatic antioxidant defense system (Abdou *et al*., 2022).

Table 3. Agronomic and physiological responses of sorghum genotype to drought stress and proline applications.

Stress type	P	Agronomic specifications			Physiological traits		
		DM (%)	RL (cm)	RDW (g.plant^{-1})	RWC (%)	LCC (SPAD)	LDD (1-10)
I100	0	11.45±0.16^{fg}	52.81±1.54^{j}	13.33±0.55^{e}	71.10±0.65^{e}	25.70±0.62^{i}	0.33±0.33^{fg}
	200	13.06±0.33^{de}	55.11±1.51^{i}	16.38±0.68^{d}	85.81±0.98^{b}	39.48±0.75^{b}	0.00±0.00^{g}
	400	17.40±0.33^{bc}	56.30±1.71^{h}	19.67±0.59^{bc}	89.22±1.07^{a}	43.87±0.60^{a}	0.00±0.00^{g}
	600	21.83±0.78^{a}	57.68±2.21^{g}	24.48±0.65^{a}	91.89±1.89^{a}	45.75±0.69^{a}	0.00±0.00^{g}
I75	0	7.73±0.10^{i}	57.96±2.03^{g}	10.88±0.46^{f}	63.89±0.77^{gh}	22.45±0.36^{j}	0.67±0.33^{f}
	200	10.35±0.35^{gh}	59.61±2.30^{f}	13.44±0.64^{e}	75.26±1.04^{d}	30.71±1.18^{fg}	0.33±0.33^{fg}
	400	14.17±0.18^{d}	61.93±2.22^{e}	17.08±0.76^{d}	79.22±1.39^{c}	36.24±3.22^{cd}	0.00±0.00^{g}
	600	18.54±0.20^{b}	62.70±1.87^{de}	21.26±0.91^{b}	84.19±1.16^{b}	43.88±0.61^{a}	0.00±0.00^{g}
I50	0	7.70±0.48^{i}	63.70±1.59^{d}	8.77±0.39^{gh}	61.59±0.66^{h}	19.81±0.64^{j}	3.33±0.33^{c}
	200	9.47±0.45^{h}	65.66±1.43^{c}	10.39±0.35^{fg}	65.67±1.92^{fg}	27.73±0.58^{hi}	3.00±0.00^{cd}
	400	12.88±1.33^{def}	68.45±1.20^{b}	14.15±0.77^{e}	68.04±1.83^{f}	31.93±0.26^{ef}	2.67±0.33^{d}
	600	16.98±0.38^{c}	70.44±1.03^{a}	18.88±0.55^{c}	74.66±0.65^{d}	37.74±0.70^{bc}	2.00±0.00^{e}
I25	0	5.32±0.25^{j}	33.59±1.57^{l}	7.07±0.55^{i}	45.26±1.08^{k}	16.29±0.63^{k}	5.67±0.33^{a}
	200	6.95±0.23^{i}	35.74±1.62^{k}	8.40±0.52^{hi}	52.41±0.58^{j}	25.77±1.06^{i}	4.33±0.33^{b}
	400	9.21±0.12^{h}	36.74±1.76^{k}	11.55±0.76^{f}	54.45±0.62^{j}	28.80±0.68^{gh}	4.00±0.00^{b}
	600	11.89±0.67^{ef}	36.36±1.13^{k}	13.38±0.83^{e}	57.77±0.51^{i}	34.63±0.52^{de}	3.33±0.33^{c}
	Mean	**12.18**	**54.67**	**14.32**	**70.03**	**31.92**	**1.85**
	LSD	I= 0.84**	I= 1.43**	I= 1.65**	I= 1.49**	I= 2.09**	I= 0.58**
		P= 0.72**	P= 0.49**	P= 0.80**	P= 1.50**	P= 1.42**	P= 0.28**
		I*P= 1.45*	I*P= 0.98**	I*P= 1.62*	I*P= 2.99**	I*P= 2.84**	I*P= 0.57**

*,** significant at 0.05 and 0.01 level, I:irrigation treatments, P: proline aplications, I100: Full Irrigation, I75: Upper Medium, I50: Medium, I25: Severe stress, DM: dry matter (%), RL: root length (cm), RDW: root dry weight (g.plant^{-1}), RWC: relative water content (%), LCC: leaf chlorophyll content (SPAD), LDD: leaf drying degree (1 - 10).

Table 4. Forage characteristics of 8-leaf seedlings *in vitro* under drought stress and proline applications.

Stress type	P	Forage quality features		
		NDF (%)	ADF (%)	ADL (%)
I100	0	33.48±0.56^{fg}	23.51±0.74^{de}	2.66±0.36^{j}
	200	29.22±1.61^{hi}	15.81±1.02^{g}	2.56±0.35^{k}
	400	23.04±0.89^{j}	13.15±0.57^{h}	2.41±0.35^{l}
	600	18.25±0.90^{k}	9.42±0.34^{i}	2.35±0.37^{l}
I75	0	38.69±0.71^{e}	27.81±0.58^{c}	3.16±0.20^{g}
	200	34.25±1.26^{f}	22.33±1.26^{ef}	2.93±0.26^{h}
	400	28.56±0.83^{i}	16.41±0.77^{g}	2.79±0.25^{i}
	600	22.42±0.58^{j}	12.45±0.48^{h}	2.64±0.24^{jk}
I50	0	45.59±0.71^{c}	32.37±0.60^{b}	3.65±0.21^{d}
	200	41.15±0.99^{de}	25.48±1.26^{d}	3.52±0.21^{e}
	400	33.08±1.03^{fg}	20.44±0.50^{f}	3.38±0.18^{f}
	600	28.14±0.90^{i}	15.48±0.55^{g}	3.24±0.18^{g}
I25	0	53.04±0.22^{a}	35.55±0.63^{a}	4.20±0.21^{a}
	200	49.22±1.11^{b}	28.51±1.27^{c}	4.02±0.23^{b}
	400	43.70±0.77^{cd}	20.53±1.03^{f}	3.77±0.23^{c}
	600	31.44±0.42^{gh}	16.51±0.61^{g}	3.68±0.23^{d}
	Mean	**34.58**	**20.98**	**3.18**
	LSD	I= 1.17**	I= 1.25**	I= 0.25**
		P= 1.33**	P= 1.12**	P= 0.04**
		I*P= 2.70*	I*P= 2.22*	I*P= 0.08*

*,** significant at 0.05 and 0.01 level, I: irrigation treatments, P: proline aplications (0 mg.L^{-1}, 200 mg.L^{-1}, 400 mg.L^{-1}, 600 mg.L^{-1}), I100: Full Irrigation, I75: Upper Medium, I50: Medium, I25: Severe stress, NDF: neutral detergent fiber (%), ADF: acid detergent fiber (%), ADL: acid detergent lignin (%).

Enterobacteriaceae density in feed

Among all bacterial counts, 67.87 % were observed under full irrigation, while the least bacterial growth, 0.19 %, occurred under severe drought conditions. Bacterial growth under upper medium and medium treatments was 30.11 % and 1.83 %, respectively. The highest proportion of total *Enterobacteriaceae* presence was observed under the P0 treatment, accounting for 48.52 %, followed by P200 with 28.52 %. Bacterial counts under P400 and P600 were 12.81 % and 10.14 %, respectively (Figure 1).

Drought stress lowered Enterobacteriaceae density, while higher irrigation favored their proliferation. Proline treatment further reduced microbial density, contributing to improved forage quality. Similar pattern has been reported in silage study (Blessington *et al*., 2014).

Conclusions

Sorghum shows strong adaptability to heat and drought, yet water deficit markedly limits its growth and metabolism. Foliar-applied proline mitigates these effects by enhancing dry matter accumulation, root growth, and chlorophyll (SPAD) content, thus maintaining photosynthetic efficiency. It preserves cell integrity, delays senescence, and improves forage quality by reducing ADF levels. Antioxidant enzyme activity rises under drought and is further boosted by proline, strengthening oxidative defense. Drought decreases Enterobacteriaceae populations, while higher proline doses (P400, P600) reduce them by about 10 %, supporting a healthier phyllosphere. Even under severe drought (I25), proline-treated plants retain vigor, turgor, and upright growth. Proline application at the seedling stage increases water-use efficiency by 25 - 40 %, offering a practical means to enhance drought resilience. Early-stage SPAD readings provide reliable, low-cost indicators for identifying drought-tolerant sorghum genotypes in breeding programs.

Table 5. Biochemical reactions in sorghum genotype leaves subjected to drought stress and proline treatments.

Stress type	P	Antioxidant enzyme and ROS activity			
		SOD (U.g^{-1} FW)	POD (U.g^{-1} FW)	CAT (U.g^{-1} FW)	H2O2 (nmol.g^{-1} FW)
I100	0	486.54±2.75^{k}	17.46±0.49^{k}	290.48±6.44^{l}	211.29±5.78^{fg}
	200	518.15±3.53^{j}	23.92±0.22^{i}	311.38±5.97^{k}	191.15±5.03^{h}
	400	526.14±3.81^{hij}	33.97±0.76^{g}	390.63±5.45^{i}	135.63±3.00^{jk}
	600	532.66±5.13^{ghi}	44.07±0.49^{e}	491.24±5.50^{f}	123.51±2.65^{k}
I75	0	522.52±6.19^{ij}	20.89±0.41^{j}	331.28±5.56^{j}	262.57±4.50^{e}
	200	536.92±3.08^{fgh}	30.73±2.24^{h}	414.42±3.92^{h}	199.02±5.64^{gh}
	400	545.10±3.60^{ef}	39.58±0.59^{f}	482.38±5.12^{fg}	172.70±4.64^{i}
	600	555.49±4.98^{de}	51.28±0.55^{c}	559.05±7.70^{d}	143.20±4.28^{j}
I50	0	544.06±4.20^{efg}	24.41±0.39^{i}	405.75±2.98^{hi}	340.82±6.46^{c}
	200	545.71±3.23^{ef}	33.96±0.87^{g}	513.05±4.62^{e}	310.87±5.12^{d}
	400	561.05±3.46^{d}	45.69±0.48^{e}	612.67±6.79^{c}	213.59±3.66^{fg}
	600	573.07±4.37^{bc}	55.12±0.86^{b}	711.23±5.98^{b}	172.91±7.01^{i}
I25	0	566.92±3.62^{cd}	31.48±0.62^{h}	472.70±4.63^{g}	421.99±4.16^{a}
	200	575.18±4.34^{bc}	37.36±0.91^{f}	603.78±4.99^{c}	356.85±3.10^{b}
	400	584.18±4.56^{b}	48.55±1.48^{d}	714.95±4.73^{b}	224.37±5.51^{f}
	600	621.41±6.30^{a}	62.25±0.60^{a}	813.77±5.26^{a}	193.81±8.23^{h}
	Mean	**549.69**	**37.55**	**507.42**	**229.64**
	LSD	I= 7.15**	I= 1.80**	I= 9.66**	I= 7.82**
		P= 5.96**	P= 1.21**	P= 7.87**	P= 7.88**
		I*P= 11.92**	I*P= 2.43*	I*P= 15.75**	I*P= 15.77**

*,** significant at 0.05 and 0.01 level, I: irrigation treatments, P: proline aplications, I100: Full Irrigation, I75: Upper Medium, I50: Medium, I25: Severe stress, SOD: superoxide dismutase (U.g^{-1} FW), POD: Peroxidase (U.g^{-1} FW), CAT: catalase (U.g^{-1} FW), H2O2: hydrogen peroxide (nmol.g^{-1} FW).

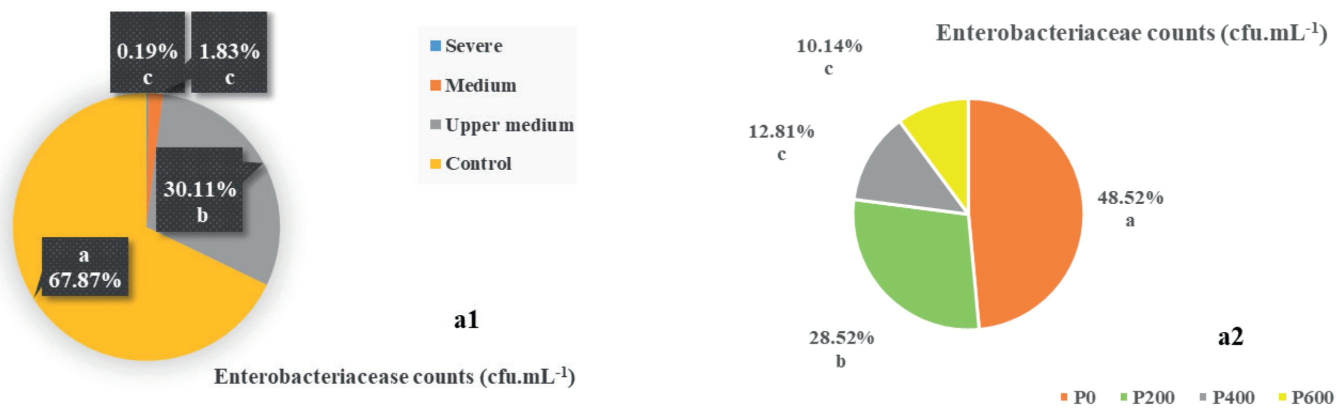

Figure 1. Enterobacteriaceae counts at different proline (a2) (P0 (Control), P200 (200 mg.L^{-1}), P400 (400 mg.L^{-1}) and P600 (600 mg.L^{-1}) and drought stress treatments (a1) (Control (I100, full irrigation), Upper medium (I75), Medium (I50) and Severe stress (I25) applied to sorghum up to V8 stage.

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