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ARTICLE

Effect of Proline Pretreatment on the Water Stress Response in "Siete Caldos" Pepper Plants

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ABSTRACT: Exogenous proline is an effective agent for increasing plant tolerance to abiotic stress in plants. In this study, we evaluated its effect on seedlings of Siete Caldos chili pepper (*Capsicum frutescens*), a semi-domesticated variety. The Capsicum genus is known for its sensitivity to water stress. We pretreated the seedlings' roots by immersing them in proline solutions (0, 2.5, 5, 7.5, and 10 mM) for 48 h. Then, we exposed them to water stress using a Hoagland nutrient solution supplemented with 10% polyethylene glycol (PEG-8000) for nine days. We analyzed key physiological and biochemical parameters, including relative water content, cell membrane stability index, electrolyte leakage, chlorophyll, and proline content. The results indicated that proline concentrations of 2.5 and 5 mM significantly increased tolerance to water stress, with 100% survival. These seedlings maintained greater hydration and cell membrane stability compared to non-pretreated seedlings. In contrast, at the highest concentrations (7.5 and 10 mM Pro), survival was 63.63% and 54.54%, respectively. This study demonstrated that exogenous proline enhances water stress tolerance in *Capsicum frutescens* seedlings by mitigating the negative impact on physiological and biochemical processes vital for survival. This theoretical foundation can be applied to improve chili seedling performance in controlled production environments.

KEYWORDS: *Capsicum frutescens*; exogenous proline; tolerance; siete caldos chili pepper

1 Introduction

The chili pepper (*Capsicum* spp.) is a horticultural crop of importance for both gastronomy and the economy since its fruits are rich in various dietary and nutritional compounds, such as capsaicinoids, vitamins, and minerals [1,2]. In 2022, its global production reached ~37 million tons [3]. However, various environmental factors, such as salinity, drought, and extreme temperatures, can affect chili productivity [4,5].

Chili pepper cultivation is particularly vulnerable to water deficit in three critical stages: vegetative, flowering, and fruit development [6]. Among the morphological changes observed are the curling of the leaves, a delay in growth, and a reduction in the number of leaves [7]. Physically, there is a decrease in water content in shoots and roots, as well as photosynthetic pigments [8]. In biochemical terms, studies show that antioxidant and osmoprotective enzymes, such as proline (Pro), increase [7,9].

The search for strategies to improve tolerance to abiotic stress conditions and increase crop yields has focused on applying compounds such as growth regulators, hormones, polyamines, antioxidants, and exogenously [10]. Researchers have highlighted proline as an amino acid known for its efficacy as an osmoprotectant and signaling molecule, essential in primary metabolism in leaf and root tissues [11]. Several investigations have demonstrated that increasing proline accumulation positively correlates with regulating cell osmosis, stabilizing proteins and enzymes, and eliminating reactive oxygen species (ROS), all contributing to stress tolerance mechanisms [12–14].

Applying exogenous Pro through seed priming, foliar spraying, and rooting/root immersion increases tolerance to abiotic stress [15]. However, its efficiency depends on the development stage, species variety, application time, and concentration [16]. Researchers have reported that applying proline under drought conditions induces an increase in the activity of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), and peroxidases (POD), as well as improvements in fresh weight yield, shoot height, sugar content, photosynthetic pigments, phenolic compounds, and a reduction in electrolyte leakage. In addition to promoting the absorption and accumulation of nitrogen (N), phosphorus (P) and potassium (K^+) [17–19].

Researchers have reported that applying exogenous Pro in *Capsicum annuum* L. culture increases tolerance to salinity and temperature stress, affecting the activity of CAT, SOD, as well as photosynthetic and transpiration rate. They have also found that it improves growth and increases the relative water content (RWC), proline concentration, and proteins [20–22]. Given the need to search for strategies to enhance the natural tolerance of plants to abiotic factors and the limited research on the application of Pro in chili pepper crops under water stress conditions, this study aimed to determine how applying proline affects tolerance to water stress in a semi-domesticated species of *Capsicum frutescens* (Siete Caldos).

2 Materials and Methods

2.1 Obtaining of Seed

The seeds of *Capsicum frutescens* L., variety "Siete Caldos", were obtained from mature fruits collected in a shade house located in the ejido El Porvenir Agrarista (16°10′02″ N, 91°50′59″ W; 1488 m a.s.l.) in the municipality of La Trinitaria, Chiapas, Mexico. This semi-domesticated chili variety is cultivated for local consumption.

2.2 Germination and Acclimatization of Seedling

The seedling of Siete Caldos chili pepper (*Capsicum frutescens* L.) germinated in polystyrene seedbeds using a mixture of peat and perlite (3:1 v/v). Thirty days after emergence, they transferred the seedlings to a hydroponic system with Hoagland nutrient (0.2 mM $MgSO_4{\cdot}7H_2O$, 0.2 mM KH_2PO_4, 0.8 mM de $Ca(NO_3)_2{\cdot}4H_2O$, 1.2 mM KNO_3, 10 µM Fe-EDTA, 0.1 µM $NiCl{\cdot}6H_2O$, 0.1 µM $(NH_4)_6Mo_3O_{24}{\cdot}2H_2O$, 0.5 µM $CuSO_4$, 1 µM $ZnSO_4{\cdot}7H_2O$, 1 µM $MnSO_4{\cdot}H_2O$, 12.5 µM H_3BO_3 and 50 µM $CaCl_2$ (Sigma-Aldrich®, Merck KGaA, Darmstadt, Germany) in distilled water) at 1/5 of its ionic strength (i.s.) [23] for acclimatization over three days. The development and growth of the seedlings took place in a growth chamber with a photoperiod of 16 h of light and 8 h of darkness, maintaining an average temperature of 25 ± 2°C and a relative humidity of 52% [24].

2.3 Application of Priming with Proline

We applied proline through root immersion [25]. We placed the seedling 33 days after emergence in Hoagland nutrient solution (1/5 i.s.), supplemented with 0, 2.5, 5, 7.5, and 10 mM of proline (Sigma-Aldrich®, HPLC grade), for 48 h.

2.4 Exposure to Water Stress

We added polyethylene glycol (PEG 8000 Sigma-Aldrich®, reactive grade) to Hoagland's nutrient solution at 0% and 10% concentrations to induce water stress. We conducted the trial using a completely randomized factorial design with three replications. Each treatment included 15 plants housed in 250 mL glass containers. We maintained the ten treatments for nine days (216 h) with constant aeration, a photoperiod of 16/8 h light/dark, a temperature of 25 ± 2°C, and a relative humidity of 52%.

2.5 Growth Variables

At the end of exposure to water deficit, we determined the shoot's height, the root system's length, the fresh and dry weight, and the presence of flower buds.

2.6 Survival Rate and Relative Water Content

To calculate the percentage of survival, we used Eq. (1) [26] based on the number of plants living (P_l) and dead (P_d).

$$\text{Survival}\ (\%) = \frac{P_l}{P_l + P_d} \tag{1}$$

The relative water content (%RWC) of both the aerial and root systems was determined using Eq. (2), based on the protocol described by Jothimani et al. [27].

$$\%\text{RWC} = \left(\frac{\text{fresh weight} - \text{dry weight}}{\text{fresh weight}}\right) \times 100 \tag{2}$$

2.7 Cell Membrane Stability Index and Electrolyte Leakage

We strained three discs of fresh leaf tissue with a 1 cm diameter and 25 mg of root tissue in test tubes containing 15 mL of tri-distilled water (MEYER®, Mexico). We measured the initial electrical conductivity (EC_1) after two hours of incubation at room temperature. Then, we subjected the samples to a water bath at 120°C for 20 min to measure the final conductivity (EC_2). We measured electrical conductivity using the CON-BTA Vernier® conductivity probe. We used Eq. (3), described by Semida et al. [28], to determine the cell membrane stability index (MSI) and Eq. (4) to calculate electrolyte leakage Restrepo et al. [29].

$$\%\text{MSI} = \left(1 - \frac{EC_1}{EC_2}\right) \times 100 \tag{3}$$

$$\%\text{electrolyte leakage} = \left(\frac{EC_1}{EC_2}\right) \times 100 \tag{4}$$

2.8 Chlorophyll Content

We determined the total chlorophyll photosynthetic pigment following the protocol Inskeep et al. [30] described, with some modifications. We ground 50 mg of fresh leaf in 1.5 mL of 80% acetone (MEYER®, Mexico) and incubated at 4°C in the dark for 60 min. Next, we centrifuged the samples at 10,000 rpm for 5 min. We measured the absorbance at wavelengths (λ) of 664 and 647 nm using a HACH® DR 5000 spectrophotometer. Finally, we calculated the total chlorophyll concentration ($\mu g \cdot mL^{-1}$) using Eq. (5).

$$\text{Chl a} = 12.634A_{664} - 2.52A_{647} \tag{5}$$

2.9 Proline Content

To measure the proline content in roots and leaves, the methodology of Bates et al. [31] was followed, with modifications by Escalante-Magaña [32]. We transferred the supernatant obtained from fresh leaf and root tissue into test tubes containing a mixture of glacial acetic acid (MEYER®, Mexico) and acidic ninhydrin (Sigma-Aldrich®, Merck KGaA, Darmstadt, Germany). We incubated the samples in a water bath at 96°C for 60 min. Afterward, we added toluene (MEYER®, Mexico) and measured the absorbance of the organic phase at λ 520 nm. We used Eq. (6) to calculate the proline concentration.

$$\text{Pro}\ \left(\mu\,\text{moles} \times \text{g}^{-1}\right) = \left[\frac{\frac{(\mu\,\text{g proline} \times \text{mL}^{-1})(\text{mL toluene})}{115.5\,\mu \times \mu\,\text{mol}^{-1}}}{\frac{\text{g sample}}{5}}\right] \tag{6}$$

2.10 Statistical Analysis

To perform the statistical analysis, we used a complete factorial design. We analyzed the obtained data through an analysis of variance (ANOVA) and applied the LSD test with 95% reliability using the Statgraphics Centurion XIX software (Statgraphics Technologies, Inc., Madrid, Spain).

3 Results

3.1 Effect of Water Stress on Growth Parameters

The water stress induced by exposure to PEG (10%) negatively affected the growth of chili pepper plants. In Table 1, shoot height in plants without proline pretreatment [0 mM] and stress exposure decreased by ~16%. However, in the pretreated plants, no statistically significant difference appeared compared to the non-stressed plants. Regarding root length, under stress conditions without pretreatment, we observed a reduction of ~30%, while those pretreated with 2.5 mM of proline increased their length by ~20%, indicating a protective effect. The application of proline (5, 7.5, and 10 mM) under non-stress conditions decreased root length, but under stress conditions, we observed an improvement compared to plants without pretreatment. Additionally, water stress induced early flowering in plants without pretreatment and those pretreated with 7.5 and 10 mM Pro (Table 1).

Table 1: Effect of proline pretreatment on height, length, and flower bud number in chili pepper plants exposed to PEG

Ptt [mM Pro]	Shoot height (cm)		Root length (cm)		# Floral buds	
	%PEG					
	0	10	0	10	0	10
0	12.64 ± 1.12^{Aa}	10.6 ± 1.12^{Ba}	9.60 ± 1.40^{Aa}	6.65 ± 0.54^{Ac}	0^{B}	6^{Aa}
2.5	11.07 ± 1.18^{Aa}	11.02 ± 0.89^{Aa}	8.01 ± 0.46^{Bab}	10.11 ± 0.83^{Aa}	0^{B}	0^{Ad}
5	11.86 ± 0.82^{Aa}	12.31 ± 1.00^{Aa}	7.34 ± 0.25^{Ab}	8.25 ± 0.59^{Ab}	0^{B}	0^{Ad}
7.5	12.76 ± 0.83^{Aa}	12.08 ± 0.75^{Aa}	7.09 ± 0.71^{Ab}	7.64 ± 0.47^{Ab}	0^{B}	3^{Ab}
10	10.69 ± 0.72^{Aa}	11.71 ± 1.02^{Aa}	7.43 ± 0.92^{Aab}	7.62 ± 0.67^{Ab}	0^{B}	2^{Ac}

Note: Ptt: pretreatment; Pro: proline; PEG: polyethylene glycol. Distinct uppercase letters between columns represent statistically significant differences between treatments (%PEG), while different lowercase letters between rows indicate statistical differences between pretreatments (mM Pro). LSD test ($p \leq 0.05$), $n = 15$.

Fresh and dry shoot and root biomass parameters were reduced by ~50% in stressed plants without pretreatment [0 mM] compared to plants without stress (Table 2). Pretreatment with proline mitigated the adverse effects of osmotic stress in chili pepper plants at concentrations of 2.5 and 5 mM, showing a significant protective effect on fresh weight under stress conditions.

Table 2: Effect of proline pretreatment on fresh weight in chili pepper plants exposed to PEG

Ptt [mM Pro]	Shoot		Root	
	%PEG			
	0	10	0	10
0	0.63 ± 0.12^{Aa}	0.30 ± 0.10^{Bc}	0.07 ± 0.01^{Aa}	0.03 ± 0.004^{Bc}
2.5	0.44 ± 0.10^{Aab}	0.52 ± 0.06^{Aab}	0.05 ± 0.007^{Bab}	0.12 ± 0.02^{Aa}
5	0.37 ± 0.05^{Bb}	0.59 ± 0.08^{Aa}	0.03 ± 0.001^{Bb}	0.12 ± 0.01^{Aa}
7.5	0.41 ± 0.06^{Aab}	0.31 ± 0.03^{Abc}	0.03 ± 0.002^{Bb}	0.08 ± 0.01^{Ab}
10	0.32 ± 0.03^{Ab}	0.36 ± 0.06^{Abc}	0.03 ± 0.004^{Bb}	0.07 ± 0.01^{Abc}

Note: Ptt: pretreatment; Pro: proline; PEG: polyethylene glycol. Distinct uppercase letters between columns represent statistically significant differences between treatments (%PEG), while different lowercase letters between rows indicate statistical differences between pretreatments (mM Pro). LSD test ($p \leq 0.05$), $n = 15$.

3.2 Effect of Water Stress on Physiological and Biochemical Parameters

Pretreatments with a 2.5 and 5 mM proline positively affected the survival rate by maintaining 100% in plants exposed to water stress (Table 3). For their part, all proline pretreatments [2.5, 5, 7.5, and 10 mM] increased the chlorophyll concentration compared to untreated plants [0 mM Pro] and exposed to stress (10% PEG); under these stress conditions, untreated plants [0 mM] exhibited a drastic decrease of ~37% compared to the control plants.

Table 3: Effect of proline pretreatment on survival percentage and total chlorophyll concentration in chili pepper plants exposed to PEG

Ptt [mM Pro]	%Survival		Total Chlorophyll ($\mu g \cdot mL^{-1}$)	
	%PEG			
	0	10	0	10
0	100^{Aa}	45.45^{Bd}	33.11 ± 0.56^{Abc}	20.60 ± 0.95^{Bc}
2.5	100^{Aa}	100^{Aa}	34.76 ± 0.22^{Aab}	32.33 ± 0.78^{Ba}
5	100^{Aa}	100^{Aa}	35.21 ± 0.60^{Aa}	31.58 ± 0.54^{Bab}
7.5	100^{Aa}	63.63^{Bb}	32.73 ± 0.88^{Ac}	30.39 ± 1.03^{Aab}
10	100^{Aa}	54.54^{Bc}	32.67 ± 0.73^{Ac}	29.67 ± 0.63^{Bb}

Note: Ptt: pretreatment; Pro: proline; PEG: polyethylene glycol. Distinct uppercase letters between columns represent statistically significant differences between treatments (%PEG), while different lowercase letters between rows indicate statistical differences between pretreatments (mM Pro). LSD test ($p \leq 0.05$), $n = 15$.

Table 4 presents the relative water content (RWC) in the shoot and root of chili pepper plants under stress conditions. The values obtained show that the plants pretreated with 2.5 and 5 mM maintained an

RWC similar to that of the non-stressed plants, both in the shoot (86.23% and 86.31%) and in the root (78.40% and 79.34%), indicating more excellent resistance to PEG-induced dehydration. Pretreatments with 7.5 and 10 mM reduced the RWC in both the shoot and root, but the reduction was less severe than in plants without pretreatment.

Table 4: Effect of proline pretreatment on relative water content (%RWC) in chili pepper plants exposed to PEG

Ptt [mM Pro]	Shoot		Root	
	%PEG			
	0	10	0	10
0	85.66 ± 0.81^{Aa}	75.74 ± 0.81^{Bc}	80.66 ± 0.52^{Aa}	57.55 ± 1.93^{Bc}
2.5	86.30 ± 1.23^{Aa}	86.23 ± 0.83^{Aa}	80.37 ± 0.81^{Aa}	78.40 ± 0.92^{Aa}
5	86.54 ± 1.10^{Aa}	86.31 ± 0.33^{Aa}	80.29 ± 0.80^{Aa}	79.34 ± 0.84^{Aa}
7.5	85.05 ± 1.80^{Aa}	80.79 ± 1.83^{Ab}	79.04 ± 0.85^{Aa}	71.04 ± 1.95^{Bb}
10	85.95 ± 0.81^{Aa}	78.61 ± 1.82^{Abc}	80.21 ± 0.58^{Aa}	70.95 ± 1.97^{Bb}

Note: Ptt: pretreatment; Pro: proline; PEG: polyethylene glycol. Distinct uppercase letters between columns represent statistically significant differences between treatments (%PEG), while different lowercase letters between rows indicate statistical differences between pretreatments (mM Pro). LSD test ($p \leq 0.05$), $n = 15$.

Table 5 shows electrolyte leakage values are higher in root tissue than in shoot. Under stress-free conditions, electrolyte leakage in the shoots and roots of chili pepper plants ranged from ~24% to 35%, respectively. However, treatment with 5 mM Pro showed the lowest electrolyte leakage in both tissues compared to non-stress plants. Under stress conditions, plants without pretreatment experienced an increase of ~105% compared to control plants, indicating more significant cell damage. The shoots of the plants treated with 7.5 and 10 mM Pro showed an increase of ~59.66% and 88.86%, respectively, while in the roots, it was ~75.24% and 81.66% compared to the non-stressed plants. In contrast, shoots and roots pretreated with 2.5 and 5 mM Pro exhibited values similar to control plants.

Table 5: Effect of proline pretreatment on electrolyte leakage in chili pepper plants exposed to PEG

Electrolyte leakage (%)				
Ptt [mM Pro]	Shoot		Root	
	%PEG			
	0	10	0	10
0	24.22 ± 0.82^{Ba}	54.08 ± 1.03^{Aa}	34.86 ± 0.70^{Bab}	70.79 ± 1.18^{Aa}
2.5	22.62 ± 0.68^{Aab}	24.63 ± 0.91^{Ad}	32.68 ± 1.21^{Aab}	35.17 ± 1.72^{Ac}
5	20.64 ± 0.71^{Bb}	24.36 ± 0.50^{Ad}	30.63 ± 0.80^{Bac}	34.97 ± 1.19^{Ac}
7.5	24.80 ± 1.39^{Ba}	39.63 ± 1.33^{Ac}	35.38 ± 1.64^{Ba}	61.99 ± 2.43^{Ab}
10	25.01 ± 0.40^{Ba}	47.22 ± 0.62^{Ab}	35.97 ± 1.92^{Ba}	65.21 ± 1.56^{Ab}

Note: Ptt: pretreatment; Pro: proline; PEG: polyethylene glycol. Distinct uppercase letters between columns represent statistically significant differences between treatments (%PEG), while different lowercase letters between rows indicate statistical differences between pretreatments (mM Pro). LSD test ($p \leq 0.05$), $n = 15$.

Since the cell membrane stability index (MSI) inversely correlates with electrolyte leakage, an increase in leakage means a decrease in membrane stability. Under stress-free conditions, plants exhibited an MSI greater than 74% in shoots and 64% in roots. Under stress, the lowest MSI values appeared in plants without pretreatment. In contrast, plants pretreated with 2.5 mM of Pro maintained MSI values comparable to those without stress in both tissues, suggesting that this concentration of Pro effectively preserves membrane stability during water stress (Table 6).

Table 6: Effect of proline pretreatment on the cell membrane stability index (MSI) in chili pepper plants exposed to PEG

Membrane stability index (%)				
Ptt [mM Pro]	**Shoot**		**Root**	
	%PEG			
	0	**10**	**0**	**10**
0	75.77 ± 0.82^{Ab}	45.91 ± 1.03^{Bd}	65.13 ± 0.70^{Aab}	29.20 ± 1.18^{Ba}
2.5	77.37 ± 0.68^{Aab}	75.36 ± 0.91^{Aa}	67.31 ± 1.21^{Aab}	67.31 ± 1.72^{Ac}
5	79.35 ± 0.71^{Aa}	75.63 ± 0.50^{Ba}	69.36 ± 0.80^{Aac}	65.02 ± 1.19^{Ac}
7.5	75.19 ± 1.39^{Ab}	60.36 ± 1.33^{Bb}	64.61 ± 1.64^{Aa}	38.01 ± 2.43^{Bb}
10	74.98 ± 0.40^{Ab}	52.77 ± 0.62^{Bc}	64.02 ± 1.92^{Aa}	34.78 ± 1.56^{Bb}

Note: Ptt: pretreatment; Pro: proline; PEG: polyethylene glycol. Distinct uppercase letters between columns represent statistically significant differences between treatments (%PEG), while different lowercase letters between rows indicate statistical differences between pretreatments (mM Pro). LSD test ($p \leq 0.05$), $n = 15$.

The concentration of proline in leaves and roots increased markedly with pretreatment and with water stress (Table 7). Under stress-free conditions, the 2.5 and 5 mM doses of Pro markedly increased Pro levels by ~4.15 (leaves) and ~3.26 (roots) times compared to non-stressed plants. In contrast, at 7.5 and 10 mM, the increase was ~2 times the basal content in both tissues. In the presence of PEG, the data analysis shows a statistically significant difference in the endogenous proline content between the pretreatments, being more lavish with 2.5 and 5 mM Pro in both tissues.

Table 7: Effect of proline pretreatment on endogenous proline content (μmoles Pro·gPF·mL^{-1}) in chili pepper plants exposed to PEG

Ptt [mM Pro]	**Leaves**		**Root**	
	%PEG			
	0	**10**	**0**	**10**
0	38.45 ± 3.22^{Bd}	202.39 ± 3.26^{Ad}	31.80 ± 1.59^{Bab}	152.18 ± 5.73^{Ad}
2.5	161.41 ± 5.59^{Ba}	472.42 ± 3.91^{Aa}	105.11 ± 4.81^{Bab}	364.78 ± 5.37^{Aa}
5	158.73 ± 3.69^{Ba}	469.91 ± 2.78^{Aa}	103.72 ± 3.34^{Bac}	362.62 ± 4.08^{Aa}
7.5	87.98 ± 3.99^{Bb}	311.00 ± 1.89^{Ab}	69.46 ± 3.12^{Ba}	217.16 ± 3.06^{Ab}
10	73.55 ± 4.40^{Bc}	231.93 ± 3.93^{Ac}	57.30 ± 2.57^{Ba}	209.92 ± 4.51^{Ac}

Note: Ptt: pretreatment; Pro: proline; PEG: polyethylene glycol. Distinct uppercase letters between columns represent statistically significant differences between treatments (%PEG), while different lowercase letters between rows indicate statistical differences between pretreatments (mM Pro). LSD test ($p \leq 0.05$), $n = 15$

4 Discussion

During water stress, physiological and biochemical processes are affected, severely affecting crop growth and development [33]. Researchers have identified proline as one of the metabolites contributing to plant stress tolerance mechanisms. Exogenous proline can alleviate the damage caused by abiotic stress and improve growth [15,34,35]. Our results showed that the Siete Caldos chili pepper plants (*Capsicum frutescens* L.) presented a remarkable susceptibility to the water deficit induced by PEG-8000. At the same time, the pretreatment with proline significantly improved their tolerance, evidencing its efficacy in mitigating the adverse effects of stress.

Previous studies have shown that water stress hurts the growth of various crops [36–38]. A typical response to water deficit is the reduction of fresh biomass due to the inhibition of cell expansion that limits the growth of leaves, stems, and roots [39]. In this study, applying different proline concentrations to non-stressed plants did not result in significant height or root length changes, possibly because we assessed the plants nine days after the treatments were applied. However, exposure to 10% PEG-8000 affected shoot height and root length to some degree, a ~50% decrease in fresh biomass of the aerial part compared to control plants (Tables 1 and 2). These results are consistent with those described by Pino et al. [39], who observed a ~82% and 92% decrease in total weight in two wild potato species (*Solanum tuberosum* and *Solanum commersonni*), induced *in vitro* drought with PEG-4000 at 4% and 8%. Other studies have reported similar reductions in fresh and dry weight in different tomato varieties (*Solanum lycopersicum*) exposed to PEG-6000 at 2%, 4%, 6%, 8%, and 10%, highlighting that the magnitude of the growth reduction depends on the tolerance of each variety [40,41]. Xu et al. [42] attributed a ~16% reduction in fresh and dry biomass in tobacco plants (*Nicotiana tabacum* L. K326) exposed to 20% PEG-6000 due to cell damage from increased reactive oxygen species. We observed that parameters related to dehydration escape mechanisms were activated nine days after the onset of stress. These results are consistent with the evidence observed in crops such as rice, corn, barley, wheat, chili, and tomato that show that drought accelerates flowering in some plants to complete their life cycle before conditions become more adverse [43–45].

Studies have shown that treatment with appropriate concentrations of exogenous proline can effectively reduce the adverse effects of abiotic stress [25]. High doses negatively impact plant growth by significantly reducing the effect of the root's total soluble protein or causing toxic effects [46–48]. In our study, proline pretreatment indicated an optimal dose effect on tolerance to water stress tolerance and an optimal dose effect on tolerance to water stress tolerance in this Capsicum variety. The effects of exogenous Pro application vary depending on the species, growth stage, and concentration. Proline pretreatment at 2.5 and 5 mM significantly improved the parameters related to the stress response. In contrast, though beneficial, the positive effects of 7.5 and 10 mM treatments were not drastic. Our results are consistent with previous reports. Alkahtani et al. [19] observed that foliar application of 10 mM Pro significantly increased the fresh and dry weight of the shoot and root in sugar beet plants under drought conditions. Similarly, Elewa et al. [17] found that doses of 12.5 and 25 mM Pro mitigated drought effects in quinoa (*Chenopodium quinoa*), improving shoot weight, particularly with the 25 mM dose, due to enhanced tissue water status. In wild tobacco (*Nicotiana tabacum*), cultivating Petit Havana SR1 [49] reported three foliar applications of 10 mM Pro before a water deficit increased shoots and roots biomass. This effect resulted from increased ATP production generated by Pro metabolism, which promotes growth and stress tolerance.

A plant's survival rate closely depends on its relative water content (RWC), which reflects its water status [50]. In this study, we observed a decrease in survival when the RWC dropped in the tissues of chili plants exposed to PEG. Proline application at doses below 5 mM significantly improved both parameters, likely by maintaining turgor and osmotic balance, as Ibrahim et al. [16] reported for maize with 2 and 4 mM Pro application. Abdelaal et al. [51] reported that 10 mM of proline mitigated RWC reduction in barley under

stress conditions. Similar results have been reported in sugar beet (*Beta vulgaris* L. cv. Samba) and two wheat varieties (*Triticum aestivum* L.) [18,19].

Although higher doses were less effective than lower ones, they positively influenced chlorophyll content in chili plants, even without stress. Water stress damages photosystems and triggers the overproduction of reactive oxygen species (ROS), reducing chlorophyll content and photosynthetic rate [27,52,53]. Exogenous proline regulates chlorophyll synthesis by enhancing enzymatic activity and adjusting genetic and hormonal regulation, thereby contributing to photosynthetic stability [54]. Demiralay et al. [25] found that in maize plants, a low dose of proline [1 mM] was more effective than a high dose [10 mM] in mitigating photosystem damage. This improvement resulted from enhanced gas exchange, transpiration rate, substomatal CO_2 levels, and stomatal conductance [17].

The exogenous application of proline induced an increase in endogenous proline, as observed in this study, where the endogenous proline content in chili plants increased across all treatments except in the control plants (without pretreatment and stress). Exogenous proline stimulates metabolic regulation by activating genes responsible for its synthesis, enhancing osmoregulation, and maintaining osmotic balance [55,56]. These coordinated actions not only provide a direct supply of proline but also modulate the plant's physiology to increase endogenous proline content, even in the absence of external stress [15,54,57]. Under stress conditions, we observed an increase in endogenous proline (Table 7), which aligns with the findings of Landi et al. [52] in tomato plants (*Solanum lycopersicum* L., cultivar Red Setter 1753) subjected to 15% PEG-8000 for 48 h. These authors reported endogenous proline concentrations 27 times higher (~6 $mg \cdot g^{-1}$ FW) than in control plants. They found that proline synthesis correlated with the overexpression of the *P5CS* gene, thereby enhancing drought tolerance. Similarly, Cacefo et al. [58] observed an increase in proline concentrations in the leaves and roots of wild tobacco pretreated with 10 mM proline and exposed to drought. Elewa et al. [17] also reported increased proline and free amino acid content in quinoa plants under water deficit, improving osmotic adjustment. Farooq et al. [18] highlighted that proline accumulation could result from increased precursors such as ornithine, glutamic acid, and arginine. This response helps mitigate drought effects and sustain growth under adverse conditions [59], as observed in this study's pretreated chili plants under stress conditions.

5 Conclusion

Pretreatment with proline is an effective strategy to increase the resilience of Siete Caldos (*Capsicum frutescens* L.) chili pepper plants against water stress; concentrations of 2.5 and 5 mM of proline proved to be particularly effective in this semi-domesticated species, significantly improving cell membrane stability, water retention, biomass and reducing electrolyte leakage under conditions of water deficit. Because higher proline concentration did not improve the system, it reaffirms the importance of establishing accurate dosing to optimize osmoprotective effects.

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