

## Exogenous glutathione-mediated tolerance to deficit irrigation in salt-affected *Capsicum frutescense* (L.) plants is connected with higher antioxidant content and ionic homeostasis

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### Abstract

As an important medicinal plant used in traditional and modern medicine, chili peppers are sensitive or moderately sensitive to drought or salt stress, respectively. Therefore, potential changes due to foliar-applied glutathione (GSH; 0, 0.4 and 0.8 mM) response on growth, yield, and physio-biochemical attributes, as well as water use efficiency (WUE) and fruit alkaloid capsaicin of chili pepper plants were investigated when grown under deficit irrigation in salt-affected soil ( $EC = 6.74 \text{ dS m}^{-1}$ ). Two deficit irrigation water (DiW) regimes (80% and 60% of soil field capacity; FC) were used versus 100% of FC as a control. Both DiW treatments negatively affected growth and yield parameters, SPAD chlorophyll index, nutrient status,  $K^+/Na^+$  ratio, and plant anatomical features. In contrast, osmoprotectants, ascorbate, glutathione, capsaicin, and phenolic contents, as well as WUE were increased in association with higher  $Na^+$  and  $Cl^-$  contents. However, exogenously-applied GSH caused significant increases in the above-mentioned parameters along with an additional increase in osmoprotectants, antioxidants, and capsaicin contents, and a decrease in  $Na^+$  and  $Cl^-$  levels compared to corresponding controls. The highest WUE, growth, and fruit yield responses were recorded at 0.8 mM GSH applied to plants under DiW at 80% FC + salinity ( $6.74 \text{ dS m}^{-1}$ ). Therefore, this study suggested the use of leafy-applied GSH at 0.8 mM for satisfactory growth and yield with the highest WUE of chili pepper plants grown under salt-affected conditions with deficit irrigation.

**Keywords:** anatomy; capsaicin; chili pepper; environmental stresses; exogenous antioxidant applications; nutrient

## Introduction

During their life cycles, higher plants always face environmental stress factors, the most dangerous of which are the shortage of fresh water for irrigation and soil salinity. Deficiency of irrigation water (DiW) and soil salinity stress (SsS) solely or in combination severely affect the growth and productivity of most plant species (Abd El-Mageed *et al.*, 2018; Yang *et al.*, 2019; Abid *et al.*, 2020). As freshwater is the most important resource and a limiting factor for agricultural development, its diminishing availability has become a global problem that requires the development of alternative resources for agricultural use. Therefore, practices that increase water use efficiency (WUE) and reduce the amount of added water are important for water conservation. Besides, the expected soil salinization due to DiW must also be addressed by applying useful techniques in this regard.

Through some efforts, plants are participating in a series of changes in morphology, physiology, biochemistry, and molecular biology to overcome such adversities from these stressors. Understanding the response of plants to combined stressors is unavoidable to improve plant adaptations under open field conditions (Abd El-Mageed *et al.*, 2018; Desoky *et al.*, 2019; Rady *et al.*, 2019; Alharby *et al.*, 2020). In particular, SsS and DiW are often combined either due to the presence of salt naturally in the soil or to an increase in its accumulation due to a lack of salt washing as a result of a DiW. Thus, the low washing of salts from agricultural land due to the DiW is one of the main reasons for increasing soil salinization. Besides the DiW, poor management of available water in a hot climate of low-rainfall agricultural regions also contributes to soil salinization (Tester and Bacic, 2005; Yang *et al.*, 2019; Alharby *et al.*, 2020; Osman *et al.*, 2020). Therefore, to prevent the loss of agricultural products, more research should be developed to adopt uncomplicated techniques for agricultural producers to use for stressful crop plants to cope with stresses that worsen day by day.

In a similar fashion to DiW, plants respond to SsS. Like drought, osmotic mechanisms related to SsS and restricted the availability of water and thus decreased plant growth along with cellular metabolic alterations (Munns, 2002; Abid *et al.*, 2020). The SsS and/or DiW strictly reduces the plant's ability to use water, which disrupts cell water content and turgor. Cell enlargement, nutrient homeostasis, photosynthetic machinery, and other metabolic processes are also disrupted due to these two stresses. Also, enzyme catalysts including Rubisco enzymes are inhibited, and specific toxic (e.g. Na<sup>+</sup> and Cl<sup>-</sup>) ions are raised, and plant death is finally unavoidable (Munns, 2002; Farooq *et al.*, 2017; Hussain *et al.*, 2018; Alharby *et al.*, 2020). SsS threatens the existence of plants, especially those sensitive to high salt levels (Horie *et al.*, 2011). Regardless of whether DiW is present, SsS causes "physiological drought" and ion toxicity, affecting plant growth, yield, photosynthesis, osmoprotectants, antioxidants, nutrient status, anatomy, and WUE (Abd El-Mageed *et al.*, 2018; Shahid *et al.*, 2018; Alharby *et al.*, 2020). Additionally, SsS-DiW-plant relations often restrict cellular normal physiological activities and the productive capacity of crop plants (Alharby *et al.*, 2020).

To cope with stress, plants develop and/or adopt the preservative antioxidant system, including soluble sugars, free proline, glutathione, ascorbate, phenolic compounds, capsaicin, etc. (Abdel Latef *et al.*, 2017a, b, c, 2018, 2019a, b, 2020; Caliskan *et al.*, 2017; Rady *et al.*, 2019; Alharby *et al.*, 2020; Taha *et al.*, 2020). In most cases, the plant antioxidant system is not sufficient to enable it to withstand high stress (e.g., SsS + DiW). Therefore, exogenous supplementation of antioxidants such as glutathione (GSH) should be used to raise plant tolerance to the combined stress under study (Rady and Hemida, 2016; Hasanuzzaman *et al.*, 2017; El-Beltagi *et al.*, 2020).

Antioxidants protect plants from abiotic stress-induced oxidative damage (Rady and Hemida, 2016; El-Beltagi *et al.*, 2020). Among them, GSH (c-glutamyl-cysteinyl-glycine) is a low molecular weight and water-soluble thiol compound, which is widely distributed in most plant tissues. Regardless of its role in storing and transporting reduced sulfur, GSH is involved in the detoxification of reactive oxygen species -ROS (Foyer and Noctor, 2005a, b). It acts as a co-factor in different biochemical reactions. It interacts with hormones, signaling molecules, and its redox state triggers signal transduction (Foyer and Noctor, 2005a, b). It also plays a vital role

in detoxifying toxic metals/metalloids and xenobiotics (Srivalli and Khanna-Chopra, 2008). Thus, GSH can participate in plant growth and development under conditions of both normal growth and stress. Exogenously-applied GSH can enhance plant tolerance to different abiotic stresses, including salinity and drought (Hasanuzzaman *et al.*, 2017).

Pepper (*Capsicum* spp.) is one of the most important, most popular, and most favourite vegetable crops cultivated in both greenhouses and open fields worldwide, especially in the warm-climate, including Egypt. Medicinally, fruits of chili pepper (*Capsicum frutescense* L.) are an excellent source of bioactive products, natural colors, and antioxidant compounds, especially the alkaloid 'capsaicin', which is linked to the plant's response to stressful conditions (Phimchan and Techawongstien, 2012; Kpinkoun *et al.*, 2019). Chili peppers are sensitive or moderately sensitive to drought or salt stress, respectively (Lee, 2006; Ben-Gal *et al.*, 2008; Abdel Latef and Chaoxing, 2014). The growth and productivity of peppers are negatively affected under SsS and DiW (Mardani *et al.*, 2017; Taha *et al.*, 2018; Abd El-Mageed *et al.*, 2020). The fruit alkaloid 'capsaicin' content as an important bioactive compound in peppers has been reported to increase significantly under DiW (Kopta *et al.*, 2020) and SsS (Kpinkoun *et al.*, 2019).

As we know, there are no studies available on the effect of combined stress (DiW + SsS) on growth, fruit yield, and fruit alkaloid capsaicin content in chili pepper and mitigating the combined stress effects by leafy-applied GSH. Therefore, the main objective of the present study was to evaluate the potential positive impacts of GSH applied as a foliar spray to chili pepper plants stressed with combined SsS and DiW on growth, yield, and fruit capsaicin content. The possibility of reaching the ion homeostasis due to the modification of osmoprotectants and antioxidants, including phenols and capsaicin by the application of GSH was also evaluated under the tested combined stress.

## Materials and Methods

### *Experimental location, climatic conditions, and soil analysis*

A pot experiment was conducted three times simultaneously in the 2018/2019 summer season using an open greenhouse in the experimental farm (Southeast of Fayoum; 29° 17'N; 30° 53'E) at the Faculty of Agriculture, Fayoum University, Egypt. Average climatic conditions throughout the experimental period (March 28-June 28) were  $32 \pm 3/18 \pm 2$  °C for average day/night temperatures,  $65 \pm 4\%$  for average relative humidity, and 13 h for average daylight length. For light intensity, natural sunlight was suitable for all chili pepper growth stages. Soil physical and chemical properties (Wilde *et al.*, 1985) indicated that the soil electrical conductivity (EC) was  $6.74 \text{ dS m}^{-1}$ , indicating that it was saline soil (Dahnke and Whitney, 1988). It is characterized as a loamy clay with 22.4% for field capacity, 7.57 for pH, 1.02% as organic matter content, 22.4, 25.8, and 21.1 meq L<sup>-1</sup> for Ca<sup>2+</sup>, Na<sup>+</sup> and Cl<sup>-</sup> concentrations, and 15.9, 124.0, and 372.0 mg kg<sup>-1</sup> dry soil for N, P, and K<sup>+</sup>, respectively.

### *Plant material, experimental layout, and cultural practices*

Chili pepper seeds were obtained from the Agricultural Research Centre (ARC), Giza, Egypt. They were incubated for germination on January 25 in a private nursery, then the standardized transplants (with 2-3 pairs of true expanded leaves) were transplanted into pots (40 cm in diameter, each 12 kg of air-dried soil) on March 28 at a rate of two transplants per pot. Before transplantation, treatments were arranged in a completely randomized layout in a factorial design. Regardless of the irrigation system as an experimental factor, standard agronomic practices were applied including pest and disease control, and fertilization program as recommended for commercial peppers production. Before transplanting, 2.4 g of NPK fertilizer (20, 20, and 20% of N, P<sub>2</sub>O<sub>5</sub>, and K<sub>2</sub>O, respectively) was allocated for each pot (12 kg soil). After transplanting, foliar fertilization was applied once a week, regularly for all plants with 1g Kristalon L<sup>-1</sup> (YARA Agri, Staré Me<sup>o</sup>sto, Czech Republic),

which contains 20, 5, 10, and 2% of N, P<sub>2</sub>O<sub>5</sub>, K<sub>2</sub>O, and Mg, respectively starting from 20 days after transplanting (DAT) until the end of the flowering stage.

#### *Treatments and other practices*

With saline soil ( $EC_e = 6.74 \text{ dS m}^{-1}$ ), two factors were applied. Irrigation water was applied until the end of the experiment (28 June) in 3 regimes (60%, 80%, and 100%) based on soil field capacity (FC) each of 60 pots. The pots for each water regime were divided into three groups, each of 20 pots, intended for one of three levels of GSH; 0 (distilled water as a control), 0.4, and 0.8 mM. Therefore, a total of 9 treatments (3 water regimes  $\times$  3 GSH levels), all of them applied under combined stress (DiW + SsS;  $6.74 \text{ dS m}^{-1}$ ). The GSH concentrations used in this study were selected based on a preliminary study in which the concentrations of 0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mM GSH were applied, and the concentration of 0.8 mM produced the best response (data not shown), therefore, it was chosen along with 0.4 mM for this study.

Once the transplanting was completed, the GSH solutions were sprayed using Dorsal Spray Machine (20 L). The seedlings were allowed to establish for 14 days after transplantation (DAT), then irrigation water treatments were started. At the same time (14 DAT), the plants were sprayed again with GSH solutions. The spraying was then repeated with GSH solutions at 28 and 42 DAT. To avoid runoff and to ensure penetration of spray solutions into leaf tissues, Tween-20 (1%) was added to the spraying solutions as a wetting agent. Using time-domain reflectometry (TDR200, Soil Moisture Equipment, Spectrum, USA), monitoring and measuring water deficits were implemented for different water treatments by inserting a waveguide probe with a length of 20 cm in the upper 20-cm soil. After the daily weight, the pots were supplied with the targeted amount of irrigation water based on the measured DiW and the tested treatments. Depending on the location in the greenhouse, the pots were rotated every 48 h to ensure fair distribution of daylight and sunlight intensity for all experimental plants.

#### *Growth and yield characteristics*

Ninety DAT, five plants with root systems were randomly selected from each treatment to assess growth characteristics. Plant height was measured, main branches and leaves were counted, and fresh weight of shoot and root systems were recorded. The shoot and root systems were then dried at 70 °C until a constant dry weight was obtained. Twenty-five plants were dedicated to the chili pepper fruit yield. The fruits were harvested for the average number, fresh and dry weights for each plant, and capsaicin content.

#### *Physio-biochemical analyses*

Ninety DAT, five plants were randomly selected from each treatment for physio-biochemical assessments. Using the fully expanded third upper leaf, the concentration of total chlorophylls was measured as SPAD units using the Minolta chlorophyll meter (SPAD 502 model). Using dry matter of the fully expanded third leaf, the contents of free proline and total soluble sugars were assessed colorimetrically according to the procedures detailed in Bates *et al.* (1973) and Irigoyen *et al.* (1992), respectively. Using the methanolic extract of the same leaf material, total phenolic content was determined using the Folin-Ciocalteu colorimetric method described by Singleton and Rossi (1965). Total capsaicin content ( $\text{mg g}^{-1} \text{ DW}$ ) in dry fruits was extracted and estimated according to the method described in Augusto and Carloswild (1973). Using the same leaf material, N content was determined colorimetrically by using the Orange G dye (Rady, 2012). The content of P was quantitatively measured using the molybdenum-reduced molybdophosphoric blue color method (Jackson, 1967). The contents of K<sup>+</sup> and Na<sup>+</sup> were measured using a Perkin-Elmer Model 52-A Flame Photometer (Wilde *et al.*, 1985). The contents of Ca<sup>2+</sup>, Mg<sup>2+</sup>, Fe<sup>2+</sup>, Zn<sup>2+</sup>, Mn<sup>2+</sup>, and Cl<sup>-</sup> were measured using a Perkin-Elmer Atomic Absorption Spectrophotometer (Higinbotham *et al.*, 1967).

*Water use efficiency (WUE, %)*

Irrigation WUE was calculated according to Guang-Cheng *et al.* (2008). Water-saving (%) was calculated from the following equation: water-saving (%) = [(water consumption of control – water consumption of treatment)/water consumption of control] × 100.

*Anatomical study*

Forty-five DAT, five plants were randomly selected from each treatment for anatomical studies. Stem and leaf samples were taken from the middle of the fourth leaf from the apex. They were killed and fixed in FAA solution (50 ml, 95% ethyl alcohol + 10 ml formalin + 5 ml glacial acetic acid + 35 ml distilled water) for 48 h. They were then washed in 50% ethyl alcohol, dehydrated, and cleared in tertiary butyl alcohol series, embedded in paraffin wax of 54–56 °C m.p. Cross-sections, 20 μ thick, were cut by a rotary microtome, adhered by Haupt's adhesive, stained with the crystal violet-erythrosin combination (Nassar and El-Sahhar, 1998), cleared in carbol-xylene and mounted in Canada balsam. The sections were observed and documented using an upright light microscope (AxioPlan, Zeiss, Jena, Germany). A micrometer eyepiece was used for measurements.

*Statistical analysis*

The data are presented as means ± standard errors (SE). They were subjected to a two-way analysis of variance (ANOVA), and Duncan's Multiple Range Test was used to identify the significant variations among means, comparing at  $P \leq 0.05$ . The COSTAT computer software (CoHort Software version 6.303, Berkeley, CA, USA) was employed for data analysis.

**Results***Amount of water applied and WUE*

Throughout each experiment, thirty irrigation actions; a total of 30.6, 24.5, and 18.4 L of water per plant were applied to 100, 80, and 60% of FC irrigation treatments, respectively. Therefore, 80 and 60% of FC irrigation treatments saved approximately 19.9 and 39.9% of water, respectively compared to the 100% of FC irrigation treatment.

For irrigation regimes, WUE (%) increased significantly (by 98.2%) with a decrease in water supply by 20%, while significantly decreased (by 19.7%) with a water supply decrease by 40% of FC compared to full irrigated plants (Table 1).

Regarding GSH treatments, GSH significantly increased WUE (by 98.8%) when applied at 0.4 mM, and further increased WUE (by 237.1%) when applied at 0.8 mM compared to the control (foliar spray with water) (Table 1).

The interaction effect between irrigation treatments and GSH applications was significant for WUE (Figure 1). The highest WUE (215.4%) was obtained from the interactive treatment of the irrigation regime of 80% of FC × GSH at 0.8 mM followed by the irrigation regime of 80% of FC × GSH at 0.4 mM (WUE = 130%), then the irrigation regime of 100% of FC × GSH at 0.8 mM (WUE = 110%), while the lowest WUE (30.4 or 30.3%) was obtained from the interactive treatments of the irrigation regimes of 100 or 60% of FC × GSH at 0 mM (water), respectively.

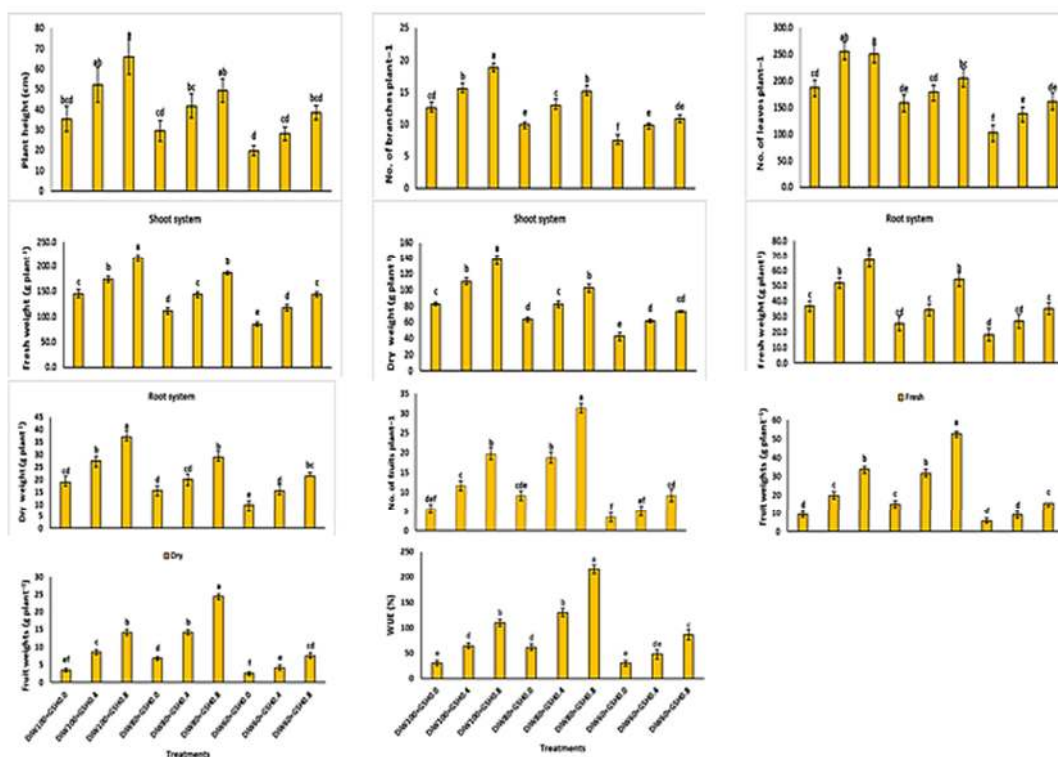
*Growth parameters and fruit yield components*

For irrigation treatments, chili pepper growth parameters (e.g., No. of branches plant<sup>-1</sup>, No. of leaves plant<sup>-1</sup>, fresh and dry weights of shoot and root systems), and fruit yield components (e.g., No. of fruits plant<sup>-1</sup>, fresh and dry weights of fruits plant<sup>-1</sup>) were significantly decreased with a decrease in irrigation water by 20% (i.e., 80% of soil field capacity; FC) or by 40% (i.e., 60% of FC) compared to the control (irrigation at

100% of FC) (Table 1). Irrigation water provided at 80 or 60% of FC decreased plant height by 21.5 or 43.8%, No. of branches plant<sup>-1</sup> by 18.6 or 40.4%, No. of leaves plant<sup>-1</sup> by 18.2 or 39.3%, fresh weight of plant shoot by 18.0 or 35.5%, fresh weight of plant root by 26.6 or 48.3%, dry weight of plant shoot by 25.1 or 46.4%, dry weight of plant root by 22.9 or 44.3%, respectively, compared to the control. Regarding yield components, while irrigation by 80% of FC increased No. of fruits plant<sup>-1</sup>, fresh weight of fruits plant<sup>-1</sup>, and dry weight of fruits plant<sup>-1</sup> by 58.9, 58.9, and 71.6%, respectively, irrigation by 60% of FC decreased these yield parameters by 52.4, 51.7, and 46.6%, respectively compared to the control.

Concerning glutathione (GSH) foliar treatments, the above growth and fruit yield parameters of the chili pepper plant increased significantly with a foliar spray of 0.4 mM GSH, and further increased with 0.8 mM GSH compared to the control (foliar spray with water) (Table 1). GSH leafy-provided at 0.4 or 0.8 mM increased plant height by 44.3 or 81.6%, No. of branches plant<sup>-1</sup> by 39.6 or 63.7%, No. of leaves plant<sup>-1</sup> by 21.3 or 37.8%, fresh weight of plant shoot by 27.5 or 60.3%, fresh weight of plant root by 41.0 or 94.0%, dry weight of plant shoot by 35.4 or 66.4%, dry weight of plant root by 41.6 or 96.6%, No. of fruits plant<sup>-1</sup> by 98.3 or 235.0%, plant fresh fruits by 102.0 or 241.0%, and plant dry fruits by 109.3 or 258.1%, respectively, compared to the control.

The interaction effect between irrigation treatments and GSH applications was significant for chili pepper plant growth and yield parameters (Figure 1). In general, the highest growth parameters were obtained from the interactive treatment of the irrigation regime of 80 or 100% of FC × GSH at 0.8 mM followed by the irrigation regime of 80 or 100% of FC × GSH at 0.4 mM, while the lowest growth parameters were obtained from the interactive treatments of the irrigation regime of 60% of FC × GSH at 0 mM (water).



**Figure 1.** The effect of the interaction between deficiency of irrigation water (DiW) and spraying of glutathione (GSH) on growth characteristics and fruit yield components of chili pepper plants grown in saline soil (EC = 6.74 dS m<sup>-1</sup>)

**Table 1.** The effect of deficiency of irrigation water (DiW) and spraying of glutathione (GSH) on growth characteristics and fruit yield components of chili pepper plants grown in saline soil (EC = 6.74 dS m<sup>-1</sup>)

Treatments	Plant height (cm)	No. of branches plant <sup>-1</sup>	No. of leaves plant <sup>-1</sup>	Fresh weight (g)		Dry weight (g)	
				Shoot system	Root system	Shoot system	Root system
DiW (FC%)	*	*	*	*	*	*	*
100	51.2±7.8a	15.6±0.8a	221.1±17.2a	178.7±7.0a	51.8±3.9a	110.4±4.1a	28.0±2.1a
80	40.2±5.6b	12.7±0.7b	180.8±15.8b	146.6±4.8b	38.0±4.4b	82.7±3.9b	21.6±2.1b
60	28.8±3.0c	9.3±0.6c	134.3±14.0c	115.2±5.2c	26.8±4.1c	59.2±2.8c	15.6±1.9c
GSH (mM)	*	*	*	*	*	*	*
0.0 (Control)	28.2±4.5c	09.1±0.7c	149.3±14.8c	113.6±6.0c	26.8±4.0c	62.8±3.5c	14.9±1.9c
0.4	40.7±5.9b	12.7±0.7b	181.1±14.6b	144.8±5.8b	37.8±4.0b	85.0±3.4b	21.1±2.2b
0.8	51.2±6.0a	14.9±0.7a	205.7±17.8a	182.1±5.2a	52.0±4.4a	104.5±3.9a	29.3±2.1a
DiW × GSH	*	*	*	*	*	*	*

Treatments	No. of fruits plant <sup>-1</sup>	Fresh fruit weights (g plant <sup>-1</sup> )	Dry fruit weights (g plant <sup>-1</sup> )	WUE (%)
DiW (FC%)	*	*	*	*
100	12.4±1.3b	20.9±1.8b	8.8±0.7b	68.4±5.7b
80	19.7±1.3a	33.2±1.9a	15.1±0.7a	135.6±7.6a
60	5.9±1.3c	10.1±1.5c	4.7±0.7c	54.9±8.1c
GSH (mM)	*	*	*	*
0.0 (Control)	6.0±1.2c	10.0±1.4c	4.3±0.5c	40.7±5.7c
0.4	11.9±1.3b	20.2±1.8b	9.0±0.7b	80.9±7.5b
0.8	20.1±1.4a	34.1±2.0a	15.4±0.8a	137.2±8.3a
DiW × GSH	*	*	*	*

\*\* and \* indicate, respectively, differences at  $P \leq 0.01$  and  $P \leq 0.05$  probability level, and "ns" indicates not a significant difference. Means ( $\pm$  SE) followed by the same letter in each column are not significantly different according to the LSD test ( $P \leq 0.01$  and  $P \leq 0.05$ ). EC = electrical conductivity, FC = field capacity, and mM = millimole.

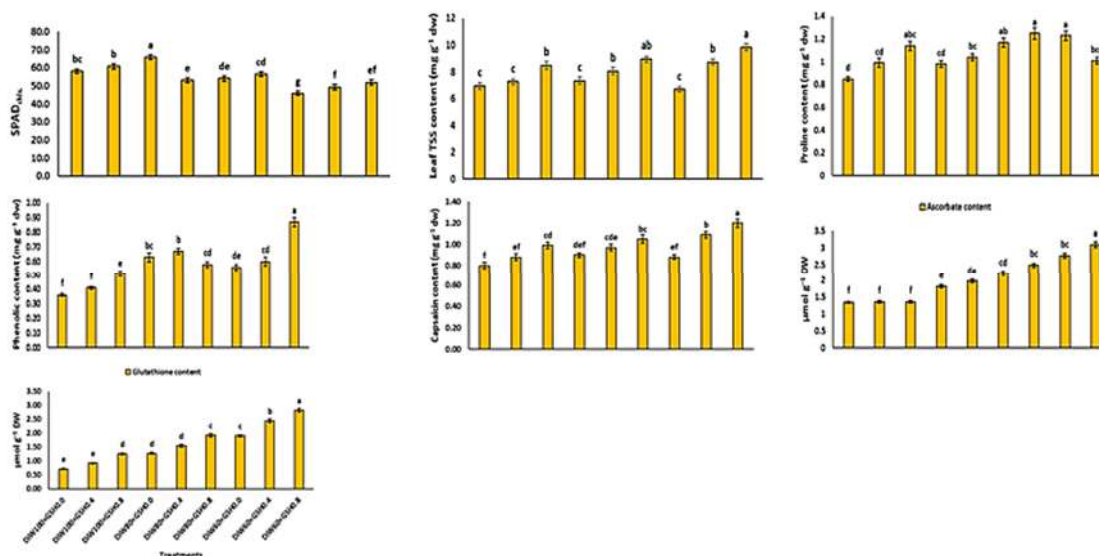
#### *Total chlorophyll, some osmoprotectant, and antioxidant contents*

Regarding irrigation treatments, deficit irrigation water treatments did not affect the soluble sugars content, but significantly decreased the chlorophyll content (measured as a SPAD index), while they significantly increased the contents of proline, phenols, capsaicin, ascorbic acid (AsA), and glutathione (GSH) compared to full irrigation treatment (Table 2). Irrigation water provided at 80 or 60% of FC decreased SPAD index by 11.5 or 20.6%, while increased proline content by 18.9 or 28.9%, phenolic content by 46.5 or 55.8%, capsaicin content by 9.0 or 19.1%, AsA content by 48.1 or 105.9%, and GSH content by 64.6 or 149.0% compared to the control.

As for GSH foliar treatments, 0.4 or 0.8 mM GSH did not affect the proline content, while they significantly increased the contents of SPAD index, proline, phenols, capsaicin, ascorbic acid (AsA), and glutathione (GSH) compared to the control (foliar spray with water) (Table 2). GSH foliar-sprayed at 0.4 or 0.8 mM increased SPAD index by 4.4 or 11.1%, soluble sugars content by 14.7 or 30.2%, phenolic content by 7.7 or 25.0%, capsaicin content by 14.0 or 25.6%, AsA content by 8.0 or 18.1%, and GSH by 26.4 or 55.0% compared to the control.

The interaction effect between irrigation treatments and GSH applications was significant for the SPAD index and all tested osmoprotectant and antioxidant parameters (Figure 2). In general, the highest values of osmoprotectant and antioxidant parameters were obtained from the interactive treatment of the irrigation

regime of 60% of FC × GSH at 0.8 mM followed by the irrigation regime of 60% of FC × GSH at 0.4 mM, then the irrigation regime of 80% of FC × GSH at 0.8 mM.



**Figure 2.** The effect of the interaction between deficiency of irrigation water (DiW) and spraying of glutathione (GSH) on SPADchl.s, some osmoprotectant and antioxidant contents in dry leaves of chili pepper plants grown in saline soil (EC = 6.74 dS m<sup>-1</sup>)

**Table 2.** The effect of deficiency of irrigation water (DiW) and spraying of glutathione (GSH) on SPADchl.s, some osmoprotectant and antioxidant contents in dry leaves of chili pepper plants grown in saline soil (EC = 6.74 dS m<sup>-1</sup>)

Treatments	SPAD chls.	Leaf TSS content	Leaf Proline content	Leaf Phenolic content	Fruit Capsaicin content	Leaf Ascorbate content	Leaf Glutathione content
DiW (FC%)	*	ns	*	**	*	*	**
100	61.7±1.4a	7.56±0.26	0.90±0.03c	0.43±0.01c	0.89±0.03c	1.35±0.03c	0.96±0.03c
80	54.6±1.4b	8.12±0.25	1.07±0.03	0.63±0.02b	0.97±0.03b	2.00±0.06	1.58±0.05b
60	49.0±1.3c	8.43±0.23	1.16±0.04a	0.67±0.03a	1.06±0.03a	2.78±0.07a	2.39±0.06a
GSH (mM)	*	*	ns	*	*	*	*
0.0 (Control)	52.4±1.3c	6.99±0.23c	1.03±0.03	0.52±0.02c	0.86±0.02c	1.88±0.05c	1.29±0.03c
0.4	54.7±1.5b	8.02±0.24b	1.09±0.04	0.56±0.02b	0.98±0.03b	2.03±0.05	1.63±0.05b
0.8	58.2±1.4a	9.10±0.26a	1.11±0.04	0.65±0.02a	1.08±0.04a	2.22±0.06a	2.00±0.05a
DiW × GSH	*	*	*	*	*	*	*

\*\* and \* indicate, respectively, differences at  $P \leq 0.01$  and  $P \leq 0.05$  probability level, and "ns" indicates not a significant difference. Means ( $\pm$  SE) followed by the same letter in each column are not significantly different according to the LSD test ( $P \leq 0.01$  and  $P \leq 0.05$ ). EC = electrical conductivity, FC= field capacity, mM= millimole, Chls= chlorophylls, and TSS= total soluble sugars.

*Leaf macro-and micro-nutrients status, Na<sup>+</sup> and Cl<sup>-</sup> contents, and K<sup>+</sup>/Na<sup>+</sup> ratio*

As for irrigation treatments, leaf macro-and micro-nutrients contents (e.g., N, P, K, Ca, Mg, Fe, Zn, and Mn) and K<sup>+</sup>/Na<sup>+</sup> ratio were significantly decreased, while the contents of Na<sup>+</sup> and Cl<sup>-</sup> were significantly increased with a decrease in irrigation water by 20% (i.e., 80% of FC) or by 40% (i.e., 60% of FC) compared to



full irrigation (100% of FC) (Table 3). Irrigation water provided at 80 or 60% of FC decreased N content by 10.7 or 15.7%, P content by 16.1 or 32.3%, K content by 13.2 or 32.9%, Ca content by 6.4 or 24.8%, Mg content by 7.3 or 13.5%, Fe content by 8.0 or 17.7%, Zn content by 17.1 or 26.8%, Mn content by 7.7 or 20.5%, and  $K^+/Na^+$  ratio by 18.5 or 40.2%, while increased  $Na^+$  content by 9.1 or 13.6%, and  $Cl^-$  content by 9.5 or 25.5%, respectively, compared to the control.

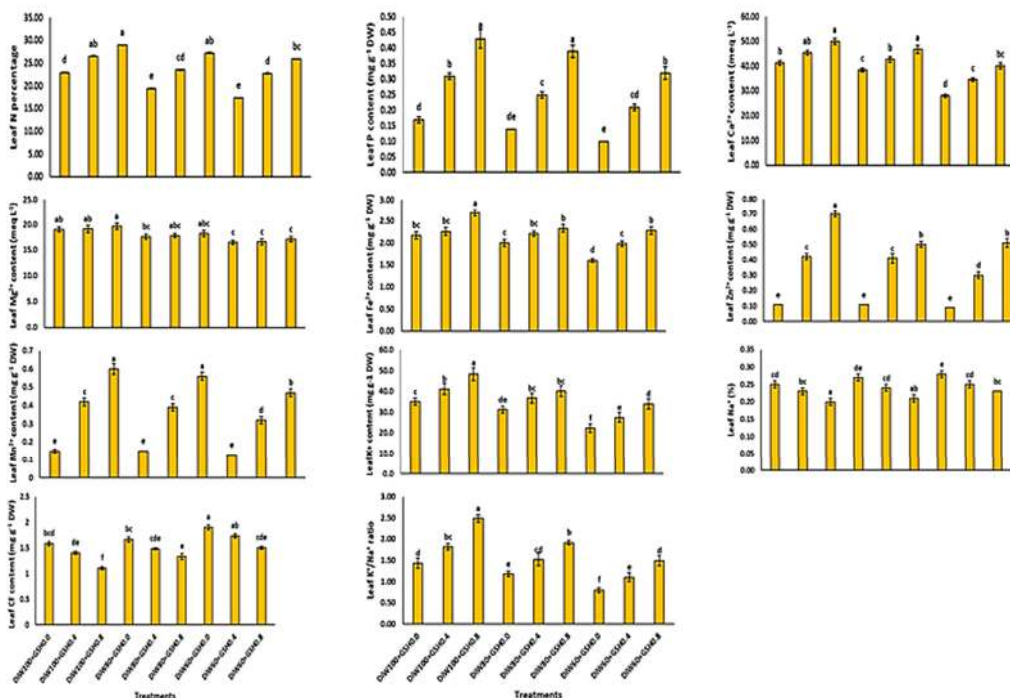
Regarding GSH foliar treatments, the above leaf macro- and micro-nutrients contents and  $K^+/Na^+$  ratio were significantly increased, while  $Na^+$  and  $Cl^-$  contents were significantly decreased with foliar spray of 0.4 or 0.8 mM GSH compared to the control (foliar spray with water) (Table 3). GSH leafy-provided at 0.4 or 0.8 mM increased N content by 21.6 or 37.2%, P content by 85.7 or 171.4%,  $K^+$  content by 18.9 or 38.2%, Ca content by 13.6 or 27.0%, Mg content by 0.6 or 3.4%, Fe content by 12.0 or 26.6%, Zn content by 280.0 or 470.0%, Mn content by 171.4 or 285.7%, and  $K^+/Na^+$  ratio by 29.8 or 71.1%, while decreased  $Na^+$  content by 7.7 or 19.2%, and  $Cl^-$  content by 9.9 or 23.3%, respectively, compared to the control.

The interaction effect between irrigation treatments and GSH applications was significant for leaf macro- and micro-nutrient status,  $Na^+$  and  $Cl^-$  contents, and  $K^+/Na^+$  ratio of the chili pepper plant (Fig. 3). Generally, the highest nutrient contents and  $K^+/Na^+$  ratio, and the lowest  $Na^+$  and  $Cl^-$  contents were obtained from the interactive treatment of the irrigation regime of 80% of FC  $\times$  GSH at 0.8 mM followed by the irrigation regime of 100% of FC  $\times$  GSH at 0.8 mM, then the irrigation regime of 100% of FC  $\times$  GSH at 0.4 mM.

**Table 3.** The effect of deficiency of irrigation water (DiW) and spraying of glutathione (GSH) on macro- and micro-nutrients,  $Na^+$ , and  $Cl^-$  contents, as well as  $K^+/Na^+$  ratio in dry leaves of chili pepper plants grown in saline soil ( $EC = 6.74 \text{ dS m}^{-1}$ )

Treatments	N	P	$Ca^{2+}$	$Mg^{2+}$	$Fe^{2+}$	$Zn^{2+}$
	$\text{mg g}^{-1} \text{ DW}$					
DiW (FC%)	*	*	*	*	*	*
100	26.1±0.8a	0.31±0.02a	45.5±1.1a	19.3±0.6a	2.37±0.8a	0.41±0.01a
80	23.3±0.6b	0.26±0.01b	42.6±1.1b	17.9±0.5b	2.18±0.8b	0.34±0.02b
60	22.0±0.7b	0.21±0.01c	34.2±0.9c	16.7±0.5c	1.95±0.6c	0.30±0.02c
GSH (mM)	*	**	*	*	*	**
0.0 (Control)	19.9±0.5c	0.14±0.00c	35.9±0.8c	17.7±0.5b	1.92±0.7c	0.10±0.00c
0.4	24.2±0.8b	0.26±0.01b	40.8±1.0b	17.8±0.6b	2.15±0.7b	0.38±0.02b
0.8	27.3±0.8a	0.38±0.02a	45.6±1.3a	18.3±0.6a	2.43±0.8a	0.57±0.02a
DiW $\times$ GSH	*	*	*	*	*	*
Treatments	$Mn^{2+}$	$K^+$	$Na^+$	$Cl^-$	$K^+/Na^+$ ratio	
	$\text{mg g}^{-1} \text{ DW}$					
DiW (FC%)	*	*	*	*	*	
100	0.39±0.02a	41.6±2.5a	0.22±0.01b	1.37±0.03c	1.89±0.10a	
80	0.36±0.01b	36.1±2.3b	0.24±0.01a	1.50±0.04b	1.54±0.09b	
60	0.31±0.01c	27.9±2.3c	0.25±0.006a	1.72±0.04a	1.13±0.09c	
GSH (mM)	**	*	*	*	*	
0.0 (Control)	0.14±0.00c	29.6±1.8c	0.26±0.01a	1.72±0.05a	1.14±0.08c	
0.4	0.38±0.02b	35.2±2.5b	0.24±0.01b	1.55±0.03b	1.48±0.11b	
0.8	0.54±0.02a	40.9±2.8a	0.21±0.006c	1.32±0.04c	1.95±0.09a	
DiW $\times$ GSH	*	*	*	*	*	

\*\* and \* indicate, respectively, differences at  $P \leq 0.01$  and  $P \leq 0.05$  probability level, and "ns" indicates not a significant difference. Means ( $\pm$  SE) followed by the same letter in each column are not significantly different according to the LSD test ( $P \leq 0.01$  and  $P \leq 0.05$ ). EC= electrical conductivity, FC= field capacity, mM= millimole, DW= dry weight, N= nitrogen, P= phosphorus, Ca= calcium, Mg= magnesium, Fe= iron, Zn= zinc, Mn= manganese, K= potassium, Na= sodium, and Cl= chlorine.



**Figure 3.** The effect of the interaction between deficiency of irrigation water (DiW) and spraying of glutathione (GSH) on macro-and micro-nutrients, Na<sup>+</sup>, and Cl<sup>-</sup> contents, as well as K<sup>+</sup>/Na<sup>+</sup> ratio in dry leaves of chili pepper plants grown in saline soil (EC = 6.74 dS m<sup>-1</sup>)

*Leaf and stem anatomy*

As for irrigation treatments, the reduction in irrigation water by 20 or 40% (i.e., 80 or 60% of FC, respectively) resulted in a marked decrease in all features of leaf and stem anatomy of chili pepper plant compared to full irrigation treatment (Table 4 a,b). Declines in leaf and stem anatomy features were more pronounced under deficit irrigation at 60% of FC than deficit irrigation at 80% of FC.

Concerning GSH foliar treatments, 0.4 or 0.8 mM GSH significantly recovered all features of leaf and stem anatomy compared to the control (foliar spray with water) (Table 4 a,b). Improvements in leaf and stem anatomy features were more noticeable with the application of GSH at 0.8 mM compared to 0.4 mM.

The interaction effect between irrigation treatments and GSH applications was significant for the tested features of leaf and stem anatomy (Table 4 a,b). Generally, the highest values of osmoprotectant and antioxidant parameters were obtained from the interactive treatment of the irrigation regime of 100% of FC × GSH at 0.8 mM followed by the irrigation regime of 80% of FC × GSH at 0.8 mM, while the lowest growth parameters were obtained from the interactive treatments of the irrigation regime of 60% of FC × GSH at 0 mM (water).

**Table 4a.** The effect of deficiency of irrigation water (DiW) and spraying of glutathione (GSH) on leaf of chili pepper plants grown in saline soil (EC = 6.74 dS m<sup>-1</sup>)

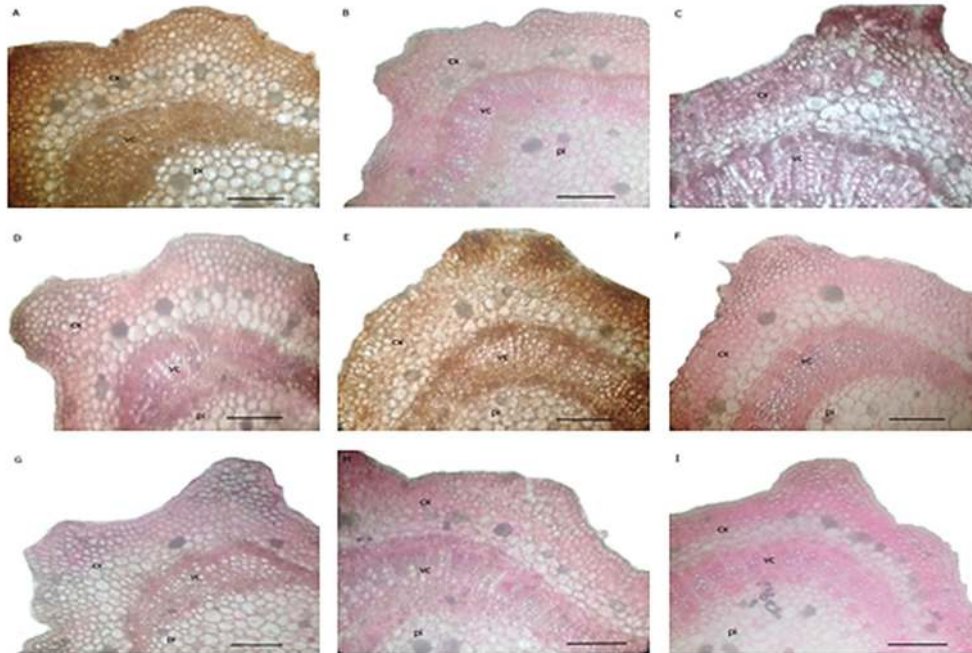
Treatments	Leaf							
	Midvien		Median vb		Average Mx vessels diameter (µm)	Blade thick. (µm)	Palisade thick. (µm)	Spongy thick. (µm)
	Width (µm)	Length (µm)	Width (µm)	Length (µm)				
DiW (FC%)	*	*	*	ns	*	*	ns	*
100	767±67a	560±43a	490±42a	123±9	12±1a	213±17a	70±5	113±9a
80	653±57b	567±50a	383±37b	137±13	10±1b	193±17b	70±6	100±6b
60	537±48c	507±43b	240±18c	123±10	10±1b	203±22ab	67±6	100±7b
GSH (mM)	*	*	*	*	*	*	*	*
0.0 (Control)	613±53c	533±43b	367±32b	133±11a	10±1b	193±18b	70±7a	97±7b
0.4	653±62b	510±40b	347±30c	107±8b	12±1a	193±17b	63±5b	97±7b
0.8	690±57a	590±53a	400±35a	143±12a	10±1b	223±20a	73±6a	120±8a
DiW × GSH	*	*	*	*	*	*	*	*
DiW <sub>100</sub> ×GSH <sub>0.0</sub>	680±55c	500±35c	450±30b	130±11ab	10±1b	180±15c	60±5c	100±8bc
DiW <sub>100</sub> ×GSH <sub>0.4</sub>	770±70b	530±40c	470±45b	100±8c	15±2a	220±15ab	70±4b	110±10b
DiW <sub>100</sub> ×GSH <sub>0.8</sub>	850±75a	650±55a	550±50a	140±9ab	10±1b	240±20a	80±6a	130±10a
DiW <sub>80</sub> ×GSH <sub>0.0</sub>	640±55c	600±50b	400±45c	150±13a	10±1b	200±20bc	80±8a	100±7bc
DiW <sub>80</sub> ×GSH <sub>0.4</sub>	650±60c	500±40c	350±30d	120±10bc	10±1b	180±15c	60±5c	100±6bc
DiW <sub>80</sub> ×GSH <sub>0.8</sub>	670±55c	600±60b	400±35c	140±15ab	10±1b	200±15bc	70±6b	100±5bc
DiW <sub>60</sub> ×GSH <sub>0.0</sub>	520±50d	500±45c	250±20e	120±10bc	10±0b	200±20bc	70±7b	90±6cd
DiW <sub>60</sub> ×GSH <sub>0.4</sub>	540±55d	500±40c	220±15e	100±7c	10±1b	180±20c	60±5c	80±6d
DiW <sub>60</sub> ×GSH <sub>0.8</sub>	550±40d	520±45c	250±20e	150±12a	10±1b	230±25a	70±5b	130±10a

\*\* and \* indicate, respectively, differences at  $P \leq 0.01$  and  $P \leq 0.05$  probability level, and "ns" indicates not a significant difference. Means ( $\pm$  SE) followed by the same letter in each column are not significantly different according to the LSD test ( $P \leq 0.01$  and  $P \leq 0.05$ ). EC= electrical conductivity, FC= field capacity, mM= millimole, µm= micrometer, vb= vascular bundle, Mx= metaxylem, thick= thickness.

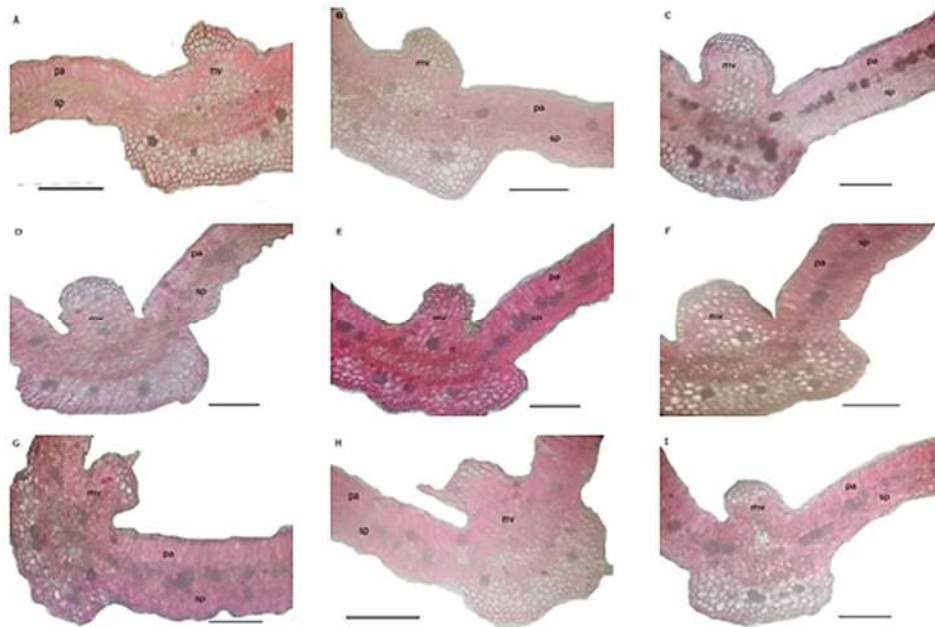
**Table 4b.** The effect of deficiency of irrigation water (DiW) and spraying of glutathione (GSH) on stem anatomic features of chili pepper plants grown in saline soil (EC = 6.74 dS m<sup>-1</sup>)

Treatments	Stem						
	Section diameter (µm)	Cortex thick. (µm)	Cortical cell No.	Thick. of vascular cylinder (µm)	Diameter of Mx vessels (µm)	Diameter of pith cell (µm)	Diameter of pith (µm)
DiW (FC%)	*	*	ns	*	*	ns	*
100	4410±310a	290±18a	8.0±0.4	1593±148a	20±2a	47±4	2307±180a
80	3383±272b	210±18b	7.7±0.5	1160±117b	17±2b	43±4	1733±152b
60	2983±215c	193±16c	8.0±0.5	1070±93b	17±2b	43±4	1500±113c
GSH (mM)	*	*	ns	*	*	*	*
0.0 (Control)	3250±252c	210±17c	8.0±0.4	1117±110c	17±1b	40±3b	1707±145c
0.4	3650±260b	233±17b	8.0±0.5	1293±117b	20±3a	47±3a	1833±138b
0.8	3877±285a	250±18a	8.0±0.5	1413±126a	17±2b	47±5a	2000±162a
DiW × GSH	*	*	ns	*	*	*	*
DiW <sub>100</sub> ×GSH <sub>0.0</sub>	3700±265a	270±18b	8.0±0.5	1250±130b	20±2a	40±3c	1870±145c
DiW <sub>100</sub> ×GSH <sub>0.4</sub>	4650±315a	270±15b	7.7±0.4	1700±155a	20±3a	60±5a	2400±185b
DiW <sub>100</sub> ×GSH <sub>0.8</sub>	4880±350a	330±21a	8.3±0.4	1830±160a	20±2a	40±4c	2650±210a
DiW <sub>80</sub> ×GSH <sub>0.0</sub>	3350±285ab	190±20d	7.7±0.4	1150±120bc	15±1b	40±3c	1750±180c
DiW <sub>80</sub> ×GSH <sub>0.4</sub>	3400±270a	250±22bc	8.0±0.6	1150±105bc	20±2a	40±2c	1750±125c
DiW <sub>80</sub> ×GSH <sub>0.8</sub>	3400±265a	190±14d	7.7±0.5	1180±110bc	15±2b	50±6b	1700±150cd
DiW <sub>60</sub> ×GSH <sub>0.0</sub>	2700±210c	170±14d	8.0±0.4	950±80d	15±1b	40±4c	1500±110de
DiW <sub>60</sub> ×GSH <sub>0.4</sub>	2900±195bc	180±15d	8.0±0.6	1030±90cd	20±3a	40±3c	1350±105e
DiW <sub>60</sub> ×GSH <sub>0.8</sub>	3350±240ab	230±18c	8.0±0.5	1230±110b	15±2b	50±4b	1650±125cd

\*\* and \* indicate, respectively, differences at  $P \leq 0.01$  and  $P \leq 0.05$  probability level, and "ns" indicates not a significant difference. Means ( $\pm$  SE) followed by the same letter in each column are not significantly different according to the LSD test ( $P \leq 0.01$  and  $P \leq 0.05$ ). EC= electrical conductivity, FC= field capacity, mM= millimole, µm= micrometer, vb= vascular bundle, Mx= metaxylem, thick= thickness.



**Figure 4.** The effect of deficiency of irrigation water (DiW) and glutathione spraying (GSH) on stem anatomic features of chili pepper plants grown in saline soil (cx= cortex, vc= vascular cylinder, pi= pith. Line= 250 μm)



**Figure 5.** The effect of deficiency of irrigation water (DiW) and glutathione spraying (GSH) on leaf anatomic features of chili pepper plants grown in saline soil (mv= midvein, pa= palisade tissue, sp= spongy tissue. Line= 250 μm).

## Discussion

Some researchers have described the importance of GSH in regulating plant physiological responses under adverse environmental conditions in different plant species (Cao *et al.*, 2017; Sabetta *et al.*, 2017; Sohag *et al.*, 2020), but have not yet been studied in salt-stressed chili pepper plants grown under the stress of drought. In this study, GSH played a crucial role in reinforcing the tolerance to drought stress in chili pepper by regulating physiological and biochemical processes, including growth traits, chlorophylls, antioxidants, and regulation of ion influxes. The deficiency of irrigation water (DiW) negatively affects the chili pepper growth criteria, fruit production, and developmental processes (Correia *et al.*, 2001; Pinheiro and Chaves, 2011). These harmful effects are magnified under combined DiW + salt stress (Abd El-Mageed *et al.*, 2018; Yang *et al.*, 2019; Alharby *et al.*, 2020).

Under saline soil conditions ( $EC = 6.74 \text{ dS m}^{-1}$ ), in this study, a 40% decrease in required irrigation water (based on soil field capacity; FC) significantly reduced growth, especially fresh and dry weights, and fruit yield parameters of chili plants. On the other side, a 20% decrease in required irrigation water significantly increased fruit yield parameters despite lower growth criteria. Alharby *et al.* (2020) explained that plant growth and yield components are decreased under combined DiW + salt stress due to suppression in cellular expansion and division, chlorophyll content, and photosynthetic efficiency, although increases in endogenous antioxidants, phytohormones, and gene expression. Besides, reducing photosynthates partitioning in plants resulting from reducing leaf area, which leads to a decrease in dry matter and plant yields production (Hong-Bo *et al.*, 2008). However, the exogenous application of GSH restored the negatively affected growth and yield parameters that occurred due to the combined DiW + salt stress (Table 1; Figure 1). This growth and yield restoration by GSH may be attributed to its metabolic enzyme interactions with plant growth regulators, which are vital in the plant establishment scheme under normal conditions and stresses. Exogenous GSH causes higher levels of abscisic acid (ABA) to reduce the transpiration rate and regulates stomatal aperture and leaf water content, which may indicate a role in controlling leaf rolling (Chen *et al.*, 2012). GSH is supposed to regulate growth-related functions, as raising endogenous GSH level enhances cell division in the root meristematic region (Vernoux *et al.*, 2000), causing this region to be elongated, which is an important morphological adaptation to stress. Similar to our results, exogenous GSH enhanced plant growth and development grown under combined DiW + salt stress (Chen *et al.*, 2012; Patil *et al.*, 2014; El-Beltagi *et al.*, 2020).

In this study, the highest fruit yield was observed with irrigation water supplementation at 80% of FC probably because this amount of water was sufficient to meet the water requirements of the crop during the flowering stage, fruit set, and fruit development to harvest even under soil salinity ( $E_{Ce} = 6.74 \text{ dS m}^{-1}$ ). A similar result was obtained with Patil *et al.* (2014) who reported that deficit saline irrigation water had no adverse effect on bell pepper (*Capsicum annuum*) fruit yield. A 20% decrease in the water supply (80% of FC) to chili pepper plants saved water by more than 20% relative to the large increase in fruit yield and water use efficiency (WUE %) compared to full irrigation (100% of FC) (Table 1). Patil *et al.* (2014) also noted that DiW at 60% of  $ET_c$  over the entire cropping period, which saved more water, led to a significantly higher WUE than all other DiW treatments. The increase in WUE by Ismail (2010) could be attributed to the increase in mass production per unit of water. In this study, the decrease in irrigation water by 20% not only increased WUE but also leafy-applied GSH significantly elevated it, besides the enhanced chili pepper growth and physiological performance under salt stress. GSH was applied to chili pepper leaves with significantly elevated endogenous levels of GSH and ascorbate (AsA) (Table 2) to enrich the GSH-AsA cycle, and thus as Foyer and Noctor (2005a) explained, it acts to eliminate ROS and maintain redox homeostasis in the tissues of stressed plants. The GSH-AsA cycle is a central component of the network of biochemical reactions that includes various antioxidants with the properties of redox reactions to efficiently eliminate ROS, thus prohibiting ROS-mediated oxidative damage in stressed plant tissues (Foyer and Noctor, 2005a). Eventually, this leads to maintaining plant growth and satisfying yield.

The chlorophyll content of plant leaves is one of the most important factors in determining the rate of photosynthesis and dry matter production (Dai *et al.*, 2009). In this study, DiW applied to chili pepper plants damaged cell chloroplasts, which resulting in chlorophyll and photosynthesis impairment (Rady *et al.*, 2020), associating with decreased plant growth (Table 1; Figure 1). Similar results indicated that combined DiW + salt stress minimized chlorophyll content (Abd El-Mageed *et al.*, 2018; Alharby *et al.*, 2020).

Several GSH mechanisms are involved in increasing WUE and mitigating salt stress and DiW damage. Under the DiW and soil salinity stresses in this study, soluble sugars content increased due to exogenous GSH, and proline level increased due to DiW elevated stress tolerance in chili pepper plants with increased WUE (Table 2; Figure 2). As compatible solutes, soluble sugars and proline play crucial roles in reducing stress-induced cell acidification and maintaining the osmoregulation as osmoprotectant substances, which also include phenolic compounds and the alkaloid capsaicin that were significantly increased under stresses (Table 2; Figure 2). These positive results combined with the increased endogenous GSH due to exogenous GSH markedly improved plant cell water status and maintained adequate tissue water content to improve metabolic processes under stress (Gill and Tuteja, 2010; Rady and Hemida, 2016; Zaki and Mohamed, 2018; Yang *et al.*, 2019).

When antioxidants, including GSH, have many important roles as key mechanisms in mitigating the effects of abiotic stress on plants (Singh *et al.*, 2016), the optimum GSH level applied to DiW-suffered chili plants can alleviate the adverse effects of DiW and reduce the impairment of chloroplasts (Hasanuzzaman *et al.*, 2017). This result leads to increased chlorophyll contents in plant leaves even under DiW conditions, which are associated with excessive production of ROS (Rady *et al.*, 2020). The photosynthesis process can be activated by applying GSH by regulating the PSII and/or the components of the plants' defense system against damage stimulated by excessive ROS production. The positive regulation of physiological and biochemical processes by GSH results in increased chlorophyll contents, and further activation of antioxidant machinery in DiW-suffered plants, which positively affect the efficiency of photosynthesis (Hasanuzzaman *et al.*, 2017). The positive effect of GSH applied at 0.8 mM explains the insignificant differences in the chlorophyll content (measured as a SPAD index) in the chili pepper plant leaves, which was obtained under both regular and DiW treatments (Table 2; Figure 2). GSH can prevent or delay the degradation of chlorophyll caused by drought-induced oxidative stress by reducing biomarkers of oxidative stress and protecting the biosynthesis enzymes of chlorophylls, which might be related to a high chlorophyll content (Hasanuzzaman *et al.*, 2017; Alharby *et al.*, 2020). Exogenous GSH supplied to drought or salt stressed-plant restored its reduced content of chlorophylls (Nahar *et al.*, 2017; Zaki and Mohamed, 2018).

Osmoprotectant materials are accumulated in many plant species in response to various stress conditions. Our study showed significant positive changes in osmotic adjustment substances, especially soluble sugars and free proline under combined DiW + salt stress conditions (Table 2; Figure 2). Similarly, Abd El-Mageed *et al.* (2018) and Alharby *et al.* (2020) observed a significant increase in the total soluble sugars and free proline contents of plants in DiW + salt treatments. In this study, the content of total soluble sugars increased significantly with DiW by 20% (80% of FC), while it decreased under DiW by 40% (60% of FC) throughout salt stressed-pepper growth, however, the free proline content increased significantly under both DiW regimes. However, a further increase in these leaf osmoprotective substances was observed using GSH application (Table 2; Figure 2). Proline is a compatible solute, which plays a crucial role in reducing stress-induced cell acidification and maintaining osmoregulation, and acts as an osmoprotectant (Hasegawa *et al.*, 2000). It can prevent water loss or preserve leaf RWC (Abd El-Mageed *et al.*, 2018). Respectively, Merwad *et al.* (2018) and Zaki *et al.* (2019), using the soybean and cowpea plants, reported the highest levels of proline and soluble sugars by exogenous GSH under drought and saline treatments.

The main components of the ascorbate (AsA)-glutathione (GSH) cycle; AsA and GSH are low-molecular-weight (LMW) antioxidants that are synthesized within cell chloroplasts and play important roles as redox buffers to modulate processes involving in plant growth and development from mitosis and cell elongation to senescence and death (Kasote *et al.*, 2015). These LMW antioxidants increased significantly with

increasing DiW from 20 to 40% and further increased with the application of GSH to the salt-stressed chili pepper plants (Table 2; Figure 2). These compounds can activate gene expression associated with responses to biotic and abiotic stress conditions to increase the defense of sensitive plants (Kasote *et al.*, 2015). AsA is generated during aerobic metabolism and is known to be the best molecule to detoxify H<sub>2</sub>O<sub>2</sub>, especially as a substrate of ascorbate peroxidase (APX), which is an essential enzyme for the AsA-GSH cycle, found in most compartments of the plant cell. It also helps regenerate the antioxidant pigments, carotenoids, and  $\alpha$ -tocopherol (Smirnoff and Wheeler, 2000). In this concern, the high activity of the antioxidant enzymes implicated in the AsA-GSH cycle is closely related to the increase in GSH content in response to DiW (Hasanuzzaman *et al.*, 2017). The increase in endogenous AsA and GSH and their redox states supported the plant's tolerance to combined DiW + salinity stress (Alharby *et al.*, 2020).

GSH is the most abundant LMW thiol in plants (Noctor *et al.*, 2012). The sulphhydryl or thiol group (-SH) of GSH can donate an electron to free radicals. Because of its abundance and potential for negative redox, GSH contributes strongly to the redox environment, allowing cells to maintain healthy reduced redox homeostasis (Alharby *et al.*, 2020). GSH (reduced glutathione) is oxidized to the glutathyl anion radical (GS<sup>•</sup>). Two of the GS<sup>•</sup> can spontaneously bind to form glutathione disulphide (GSSG; oxydized glutathione), which can be recycled back to GSH by GR, which requires NADPH as reducing power. GSSG can form disulfides mixed with thiol-containing proteins. In this way, protein-bound GSH protects protein thiol groups from auto-oxidation to sulfonic acids (Hasanuzzaman *et al.*, 2017). GSH acts as an antioxidant by suppressing ROS and is a participant in the AsA-GSH cycle, which eliminates harmful peroxides (Kasote *et al.*, 2015). In a study conducted by Herbingier *et al.* (2002) using the *Triticum aestivum* plants, GSH concentrations are significantly increased in response to DiW up to 40% of the soil water capacity. Through the use of long-term DiW-suffered *Vigna radiata* plants, Sengupta *et al.* (2012) showed a decrease in activity and level of transcription of  $\gamma$ -glutamylcysteine synthetase in plant roots. This is incompatible with the hypothesis that tolerance to abiotic stress is associated with an increase in the level and activity of  $\gamma$ -glutamyl-cysteine synthetase with an increase in GSH and cysteine concentrations, as demonstrated under salt stress (Nazar *et al.*, 2011).

The main role of alkaloid substances is generally linked to plant defense mechanisms against predators, in addition to the important ecological factors associated with them. However, the close relationship between alkaloids and the redox processes in the plants they contain strongly suggests that these compounds play a key role in protecting plants when they are under oxidative stress (Ramos-Valdivia *et al.*, 2012). In the present study, a decrease in the water supply to the chili pepper plant root-zone significantly increased the total content of capsaicin in fruits. These results are close to those reported by Jaleel *et al.* (2007) who hypothesized that similar to phenolic compounds, an increase in the total content of indole alkaloid has been observed in shoots and roots of *Catharanthus roseus* under drought-induced stress. Additionally, higher production of alkaloids has been reported during in vitro and in vivo growth of *Hypericum polyanthemum* under drought stress through induction of ionic or osmotic stress (De-Matos *et al.*, 2014). However, the addition of GSH as foliar application further increased the total content of alkaloid capsaicin in fruits (Table 2; Figure 2) to impose a protective effect (Kotebagilu *et al.*, 2014) against tested stresses. In this study, GSH and alkaloid capsaicin functionally acted side-by-side in favor of the antioxidant defense system in the combined-stressed chili pepper plants. Capsaicin can function as an H<sub>2</sub>O<sub>2</sub> scavenging system. As a result, the increase in GSH-induced capsaicin functionally acted side-by-side to minimize the effects of combined DiW + salt stress by scavenging ROS.

As shown in Table 2; Figure 2, endogenous GSH and AsA levels were significantly elevated from GSH applied exogenously to enrich the GSH-AsA cycle to raise its efficiency to detoxify species of oxygen radicals (ROS) to suppress oxidative stress in plants. This interpretation is in agreement with Nahar *et al.* (2017), reporting that a higher level of GSH in plants efficiently removes ROS and suppresses oxidative damage. They also added that an exogenous GSH can efficiently recycle AsA to eliminate ROS. GSH can attenuate the prohibited influences of ROS-stimulated oxidative stress. Besides, the use of GSH externally boosted other non-enzymatic components of the plant antioxidant system such as proline, phenolic compounds, and

capsaicin (Table 2; Figure 2) that worked to prevent ROS-stimulated oxidative stress damage in stressed plants (Nahar *et al.*, 2017) to help maintain homeostasis of cellular redox. The exogenous use of GSH maintains the homeostasis of GSH redox in plant leaves and helps to regulate synthesis and regeneration of GSH to meet its increasing need, thus boosting the chili pepper plant's tolerance to the adverse impacts of oxidative stress (Zhou *et al.*, 2017).

An increase in DiW from 20% to 40% gradually reduced the leaf contents of macro-nutrients (e.g., nitrogen; N, phosphorus; P, potassium;  $K^+$ , calcium;  $Ca^{2+}$ , and magnesium;  $Mg^{2+}$ ) and micro-nutrients (e.g., iron; Fe, zinc; Zn and manganese; Mn), as well as the ratio of  $K^+/Na^+$ , while the leaf contents of sodium ( $Na^+$ ) and chloride ( $Cl^-$ ) were gradually increased in salt-stressed chili pepper plants compared to the control (Table 3; Figure 3). These results are confirmed by Zaki and Mohamed (2018) and Bekmirzaev *et al.* (2019) using faba bean and *Tetragonia tetragonioides* plants, respectively, under different stress conditions. However, GSH mitigated the adverse effects of DiW by increasing all investigated macro- and micro-nutrient contents and  $K^+/Na^+$  ratio, while  $Na^+$  and  $Cl^-$  contents were decreased significantly in chili pepper plants. These positive results were more pronounced with the 0.8 mM GSH level. The positive role of GSH in improving plant ion contents under DiW may be due to its effect in modifying membrane permeability and improving osmotic tolerance and/or regulation of biochemical processes (Kattab, 2007). Zaki and Mohamed (2018) obtained similar results with exogenously-applied GSH, which prohibited the deleterious effect of salinity stress and increased both the mineral ion contents (e.g., K, Ca, Mg, P, and N) and  $K^+/Na^+$  ratio, while  $Na^+$  content was decreased compared to the control. Under stress conditions as in this study, an increased level of abscisic acid (ABA) has been reported to stimulate stomatal closure (Aliniaiefard *et al.*, 2014). Under this condition (stomatal closure), the influx of water and nutrient ions dissolved in the water from roots to leaves will diminish due to the suppressed rate of transpiration, thus lowering the nutrient contents as shown in Table 3; Figure 3. Table 2; Figure 2 displays that applying GSH markedly increased the level of AsA, which has been reported to reverse stomatal closure (Chen and Gallie, 2004). AsA localized in cellular walls plays a specific role in regulating stomatal dynamics through the action of dehydroascorbate (Fotopoulos *et al.*, 2008). Thus, AsA scavenges ROS and prevents ABA-stimulated stomatal closure (Chen and Gallie, 2004), and ionic homeostasis will occur in the leaves. Besides, applying GSH considerably increased the osmoprotectant compounds like proline and soluble sugars, reinforcing the power of osmotic absorption and thus more water uptake along with the nutrients. Therefore, the increase in the contents of nutrients will occur as shown in this study (Table 3; Figure 3). In this study, there was a crucial role of different osmotic protective compounds (proline and soluble sugars) in plant tolerance to DiW because these compounds regulate a wide range of metabolic processes, including up-regulating ion transport and transpiration rate (Iqbal, 2018).

Changes in the features of leaf and stem anatomy that appeared in chili pepper plant as affecting by combined stress (DiW + salinity) may be attributed to the osmotic effect and deficiency of nutrients. The combined stress negative effects on anatomical features were due to defects that occurred in leaf midvein and blade and the decrease in stem diameter and its components, as well as due to plant cell plasmolysis and cell shrinkage due to lack of water due to stress. Barcelo *et al.* (1986) noted that one of the mechanisms of stress is the negative effects on cell elongation and wall plasticity. However, the foliar application of GSH, especially at 0.8 mM, alleviated the adverse effects of DiW + salt stress on all anatomical parameters of the leaf and stem of chili plants. This positive effect may be attributed to increased cell wall expansion along with higher turgor pressure by applying GSH (Munns and Termaat, 1986). In the present study, the foliar application of GSH maintained osmotic and ionic balance, thus improving the plant water status to resist DiW + salt stress and improving the anatomical features of the chili pepper plant.



**Table 5.** Changes (%) in plant growth, physiology, biochemistry, productivity, and water use efficiency relative to the control in chili pepper plants grown under saline soil ( $EC = 6.74 \text{ dS m}^{-1}$ ) conditions. Three color scale heatmap, yellow as the midpoint of control and parameters with insignificant values compared to control, red for changes below control values, and green for changes over control values

Parameters	Treatments					
	Irrigation regimes (FC, %)			Glutathione treatments (mM)		
	100 (control)	80	60	0 (control)	0.4	0.8
Plant height	a	-21.5b	-43.8c	C	+44.3b	+81.6a
No. of branches plant <sup>-1</sup>	a	-18.6b	-40.4c	C	+39.6b	+63.7a
No. of branches plant <sup>-1</sup>	a	-18.2b	-39.3c	C	+21.3b	+37.8a
Shoot fresh weight	a	-18.0b	-35.5c	C	+27.5b	+60.3a
Root fresh weight	a	-26.6b	-48.3c	C	+41.0b	+94.0a
Shoot dry weight	a	-25.1b	-46.4c	C	+35.4b	+66.4a
Root dry weight	a	-22.9b	-44.3c	C	+41.6b	+96.6a
No. of fruits plant <sup>-1</sup>	b	+58.9a	-52.4c	C	+98.3b	+235a
Fruit fresh weight	b	+58.9a	-51.7c	C	+102b	+241a
Fruit dry weight	b	+71.6a	-46.6c	C	+109b	+258a
Water use efficiency (WUE)	b	+98.2a	-19.7c	C	+98.8b	+237a
SPAD chlorophyll index	a	-11.5b	-20.6c	C	+4.4b	+11.1a
Soluble sugars content	a	+7.4a	+11.5a	C	+14.7b	+30.2a
Proline content	c	+18.9b	+28.9a	A	+5.8a	+7.8a
Phenolics content	c	+46.5b	+55.8a	C	+7.7b	+25.0a
Capsaicin content	c	+9.0b	+19.1a	C	+14.0b	+25.6a
Ascorbate content	c	+48.1b	+106a	C	+8.0b	+18.1a
Glutathione content	c	+64.6b	+149a	C	+26.4b	+55.0a
N content	a	-10.7b	-15.7b	C	+21.6b	+37.2a
P content	a	-16.1b	-32.3c	C	+85.7b	+171a
Ca <sup>2+</sup> content	a	-6.4b	-24.8c	C	+13.6b	+27.0a
Mg <sup>2+</sup> content	a	-7.3b	-13.5c	B	+0.6b	+3.4a
Fe <sup>2+</sup> content	a	-8.0b	-17.7c	C	+12.0b	+26.6a
Zn <sup>2+</sup> content	a	-17.1b	-26.8c	C	+280b	+470a
Mn <sup>2+</sup> content	a	-7.7b	-20.5c	C	+171b	+286a
K <sup>+</sup> content	a	-13.2b	-32.9c	C	+18.9b	+38.2a
Na <sup>+</sup> content	b	+9.1a	+13.6a	A	-7.7b	-19.2c
Cl <sup>-</sup> content	c	+9.5b	+25.5a	A	-9.9b	-23.3c
K <sup>+</sup> /Na <sup>+</sup> ratio	a	-18.5b	-40.2c	C	+29.8b	+71.1a

From the results of this study and Table 5, chili pepper plants can be irrigated with 80% of soil field capacity, saving about 20% of water with elevating the water use efficiency and increasing the obtained fruit yield components, although the growth of plants decreased. These positive results were connected with exogenous application of 0.8 mM GSH, which support growth and productivity of chili pepper plants under 20% shortage of irrigation water through the significant improvement in physiology and biochemistry, including antioxidants and nutrient homeostasis.

## Conclusions

Our results showed that 0.8 mM glutathione (GSH) applied to leaves improved the growth, physio-biochemical, and anatomical performances of chili pepper plants grown under drought and salinity. Damage

from these stresses was mitigated with the use of GSH by maintaining the osmotic homeostasis by regulating the proline, soluble sugars, and  $K^+$  contents to maximize the plant's water status and healthy metabolic processes. Plant defense system components such as proline, phenolic compounds, and the alkaloid capsaicin have also been improved, as well as nutrient homeostasis and anatomical features. Our study suggested the use of 0.8 mM GSH for satisfying growth and yield with higher WUE of chili pepper plants grown under salt-affected conditions with deficit irrigation.

### Authors' Contributions

Conceptualization: OAAIA, GFM, AAAL; Methodology: OAAIA, GFM, HAA; Validation: MMR; Formal analysis: OAAIA, GFM, HAA; Investigation: OAAIA, GFM, HAA; Data curation: OAAIA, GFM, HAA, AAAL; Funding acquisition: AAAL; Project administration: AAAL. Writing: OAAIA, GFM, HAA; Review and editing: MMR, AAAL. All authors read and approved the final manuscript.

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### Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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