IMPACT OF EXOGENOUS PROLINE APPLICATION ON PHYSIOLOGY, BIOCHEMISTRY, AND FORAGE QUALITY OF MAIZE (Zea *mays* L.) UNDER DROUGHT STRESS

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ABSTRACT

Drought is a real threat to crop production, and crops like maize, which consume high amounts of water, are adversely affected by the lack of adequate soil moisture. The information on drought tolerance mechanism of maize during the seedling stage is essential to alleviate the negative effect of drought stress. This study was conducted to determine the effects of exogenous proline application on morphology, physiology, biochemical characteristics, feed quality, and enterobacteriacae parameters of a commercial maize variety grown under drought conditions. Deficit irrigation had detrimental effects on maize growth, while exogenous proline application mitigated the negative effects of drought and improved the maize growth. The chlorophyll content of maize plants was negatively affected by drought, but proline treatments regulated chlorophyll degradation by up to 90%. The concentrations of hemicellulose, cellulose, and lignin increased under drought conditions during the seedling stage, which led to accelerated plant aging. The increase in drought intensity led to higher levels of oxidants (such as hydrogen peroxide) and increased antioxidant activities. Exogenous proline application enhanced the activities of antioxidant enzymes (superoxide dismutase, peroxidase, and catalase) and reduced the harmful effects of hydrogen peroxide. The results revealed that proline application during the seedling period reduced the water consumption of maize plantsby25- 40%. In addition, leaf chlorophyll content is a practical and cost-effective parameter for assessing maize drought stress and tolerance levels

Key words: Drought stress, Exagenous proline, antioxidant activities, Enterobacteriaceae

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INTRODUCTION

The increase in carbondioxide (CO_2) levels in the atmosphere from 280-300 ppm to 420 ppm which led to an increase of 1.5°C in the global average temperature (IPCC, 2023). These shifts have increased the drought riskboth in Turkey and worldwide, öeteorological, and hydrological droughts. The surge in global population to 8.123 billion in 2024 has increased the demand for food, needing the protection of intensively grown crops, particularly water intensive maize (*Zea mays* L.), by prioritizing genotype breeding and adopting water-efficient practices.

Drought during critical phenological stages, specifically seedling, tasseling-silking, and grain-filling, drought stress severely impairs maize development, leading to stunted growth, reproductive failure, and yield loss (Vennam et al., 2023a and 2023b). Plant-water relationships are largely governed by available soil moisture content, which affects stomatal conductance and photosynthesis (Yan et al., 2016). Physiological processes of plants grown under drought conditions are impaired and led to lower leaf development, reduced leaf area, and decreased dry weight (Rafique, 2020). Beyond their aboveground response, maize plants have significant root adaptation capabilities that favor deeper penetration to cope with drought (Guo et al., 2022; Giri et al., 2017).

Superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) are known protecting plants from oxidative-stress created under drought stress(Mishra et al., 2023). The activities of these enzymes under drought stress are upregulated to mitigate the detrimental effects of reactive oxygen species (ROS). For instance, Hameed et al. (2011) reported that restricting available water to 50% significantly increased CAT, SOD, and POD activities in wheat leaves compared to full irrigtaed conditions Despite these defenece mechanisms, prolonged drought stress significantly diminishes yield in forage crops due to impaired photosynthesis, stomatal closure, reduced CO_2 assimilation, and losses in leaf area and biomass (Qiao et al., 2024). In addition, increasing drought severity in alfalfa is reported to lower forage quality by increasing the ratio of fiber components, such as Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF), and Acid Detergent Lignin (ADL).

The accumulation of free amino acids like proline is the major physiological response of maize to drought stress. The magnititude of this response is distinct in maize, with reported concentrations reaching levels 10 and 100 times greater than those in cereal crops (wheat, rice), and dicots (sunflower tobacco), respectively (Taie et al., 2013). Similarly, Zadehbaghri et al. (2014) reported that exogenous proline application supports the natural defenese system of plants. The application of exogenous proline reduces osmotic stress and supports cell membrane integrity, improving water absorption capability (Al-Shaheen et al., 2014). Other morphological, physiological, and biochemical responses that are critical for sustaining growth and development, have also been reported in response to exogenous proline application for many other cash crops (Abbas and Alak, 2016).

The specific aims of this study are:

i) To assess the efficiency of exogenously applied proline, functioning both an amino acid and an osmoprotectant, in mitigating drought stress effect.

ii) To evaluate the water-saving potential and water use efficiency of maize plants during the seedling stage.

iii) To figure out the effects of drought stress and foliar proline application on agronomic traits, physiological mechanisms, biochemical markers, forage quality parameters, and Enterobacteriaceae counts in maize.

iv) To provide a theoretical basis and reference for future research concerning moisture stress and proline applications in other plant species.

iv) To develop practical, cost- effective, and time-efficient methods for early-stage identification of drought tolerant, sensitive genotypes, , thereby easing maize breeding programs.

MATERIALS AND METHODS

Plant material and growth conditions: The experiment was conducted between April 15 and June 15, 2024, at Kepsut Vocational School, Balıkesir University, Turkey. For maize seed germination and seedling development, the plants were grown under controlled conditions in a plant growth chamber, where light intensity was set at 12,000 lux, with a photo period of 16 hours of daylight and 8 hours of darkness. Temperature was maintained at 20/25°C (night/day) and relative humidity was kept at 65%. The biochemical analyses were conducted in the Biology Department laboratory at Çanakkale University, Turkey.

In the experiment, the registered cultivar Kale was used. The seeds were surface sterilized by soaking in a 5% sodium hypochlorite (NaClO) solution for 10 minutes, followed by four washes with distilled water. The seeds were then soaked in distilled water for one day and dried on filter paper (Naeem et al. 2017). The characteristics of soil used in the experiment are given in Table 1. The pots were irrigated to 100% field capacity and equilibrated for over two days prior to seed sowing. Subsequently, five viable maize seeds were sown per pot (50 cm diameter × 48.5 cm height; volume: 95 L) filled with 9.5 kg of soil. The physical and chemical properties experimental soil are presented in Table 1.

The experiment was conducted in a factorial arrangement based on a completely randomized design with three repplications, comprising a total of 48 pots. The treatments consisted of four drought stress levels, and four foliar proline doses. Following germination, the seedlings were thinned to three plants per pot. To minimize microclimatic variation (light, temperature, humidity), the pots were re-randomized (rotated) every three days. The physical and chemical characteristics of the potting soil were suitable for maize growth. Before sowing, basal fertilization consisting of 800 mg kg^{-1} P_2O_5 and 1000 mg kg^{-1} K_2O was homogenously incorporated into soil. Additionally, nitrogen (1600 mg kg^{-1} N) was applied in split doses during the V2, V4, and V6 vegetaion growth stages.

Drought stress treatments: Gravimetric method was used to apply drought stress treatments (Liyanage et al., 2022). Water levels were adjusted according to the following treatments: I100 (full irrigation, control), I75 (upper medium), I50 (moderate), and I25 (severe drought). The amount of water applied was reduced to 75%, 50%, and 25% of the water holding capacity based on the calculated water-holding capacity (Li et al., 2024).

Foliar application of prolin: Proline doses were applied to maize seedlings during the V2, V4, V6, and V8 vegetative stages, with the following treatments: P0 (Control, no proline), P200 (200 mg L^{-1}), P400 (400 mg L^{-1}), and P600 (600 mg L^{-1}), using foliar spraying. Prior to treatment, the volume of water required for full foliar coverage was calibrated. The proline solutions were applied using a calibrated sprayer until runoff to ensure thorough wetting of leaf surfaces. Distilled water was applied control plants. Maize plants were harvested at the V8 (eight-leaf) stage (Noein and Soleymani, 2022). Measurements were performed on the three plants in each pot and averaged to represent a single replicate.

Plant growth parameters: Plant growth parameters were determined during V8 growth stage. Plant height (PH, cm) was measured from the soil surface to the tip of the uppermost leaf (Mwadzingeni et al., 2016). The roots were carefully removed and gently washed to remove soil particles. Root length (RL, cm) was recorded using the procedure described by Kalhoro et al. (2018), dry matter **(**DM, %) was determined according to Mi et al. (2018).

Physiological measurements and Forage Quality: Physiological traits of maize plants were determined during the V8 stage. Leaf area (LA, cm^{-2}) was determined using the protocol of Plénet et al. (2000). Leaf chlorophyll content (LCC, SPAD) was measured in maize using a portable chlorophyll meter (Minolta SPAD 502). The measurement was taken six times at different parts of a fully developed leaf during the V8 stage (Zhang et al., 2022). Relative water content (RWC, %) was measured using the method established by González-Espíndola et al. (2024). The forage quality parameters, including NDF, ADF, and ADL, were determined on entire maize plants harvested at the V8 growth stage (Goering and Van Soest, 1970; Van Soest et al. 1991).

Determination of oxidant and antioxidant activities: During the V8 stage of maize under control and drought stress treatments, healthy leaves located just below the topmost leaf were harvested. These leaves were frozen in liquid nitrogen and stored in a -86°C deep freezer until enzyme activity analysis was performed. The Superoxide Dismutase (SOD, U/g FW) enzyme activity in maize leaf samples was determined according to the previously described method (Tian et al., 2023). Peroxidase (POD, U/g FW) enzyme activity in maize leaf samples was determined according to the previously described method (Jack et al., 2019). Catalase activity (CAT, U/g FW) in leaf samples was determined using the procedure of Beers and Sizer (1952). Hydrogen peroxide (H_2O_2, nmol/g FW) content in leaf samples was determined following the method of Jack et al. (2019).

Presence of enterobacteriaceae in feed: The enumeration of Enterobacteriaceae in the forage samples were conducted according to the ISO 21528-2 standard (ISO, 2018).

Statistical analysis: Statistical analyses were performed using JMP Pro software (Version 13.2.0, SAS Institute Inc., Cary, NC, USA). Prior to ANOVA, data were assessed for normality using the Shapiro–Wilk test and for homogeneity of variances using Levene's test to ensure that statistical assumptions were met. Subsequently, a two-way analysis of variance (ANOVA) was employed to evaluate the main effects of irrigation levels (I) and proline application (P), as well as their interaction (I × P), on all measured parameters. The linear statistical model used for analysis was as follows**:**

$$\boldsymbol{Y_{ijk}} = \mu + I_i + P_j + (IxP)_{ij} + \in_{ijk}$$

Y_{ijk} represents the observed value, μ is the overall mean, I_i is the effect of the i^{th} irrigation treatment, P_j is the effect of the i^{th} proline treatment, $(IxP)_{ij}$ denotes the interaction effect between irrigation and proline treatments, and ε_{ijk} is the random error term, Treatment means were compared using Fisher's Least Significant Difference (LSD) test at the $p \leq 0.05$ significance level.

RESULTS

The effects of drought and proline applications on maize variety were statistically significant ($P \leq 0.01$) for agronomic, physiological, feed quality, antioxidant, oxidative stress, and Enterobacteriaceae properties. In the drought × proline interaction, significant differences ($P \leq 0.01$) were found for parameters such as PH, DM, RL, LA, LCC, NDF, SOD, CAT, and H2O2. Differences for RWC, ADF, ADL, and POD were less significant ($P \leq 0.05$), While EBC was found to be non-significant (Table 2)

Plant growth and physiological traits: It was determined that PH was influenced by both drought stress and proline applications. Compared to the control irrigation, plant height showed a minimal decrease under Upper medium and medium irrigation treatments, with I100, I75, and I50 being in the same statistical group. However, under severe drought stress, PH decreased by 27.52%. In comparison to the P0 group, increasing proline applications (P200, P400, P600) resulted in a respective increase in PH of 11.89%, 17.72%, and 14.19%. The highest PH was observed at proline concentration of P400 Additionally, in the I75 × P400 interaction, the highest PH was achieved, while the lowest PH was observed in the severe drought × P0 interaction (Table 3).

Dry matter is an important indicator of biomass accumulation. Compared to control irrigation, the greatest biomass loss was observed under severe drought stress, with a decrease of 42.78%, followed by I50 with a 25.73% reduction and I75 with a 15.89% decrease. Increasing proline applications positively affected the DM content. Compared to the P0 treatment, proline applications at P200, P400, and P600 resulted in respective increases in DM by 17.71%, 38.89%, and 61.86%. Furthermore, compared to the control interactions (I100 × P0, I75 × P0, I50 × P0, and I25 × P0), the interactions of I100 × P600, I75 × P600, I50 × P600, and I25 × P600 showed increases in DM by 79.58%, 64.87%, 57.33%, and 35.56%, respectively (Table 3).

Compared to the non-stressed irrigation, root length increased by 8.92% and 18.08% under Upper medium and medium treatments, respectively, while it decreased by 37.47% under severe drought stress. Compared to the non-proline treatment, proline applications at P200, P400, and P600 resulted in respective increases in RL by 6.89%, 12.62%, and 15.74%. Additionally, compared to the control interactions (I100 × P0, I75 × P0, I50 × P0, and I25 × P0), the interactions of I100 × P600, I75 × P600, I50 × P600, and I25 × P600 showed increases in RL by 16.17%, 19.64%, 13.34%, and 12.94%, respectively (Table 3).

LA is an important physiological indicator for drought stress. Compared to the severe drought treatment, LA increased by 23.00%, 37.69%, and 42.54% for medium, upper medium, and control treatments, respectively, with the I100 and I75 treatments belonging to the same statistical group. Compared to the P0 treatment, LA increased by 15.88%, 21.79%, and 19.72% for the P200, P400, and P600 proline treatments, respectively. LA increased by 13.68%, 28.22%, 26.61%, and 10.26% for the I100 x P600, I75 x P600, I50 x P600, and I25 x P600 interactions, respectively, compared to the control interactions of I100 x P0, I75 x P0, I50 x P0, and I25 x P0 (Table 3).

Compared to the control, as drought stress increased, RCW, with this reduction reaching 46.42% under severe drought. Although slightly increasing proline doses lead to an increase in RCW%. Compared to the P0 treatment, RCW increased by 6.55%, 10.44%, and 14.02% for the P200, P400, and P600 proline treatments, respectively. RCW increased by 10.42%, 6.65%, 18.00%, and 29.37% for the I100 x P600, I75 x P600, I50 x P600, and I25 x P600 interactions, respectively, compared to the control interactions of I100 x P0, I75 x P0, I50 x P0, and I25 x P0 (Table 3).

Compared to control irrigation, leaf chlorophyll content decreased by 15.80%, 30.41%, and 44.85% for the upper medium, medium, and severe drought treatments, respectively. Compared to control proline treatment, LCC increased by 34.33%, 59.24%, and 79.71% for the P200, P400, and P600 treatments, respectively. LCC increased by 65.98%, 86.48%, 83.36%, and 91.97% for the I100 x P600, I75 x P600, I50 x P600, and I25 x P600 interactions, respectively, compared to the I100 x P0, I75 x P0, I50 x P0, and I25 x P0 interactions (Table 3).

Feed quality variations: Compared to the control irrigation, NDF increased by 27.09%, 63.66%, and 95.10% for the upper medium, medium, and severe drought treatments, respectively. Compared to the control proline treatment, NDF decreased by 6.64%, 11.95%, and 17.78% for the P200, P400, and P600 proline treatments, respectively. NDF decreased by 27.63%, 18.95%, 18.37%, and 11.02% for the I100 x P600, I75 x P600, I50 x P600, and I25 x P600 interactions, respectively, compared to the I100 x P0, I75 x P0, I50 x P0, and I25 x P0 interactions (Table 4).

Compared to control irrigation, ADF increased by 23.77%, 49.18%, and 91.60% for the upper medium, medium, and severe drought treatments, respectively. Compared to the control proline treatment, ADF decreased by 30.59%, 43.17%, and 62.59% for the P200, P400, and P600 proline treatments, respectively. ADF decreased by 62.66%, 69.35%, 68.33%, and 51.90% for the I100 x P600, I75 x P600, I50 x P600, and I25 x P600 interactions, respectively, compared to the I100 x P0, I75 x P0, I50 x P0, and I25 x P0 interactions (Table 4).

Compared to full irrigation, ADL increased by 21.23%, 41.15%, and 88.04% for the upper medium, medium, and severe drought treatments, respectively. Compared to the control proline treatment, ADL decreased by 5.88%, 10.59%, and 16.29% for the P200, P400, and P600 proline treatments, respectively. ADL decreased by 23.24%, 16.96%, 11.84%, and 15.16% for the I100 x P600, I75 x P600, I50 x P600, and I25 x P600 interactions, respectively, compared to the I100 x P0, I75 x P0, I50 x P0, and I25 x P0 interactions (Table 4).

Enzymatic antioxidants and oxidant activity: Compared to full irrigation, the content of superoxide dismutase increased by 8.49%, 21.50%, and 33.63% for the upper medium, medium, and severe drought treatments, respectively. Additionally, compared to the control proline treatment, SOD content increased by 16.67%, 35.54%, and 47.83% for the P200, P400, and P600 proline treatments, respectively. SOD antioxidant activity increased by 54.63%, 43.94%, 52.35%, and 41.84% for the I100 x P600, I75 x P600, I50 x P600, and I25 x P600 interactions, respectively, compared to the I100 x P0, I75 x P0, I50 x P0, and I25 x P0 interactions (Table 5).

Compared to full irrigation, Peroxidase content increased by 46.82%, 77.64%, and 110.98% for the upper medium, medium, and severe drought treatments, respectively. Additionally, compared to the control proline treatment, POD content increased by 43.92%, 72.66%, and 100.64% for the P200, P400, and P600 proline treatments, respectively. POD antioxidant activity increased by 150.78%, 109.26%, 90.62%, and 83.44% for the I100 x P600, I75 x P600, I50 x P600, and I25 x P600 interactions, respectively, compared to the I100 x P0, I75 x P0, I50 x P0, and I25 x P0 interactions (Table 5).

Compared to full irrigation, catalase content increased by 17.75%, 38.93%, and 61.37% for the upper medium, medium, and severe drought treatments, respectively. Additionally, compared to the control proline treatment, CAT content increased by 17.52%, 33.67%, and 49.36% for the P200, P400, and P600 proline treatments, respectively. CAT antioxidant activity increased by 78.76%, 62.27%, 35.70%, and 36.83% for the I100 x P600, I75 x P600, I50 x P600, and I25 x P600 interactions, respectively, compared to the I100 x P0, I75 x P0, I50 x P0, and I25 x P0 interactions (Table 5).

Compared to control irrigation, hydrogen peroxide content increased by 20.57%, 38.45%, and 64.45% for the upper medium, medium, and severe drought treatments, respectively. Additionally, compared to the control proline treatment, H2O2 content decreased by 28.00%, 40.70%, and 56.01% for the P200, P400, and P600 proline treatments, respectively. H2O2 damage decreased by 61.84%, 57.49%, 56.55%, and 50.61% for the I100 x P600, I75 x P600, I50 x P600, and I25 x P600 interactions, respectively, compared to the I100 x P0, I75 x P0, I50 x P0, and I25 x P0 interactions (Table 5).

Enterobacteriaceae density in feed: The highest bacterial growth, 98.56%, was observed under full irrigation, while the lowest growth, 0.19%, occurred under severe drought condition. Bacterial growth in both upper medium and medium drought treatments remained below 1%. The highest prevalence of total enterobacteriaceae was observed in the P0 (no proline) treatment, reaching 54%, followed by 45% in the P200 proline dose. In the P400 and P600 treatments, bacterial numbers sharply decreased and remained below 1% (Figure 1).

Table 1. Physical and chemical properties of the experimental soil

EC (dSm^{-1})	pH	Organic matter (%)	Sand (%)	Clay (%)	Silt (%)	P2O5 (kg ha^{-1})	K2O (kg ha^{-1})	Cu (mg kg^{-1})	Mn (mg kg^{-1})	Fe (mg kg^{-1})	Zn (mg kg^{-1})
0.91	7.78	1.05	34.82	18.76	46.42	57.40	2120	1.21	3.77	4.09	1.13

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

SV	DF	F ratio						
		PH	**DM**	**RL**	**LA**	**RWC**	**LCC**	**NDF**
R	2	5.12	5.89	8.77	4.68	58.42	15.06	30.30
I	3	15.73**	48.32**	212.49**	62.52**	2309.95**	75.05**	381.22**
P	3	36.17**	48.55**	129.71**	117.21**	96.23**	248.26**	265.28**
I*P	9	5.80**	2.84**	3.76**	9.07**	2.79*	2.86**	3.16**
Error	30							
CV(%)		3.98	10.19	1.92	2.76	1.96	5.23	1.74
		ADF	**ADL**	**SOD**	**POD**	**CAT**	**H2O2**	**EBC**
R	2	4.76	221.34	22.23	1.32	0.14	5.70	0.94
I	3	75.43**	166.53**	1496.54**	71.63**	437.43**	2946.36**	341.02**
P	3	156.22**	100.22**	548.95**	203.65**	1334.86**	3952.54**	194.35**
I*P	9	2.59*	2.05*	7.57**	2.60*	7.75**	25.77**	186.89^{ns}
Error	30							
CV(%)		11.03	2.37	2.48	6.73	1.60	1.89	28.60

$^{+}$) *=significant at 0.05 level of probability, ** = significant at 0.01 level of probability, ns = non-significant, SV: source of variation, DF: degree of freedom, CV: coefficient of variation, R: replication, I: Irrigation, P: proline

$^{++}$) PH: plant height (cm^{-1}), DM: dry matter (%), RL: root length (cm^{-1}), LA: leaf area (cm^{-2}), RWC: relative water content (%), LCC: leaf chlorophyll content (SPAD), NDF: neutral detergent fiber (%), ADF: acid detergent fiber (%), ADL: acid detergent lignin (%), SOD: superoxide dismutase (U/g FW), POD: Peroxidase (U/g FW), CAT: catalase (U/g FW), H_2O_2: hydrogen peroxide (nmol/g FW), EBC: *Enterobacteriaceae* counts (cfu/m

Table 3 Agronomic and physiological responses of maize to drought stress and proline applications

Stress type	**P**	**Agronomic specifications**			**Physiological traits**		
		PH (cm^{-1})	**DM (%)**	**RL (cm^{-1})**	**LA (cm^{-2})**	**RWC (%)**	**LCC (SPAD)**
I_{100} (Full irrigation)	0	55.03 ± 1.03^{d}	8.52±0.69^{e}	45.59±1.86^{h}	139.51±3.50^{de}	83.18±1.07^{c}	24.63±3.26 ^{ef}
	200	61.06 ± 1.28^{c}	10.21±1.07^{cd}	48.03±1.74^{g}	156.16±4.15^{c}	87.25±1.17^{b}	31.93±1.89^{c}
	400	64.85 ± 0.89^{b}	12.25±0.26^{b}	51.59±1.89^{f}	162.67±7.11^{b}	88.92±1.29^{b}	36.26±1.07^{b}
	600	60.18 ± 1.01^{c}	15.30±0.45^{a}	52.96±2.33^{ef}	158.60±1.81^{bc}	91.85±1.85^{a}	40.88±1.55^{a}
I_{75} (Upper Medium)	0	55.51 ± 3.51^{d}	7.43±0.33^{efg}	48.63±1.52^{g}	121.27±2.42^{f}	74.59±1.63^{f}	19.67±1.76^{hı}
	200	61.26 ± 2.13^{bc}	8.62±0.50^{e}	53.18±0.48^{e}	153.72±2.07^{c}	77.44±2.21^{e}	25.26±1.58^{e}

	400	66.42 ± 5.12^{a}	10.62 ± 0.91^{c}	55.85 ± 0.25^{d}	165.45 ± 4.37^{a}	80.55 ± 2.07^{d}	30.97 ± 1.19^{cd}
	600	56.10 ± 0.65^{d}	12.25 ± 0.57^{b}	58.18 ± 0.58^{c}	155.49 ± 4.76^{c}	79.55 ± 0.85^{de}	36.68 ± 1.34^{b}
I_{50} (Medium)	0	49.89 ± 1.82^{e}	6.82 ± 0.35^{fgh}	54.48 ± 2.24^{de}	113.21 ± 3.79^{g}	57.07 ± 3.01^{j}	15.93 ± 1.54^{j}
	200	55.93 ± 3.27^{d}	7.97 ± 0.80^{ef}	57.96 ± 1.73^{c}	135.78 ± 4.40^{e}	61.18 ± 1.17^{i}	21.91 ± 2.32^{gh}
	400	58.06 ± 3.24^{cd}	8.85 ± 1.12^{de}	59.81 ± 1.97^{b}	140.04 ± 4.27^{de}	64.11 ± 1.19^{h}	26.00 ± 1.98^{e}
	600	60.78 ± 3.07^{c}	10.73 ± 0.69^{bc}	61.75 ± 2.06^{a}	143.33 ± 5.66^{d}	67.34 ± 1.67^{g}	29.21 ± 1.60^{d}
I_{25} (Severe)	0	37.81 ± 3.68^{g}	5.40 ± 0.42^{h}	28.66 ± 0.70^{k}	102.18 ± 3.08^{l}	40.65 ± 1.56^{m}	11.83 ± 1.19^{k}
	200	43.56 ± 4.55^{f}	6.37 ± 0.22^{gh}	30.41 ± 0.62^{j}	106.16 ± 3.42^{hi}	46.37 ± 1.29^{l}	17.69 ± 1.24^{ij}
	400	44.06 ± 4.00^{f}	7.40 ± 0.54^{efg}	32.48 ± 0.45^{i}	111.80 ± 4.18^{gh}	48.58 ± 0.67^{l}	21.51 ± 1.41^{gh}
	600	49.33 ± 3.37^{e}	7.32 ± 0.49^{efg}	32.37 ± 0.37^{i}	112.66 ± 3.00^{g}	52.59 ± 1.44^{k}	22.71 ± 1.23^{fg}
	Mean	**54.99**	**9.13**	**48.24**	**136.13**	**68.83**	**25.82**
	LSD	I= 6.73^{**}	I= 1.02^{**}	I= 2.85^{**}	I= 9.00^{**}	I= 1.26^{**}	I= 2.56^{**}
		P= 1.83^{**}	P= 0.78^{**}	P= 0.78^{**}	P= 3.15^{**}	P= 1.13^{**}	P= 1.14^{**}
		I*P= 3.68^{**}	I*P= 1.56^{**}	I*P= 1.56^{**}	I*P= 6.32^{**}	I*P= 2.26^{*}	I*P= 2.28^{**}

$^{+}$) *Significant at 0.05 level of probability, **Significant at 0.01 level of probability

$^{++}$) The same letters after means ± SE in each column indicate no significant differences according to the LSD test at P ≤ 0.05

$^{+++}$)I: irrigation treatments, P: proline aplications (0 mg L^{-1}, 200 mg L^{-1}, 400 mg L^{-1}, 600 mg L^{-1}

Table 4 Forage quality in maize seedlings subjected to drought stress and proline applications using in vitro

Stress type	**P**	**Forage quality traits**		
		NDF (%)	**ADF (%)**	**ADL (%)**
I_{100} (Full irrigation)	0	22.11 ± 1.17^{l}	20.03 ± 0.78^{de}	2.41 ± 0.48^{h}
	200	20.41 ± 1.52^{m}	12.99 ± 2.02^{gh}	2.23 ± 0.46^{i}
	400	18.03 ± 1.62^{n}	10.37 ± 0.70^{hij}	2.10 ± 0.43^{j}
	600	16.00 ± 2.02^{o}	7.48 ± 0.81^{j}	1.85 ± 0.45^{k}
I_{75} (Upper Medium)	0	26.96 ± 0.93^{i}	26.30 ± 1.00^{c}	2.83 ± 0.51^{f}
	200	24.66 ± 0.96^{j}	17.33 ± 2.76^{ef}	2.67 ± 0.50^{g}
	400	23.81 ± 0.74^{k}	11.29 ± 0.73^{hij}	2.56 ± 0.47^{g}
	600	21.85 ± 0.81^{l}	8.06 ± 0.62^{ij}	2.35 ± 0.43^{hi}
I_{50} (Medium)	0	34.41 ± 1.28^{e}	29.81 ± 0.73^{ab}	3.21 ± 0.44^{d}
	200	32.41 ± 1.19^{f}	18.52 ± 1.31^{ef}	3.10 ± 0.44^{de}
	400	30.37 ± 1.63^{g}	18.14 ± 1.61^{ef}	2.98 ± 0.46^{e}
	600	28.09 ± 1.22^{h}	9.44 ± 0.36^{ij}	2.83 ± 0.45^{f}
I_{25} (Severe)	0	39.85 ± 0.80^{a}	32.81 ± 0.96^{a}	4.42 ± 0.54^{a}
	200	37.66 ± 0.64^{b}	26.79 ± 1.88^{bc}	4.11 ± 0.49^{b}
	400	36.38 ± 0.86^{c}	22.11 ± 0.95^{d}	3.87 ± 0.49^{c}
	600	35.46 ± 0.89^{d}	15.78 ± 0.51^{fg}	3.75 ± 0.45^{c}
	Mean	**28.03**	**17.95**	**2.95**
	LSD	I= 1.39^{**}	I= 1.97^{**}	I= 0.19^{**}
		P= 0.41^{**}	P= 1.64^{**}	P= 0.06^{**}
		I*P= 0.82^{**}	I*P=3.31^{*}	I*P= 0.12^{*}

$^{+}$) *Significant at 0.05 level of probability, **Significant at 0.01 level of probability

$^{++}$) The same letters after means ± SE in each column indicate no significant differences according to the LSD test at P ≤ 0.05

$^{+++}$)I: irrigation treatments, P: proline aplications (0 mg L^{-1}, 200 mg L^{-1}, 400 mg L^{-1}, 600 mg L^{-1})

Table 5 Biochemical responses in maize leaf under drought stress and proline applications

Stress type	**P**	**Antioxidant enzyme and oxidant activity**			
		SOD ((U/g FW)	**POD (U/g FW)**	**CAT (U/g FW)**	**H2O2 (nmol/g FW)**
I_{100} (Full irrigation)	0	326.48 ± 2.64^{j}	11.56 ± 0.55^{k}	238.00 ± 3.49^{k}	544.63 ± 6.29^{d}
	200	391.15 ± 5.85^{h}	17.41 ± 0.87^{j}	301.26 ± 3.62^{j}	355.68 ± 2.52^{k}

	400	431.26 ± 5.75^{f}	21.73 ± 3.23^{i}	354.04 ± 5.48^{i}	298.41 ± 5.09^{l}
	600	504.85 ± 9.64^{cd}	28.99 ± 1.74^{gh}	425.44 ± 2.55^{fg}	207.85 ± 3.26^{n}
I_{75} (Upper Medium)	0	361.01 ± 5.79^{i}	18.03 ± 0.86^{j}	297.47 ± 1.34^{j}	605.62 ± 3.63^{c}
	200	421.88 ± 5.30^{fg}	28.67 ± 2.99^{h}	354.17 ± 6.39^{i}	451.17 ± 4.69^{g}
	400	491.67 ± 5.84^{d}	32.56 ± 0.36^{fg}	418.49 ± 6.61^{g}	381.70 ± 5.43^{j}
	600	519.62 ± 8.17^{bc}	37.73 ± 0.58^{de}	482.71 ± 4.05^{d}	257.47 ± 5.88^{m}
I_{50} (Medium)	0	404.37 ± 8.47^{gh}	23.34 ± 0.88^{i}	385.15 ± 4.86^{h}	701.23 ± 6.39^{b}
	200	452.22 ± 9.44^{e}	34.48 ± 0.54^{ef}	434.15 ± 2.61^{ef}	508.86 ± 3.41^{e}
	400	536.66 ± 6.39^{b}	39.26 ± 1.07^{d}	490.19 ± 5.61^{d}	432.67 ± 6.70^{h}
	600	616.06 ± 9.75^{a}	44.49 ± 1.2^{c}	522.63 ± 5.88^{c}	304.70 ± 5.02^{l}
I_{25} (Severe)	0	441.47 ± 6.66^{ef}	29.11 ± 0.87^{gh}	444.22 ± 5.94^{e}	823.15 ± 6.11^{a}
	200	523.67 ± 4.57^{bc}	37.51 ± 2.01^{de}	514.44 ± 6.97^{c}	610.11 ± 4.84^{c}
	400	618.63 ± 7.74^{a}	48.10 ± 1.55^{b}	561.62 ± 6.65^{b}	473.34 ± 6.30^{f}
	600	626.17 ± 8.36^{a}	53.40 ± 2.02^{a}	607.82 ± 4.45^{a}	406.52 ± 3.35^{i}
	Mean	**479.20**	**31.65**	**426.99**	**460.19**
	LSD	I= 5.44^{**}	I= 3.83^{**}	I= 14.42^{**}	I= 6.11^{**}
		P= 10.01^{**}	P= 1.79^{**}	P= 5.76^{**}	P= 7.35^{**}
		I*P= 20.04^{**}	I*P= 3.58^{**}	I*P= 11.53^{**}	I*P= 14.70^{**}

$^{+}$) *Significant at 0.05 level of probability, **Significant at 0.01 level of probability

$^{++}$) The same letters after means ± SE in each column indicate no significant differences according to the LSD test at $P \leq 0.05$

$^{+++}$) I: irrigation treatments, P: proline aplications (0 mg L^{-1}, 200 mg L^{-1}, 400 mg L^{-1}, 600 mg L^{-1})

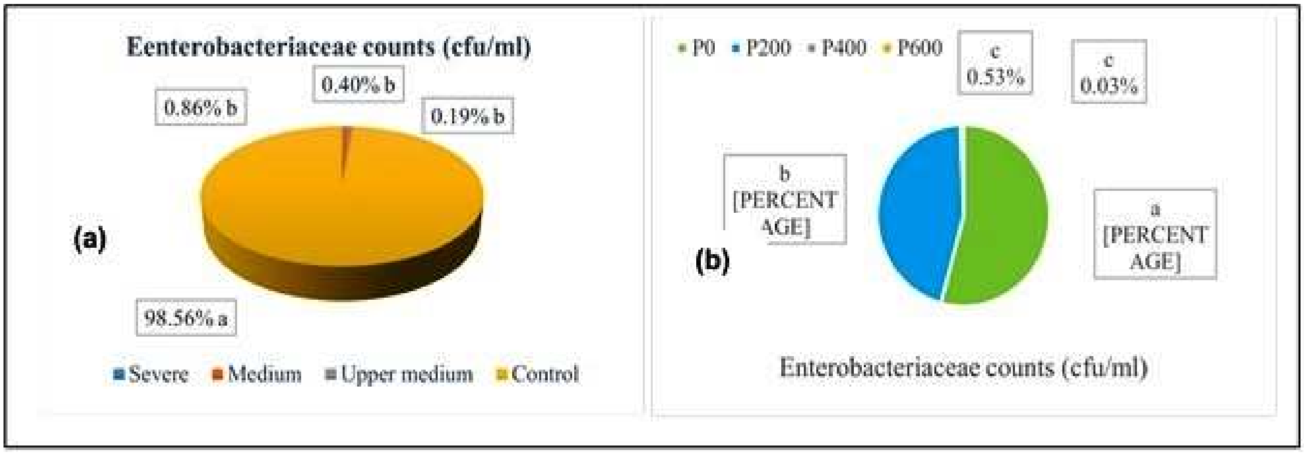

Figure 1. Enterobacteriaceae counts at different proline (a) (P0 (Control, non proline), P200 (200 mg L^{-1}), P400 (400 mg L^{-1}) and P600 (600 mg L^{-1}) and drought stress treatments (b) (Control (I_{100}, full irrigation), Upper medium (I_{75}), Moderate (I_{50}) and Severe (I_{25}) applied to maize plants up to V8 stage

DISCUSSION

Effects of exogenous proline on the agronomic of maize under drought stress: The maize variety showed tolerance up to medium drought in present study. Under severe drought, it is anticipated that the prolonged closure of stomata and reduced photosynthesis and respiration activities contributed to the decrease in PH. As the proline doses applied externally to the leaves increased, plant development and height improved. It is believed that external application of proline under drought stress conditions not only enhanced proline secretion in the roots and leaves of the plant but also supported energy production and growth by maintaining chlorophyll content and photosynthetic activity. In studies by Ibrahim et al., (2022) and Sah et al., (2020), and Shah et al., (2020), it was indicated that as drought intensity increased, plant height decreased, while external application of proline improved this condition.

All drought stress levels caused a significant DM loss. Aabsorption of water and nutrients under drought conditions is imparied, and plant growth and development are retarded. Foliar proline application positively affected DM content accross all drought stress levels, although magnitute of the effect decreased as drought severity increased. The improvement in plant growth under stress conditions can be attributed to improved cellular turgor and osmnotic balance within maize leaves. These findings agree with those reported by Sah et al. (2020), Shah et al. (2020) and Ibrahim et al. (2022).

Moderate drought stress treatments (I_{50} and I_{75}) initially stimulated root growth. This response was attributed to an adaptive strategy of maize plants to extend their root systems deeper into the soil to access moisture in deeper soil layers (Mahgoub et al., 2017). Root elongation, however, was severely inhibited or ceased completely under severe drought stress conditions. Therefore, the inhibition of maize growth under water stress can be linked to the reductions in root length and volume, which impaired water and nutrient absorption. The arithmetic increase in proline dosage also promoted root growth, with the most positive effect on root development observed under upper medium drought. Proline sprayed onto the leaves is hypothesized to facilitate osmotic balance between the roots and leaves, stimulating the release of antioxidant hormones such as proline and abscisic acid in the root zone.These results were consistent with previous studies (Sah et al., 2020; Cheng et al., 2021).

Effects of exogenous proline application on the physiology of maize plants under drought stress: Under severe drought application, LA significantly reduced, curled, and dried out. As the severity of drought decreased, LA increased, and the maize variety tolerated up to 25% water deficit. All proline applications had a positive effect on LA. The highest LA was observed under P400 and P600 proline treatments. In the I x P interaction, proline applied at all water levels positively influenced LA. The most significant effects of proline doses were recorded under upper moderate and moderate irrigation treatments. Proline enabled the plants to tolerate even 50% reduction in water availability. Turgor pressure decreased under drought stress, which caused cell expansion to cease and resulted in smaller leaf areas. The reduction in leaf area is linked to the stomal closure, which restricts carbon dioxide uptake photosynthetic activity. Additionally, drought-induced accumulation of abscisic acidnot only promotes this closure but also directly inhibits vegetative growth(Mossa et al., 2016; Ahmed et al., 2020).

Severe drought induced the highest water loss; however, foliar proline application improved RWC in a dose-dependent manner, an effect most pronounced under severe drought stress. Proline also mitigated drought-induced declines in osmotic and water potentials. This preservation of water status is attributed to stomatal closure and upregulated antioxidant enzyme activities, which maintain cellular integrity. These findings align with previous studies indicating that exogenous proline counteracts protoplasmic dehydration by regulating cell turgor and osmotic balance (Ibrahim et al., 2022; Xia et al., 2020; Li et al., 2024).

Even with minimal water deficit (I_{75}), a reduction in leaf chlorophyll content was observed, which became more pronounced as the level of water restriction increased. However, proline helped to stop the reduction in CC and even improved it. Particularly in interactions, it was noted that as water deficit increased, the positive effects of proline became more noticeable. In the study, as drought severity increased, the amounts of antioxidants such as SOD and CAT increased, suggesting that these mechanisms slow down photosynthesis and chlorophyll synthesis to conserve energy. Leaves closed their stomata to minimize water loss, which reduced both carbon dioxide and respiration, thereby negatively affecting photosynthesis and chlorophyll synthesis (Xia et al., 2020; Ibrahim et al., 2022).

Effects of exogenous proline on the forage quality of maize under drought stress: As the severity of drought increased, the NDF value also increased, with a near doubling of this increase under severe drought conditions. As water deficiency escalated, plant tissues experienced increased aging, which was reflected in the NDF values. Proline doses slightly reduce the aging in the cell walls caused by drought stress. In the I x P interaction, proline applied at all water levels had a positive effect on NDF values. The highest positive effect was observed under full irrigation, while the lowest effect was under severe drought conditions. The highest NDF values observed were ideal for a fully developed maize plant, but they were high for a seedling stage plant. Under drought stress conditions, the maize plant is thought to accelerate the production of hemicellulose, cellulose, and lignin in order to reduce water loss and strengthen the cell wall. In this study, it is hypothesized that foliar-applied proline, by promoting antioxidants like SOD and CAT, helped maintain intracellular water potential and slowed down the aging process.

As the level of drought increased, the cellulose structure and lignin content in maize tissues also increased, with the ADF value nearly doubling under severe drought. Under drought stress, proline applications showed a more significant improvement in ADF than in NDF. The applied proline doses did not hinder the increase in hemicellulose but are thought to have prevented the increase in cellulose and lignin content.

ADL is a feed quality parameter indicating lignin changes. As with other quality traits, drought stress decreased feed quality, and increased lignin content in the cell wall. This increase peaked under severe drought due to cellular damage. Proline application under drought stress slightly reduced and improved ADL values. In all drought applications, proline had a regulatory and stress-reducing effect. The forage quality results are consistent with those of other studies conducted by Kale et al., (2018), and Ferreira et al., (2021).

Effects of exogenous proline on antioxidant and oxidant traits of maize under drought stress: SOD is a potent antioxidant that reduces cellular damage in plants. SOD catalyzes the superoxide radicals (O_2^-), one of the ROS, into less harmful molecules, converting them into molecular oxygen and thereby reducing oxidative stress. In this study, as drought severity increased, the amount of hydrogen peroxide (H_2O_2) was found to rise, and correspondingly, the SOD content increased to mitigate cellular damage and enhance tolerance. Additionally, maize plants were thought to regulate osmotic balance within the cells, preventing water loss in leaf cells with the help of foliar-applied proline. In the I x P interaction, as the proline doses increased, the effectiveness of the SOD defense mechanism enhanced across all stress levels.

POD is part of the cellular defense mechanism against oxidative stress. In this study, as the drought level increased, particularly in leaf cells, POD levels increased, nearly doubling under severe drought conditions. In the I x P interaction, proline, compared to SOD, had a greater effect on increasing POD content and activity across all drought levels. POD enzymes are thought to reduce the harmful effects of H_2O_2 by converting it into less harmful compounds through reactions with water and other organic or inorganic substrates. On one hand, the increase in lignin accumulation in the cell wall due to drought accelerates aging in plants, while on the other hand, POD participates in lignin synthesis, strengthens the cell walls, and helps the plant cells prevent water loss.

Catalase is an important antioxidant enzyme induced in plants to mitigate oxidative stress. In this study, consistent with the responses of SOD and POD, CAT activity increased progressively with the severity of drought. Furthermore, foliar proline application significantly upregulated CAT activity. CAT catalyzes the dismutation of H_2O_2 into water and oxygen, thereby playing a role in cellular detoxification of homeostasisparticularly under severe drought stress conditions.

Hydrogen peroxide serves as both a strong oxidant and a signaling molecule. While SOD converts superoxide radicals into H_2O_2,it is the specific role of CAT and POD to scavenge this accumulated H_2O_2. Our results indicate that while drought stress intensified H_2O_2 accumulation in leaf cells, exogenous proline consistently mitigated this content. In the I x P interaction, proline application effectively reduced H_2O_2 levels accross all drought intensities.

The finding of antioxidant and oxidant are consistent with those of other studies. Water stress increases the formation of reactive oxygen species such as H_2O_2, which leads to oxidative damage in plants (Mansoor et al. 2022). However, the foliar application of proline may be responsible for clearing ROS and other free radicals (Kaul et al. 2008). Additionally, proline stabilizes free radicals, allows mitochondrial electron transport, enhances antioxidant enzyme activities such as SOD, POD, and CAT, improves stomatal conductance, and facilitates higher CO_2 diffusion from the leaves (Semida et al., 2020; Tariq et al., 2021; Abdou et al., 2022).

Effects of exogenous proline on enterobacteriaceae counts of maize under drought stress: Although drought stress negatively affected plant development and growth, it significantly reduced the density of Enterobacteriaceae and had a beneficial effect. It is predicted that the increase in Enterobacteriaceae numbers in forage was due to the interaction between irrigation and temperature. Another factor influencing and positively affecting the reduction of Enterobacteriaceae levels was the application of proline. There is limited research regarding Enterobacteriaceae contamination. Our study partially aligns with studies conducted on different plants, where it was reported that when temperature was fixed at 25°C and relative humidity was incrementally increased in experimental setups, the highest Enterobacteriaceae contamination in plants was found at the highest humidity levels (Weinberga et al., 2008; Blessington et al., 2014).

Conclusions: While water deficits generally negatively affected the development and growth of the plants, the exogeneous proline application attempted to mitigate the stress damage. Compared to full irrigation, in moderate water deficits such as upper moderate and moderate, the foliar application of proline tolerated the negative effects of drought on plant height. In comparison to stress-free irrigation, root length continued to grow under the Upper moderate (I75) and moderate (I50) treatments. Proline tolerated 25% water deficit for leaf area. Exogenous proline applications improved the destructive effects of drought on leaf chlorophyll content, with this improvement reaching up to 90% at the P600 dose. Drought increased the components of the cell wall and accelerated aging, but proline attempted to halt aging in the cell wall. Proline had a more positive effect on ADF compared to other forage quality parameters. Drought stress increased enzymatic antioxidant activity, which was further promoted by proline. The number of Enterobacteriaceae decreased as drought severity increased. High humidity, on the other hand, increased Enterobacteriaceae numbers. P400 and P600 proline doses reduced the Enterobacteriaceae count to below 1%. Leaf chlorophyll content was determined to be the most practical and cost-effective method for measuring plant drought stress and tolerance levels. An exciting result of the study was the effect of proline application during the maize seedling period, which contributed to between 25-40 % water saving.

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