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Leaf extract of neem (*Azadirachta indica* Adr. Juss.) modulates morphological and biochemical characteristics in maize (*Zea mays* L.) under water deficit stress

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Abstract
Foliar spray of neem (*Azadirachta indica* Adr. Juss.) leaf extract was exogenously applied at pre-flowering stage to examine the changes in growth and physio-biochemical characteristics of two maize cultivars (Sultan and Sadaf) under different water regimes (100%, 75% and 60% field capacity). Water deficit conditions significantly reduced shoot fresh weight (SFW), shoot dry weight (SDW), root fresh weight (RFW), root dry weight (RDW), shoot length, root length, chlorophyll b, total soluble proteins and free proline, but it significantly increased ascorbic acid (AsA) and total phenolics in both maize cultivars. Foliar-applied leaf neem extract significantly improved SFW, SDW, RFW, RDW, shoot length, chlorophyll b and ascorbic acid concentrations, but it significantly minimized hydrogen peroxide (H_2O_2) contents. Application of 1.0% neem extract was effective in improving chlorophyll b and AsA contents, whereas 2.0% neem extract was beneficial for total soluble proteins and MDA contents. Of both maize cultivars, cv. Sultan was found to be drought sensitive and cv. Sadaf as drought tolerant. Exogenous application of leaf neem extract was suggested to be effective in enhancing drought stress tolerance in maize by improving plant growth and oxidative defense system.

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Introduction

The life cycle of plants is usually affected by various ecological factors such as exposure to abiotic stresses which affect their growth and development, ultimately limiting their productivity. Water deficit conditions, one of the serious environmental hazards, particularly in tropical and sub-tropical regions of the world significantly cause reduction in crop yield (Fathi and Tari, 2016). Under soil desiccated conditions, plants close their stomata as a first line of defense to avoid transpiration, thereby resulting in lowering of mesophyll CO_2 fixation, and hence limiting photosynthesis, mainly due to structural distortion of leaf photosynthetic pigments (Mafakheri et al., 2010). Under stress adversaries, a significant reduction in water potential in plant tissues and increase in endogenous concentrations of abscisic acid result in altering physiological processes. To combat stress deleterious circumstances, plants tend to accumulate various compatible solutes such as phenolics, glycinebetaine, and proline in cellular compartments (Fàbregas and Fernie, 2019). Similarly, accumulation of various sugars, carotenoids, and various other osmolytes against prevailing dehydrated conditions is called osmotic adjustment (Masouleh et al., 2019). Under such drought situation in the rooting zone and within cellular tissues, various toxic secondary

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metabolites such as reactive oxygen species (ROS), malondialdehyde and hydrogen peroxide are generated, which deteriorate biological membranous integrity and impair their fluidity (Laxa et al., 2019). The free radicals of ROS initiate a signaling cascade of degradation of metabolic proteins as well as nucleic acids, thereby causing peroxidation of membranous fatty acids (Hasanuzzaman et al., 2020). To alleviate the injurious effects of severe water stress, plants accumulate various antioxidative enzymes like peroxidase, catalase and superoxide dismutase in cellular compartments such as chloroplasts and mitochondria to scavenge the most damaging ROS (Das and Roychoudhury, 2014). The intrinsic protein structural modification and denaturation often occur under high temperature and dehydrated conditions, which are mostly associated with altered gene expression. Consequently, many cellular processes are altered under stressful cues (Sara et al., 2012).

Neem (*Azadirachta indica* Adr. Juss.) is a well-known plant of tropical parts of South Asia. It has a wide medicinal importance. Its leaf extract has a variety of phytochemical ingredients which are utilized for various useful purposes. Many types of chemicals present in neem such as flavonoids and terpenoids have antibacterial and insecticidal features (Verma and Mehata, 2016). The neem seed oil has numerous biological active chemicals which are potentially effective for prevention of insect populations such as mosquitoes (Benelli et al., 2019). The leaves, roots and stems of neem have potent medicinal importance against microbial infections in humans as well as in animals as antiseptic (Subapriya and Nagini, 2005). The neem plant is considered as a village pharmacy for the local inhabitants. Its leaf extract is commonly used as homeopathic medicine and in several pharmaceutical products (Brahmachari, 2004). Its alcoholic and hexane leaf extract is applied to control crop pests and for storing grains (Nathan et al., 2007).

Maize (*Zea mays* L.) is an important fodder and grain crop. It is widely cultivated in developing countries mostly in arable areas of South Asia. It is a big source of income among low-cost farmers (FAOSTAT, 2011). It is used as a staple food in many countries of the world, specifically in Africa and South Asia (Adetiminrin et al., 2008). After the production of wheat and rice, it is the third most cultivated crop of the world (Farnham et al., 2003; McCutcheon, 2007; Mboya, 2011). The crop requires reasonable amount of water for its optimum growth and productivity (Ibrahim and Ibrahim, 2020; Sah et al., 2020), but under rain-fed or water deficit conditions the crop productivity is adversely affected. Thus, the present study was carried out to determine if foliar-applied neem extract could enhance the maize crop ability to withstand drought-hit conditions.

Materials and Methods

A pot experiment comprising maize plants arranged in a completely randomized design having three replicates was setup in the Botanical Garden of the Government College University, Faisalabad, Pakistan from March to September 2020. The seeds of two cultivars of maize (Sadaf and Sultan) were procured from the Ayub Agricultural Research Institute, Faisalabad. Each plastic pot having depth 24.5 cm and radius 10.5 cm, was filled with 7 kg of sandy loam soil. The maize seeds were soaked in distilled water for 4 h prior to sowing. The sowing of soaked seeds was done at the depth of one and half cm by the hand drill technique. Within 10 days, germination started in all experimental pots. Thinning and weeding were done from time to time to retain five almost equal sized plants per pot. After the interval of two weeks of seedling growth, three water regimes (100% F.C., 75% F.C. and 60% F.C.) were maintained. After three weeks of drought stress, three levels (0%, 1% and 2%) of neem leaf extract with 0.1% tween-20 were applied as a foliar spray treatment. After two weeks of foliar spray, the maize plants were harvested for the determination of following morphological and biochemical attributes:

Morphological parameters

Shoot and root fresh weight

The fresh uprooted plants were excised into roots and shoots, then carefully they were cleaned with a blotting paper and fresh weights were recorded.

Shoot and root dry weight

The excised shoots and roots of the maize plants were air-dried and then packed in paper bags, placed in an oven at 65 ^{o}C for 3 days. After that, their dry weights were recorded.

Shoot and root length

The lengths of maize shoots and roots were recorded.

Physiological parameters

Chlorophyll extraction

The fresh excised leaf (0.5 g) was crushed with 10 ml of 80% acetone in a pre-chilled pestle and mortar. The extracted mixture was placed overnight at 4 °C. Then the supernatant was analyzed for chlorophyll *a* and *b* following the procedure of Arnon (1949).

Biochemical attributes

Total soluble proteins

For this parameter, 0.1 g of top third leaf sample was extracted in a pre-chilled pestle and mortar containing 10 mL of phosphate buffer. In a test tube, 0.1 mL of aliquot was mixed with 2 mL of the Bradford reagent. The optical density of the reaction mixture was taken at 595 nm according to Bradford (1976).

Free proline

By using a pestle and mortar, the leaf sample of 0.25 g was homogenized in 5 mL of 3% sulfosalicylic acid. Further process of the reaction mixture was completed as instructed in the method of Bates et al. (1973) by taking final absorbance at 520 nm.

Ascorbic acid (AsA)

Fresh leaf (0.1 g) was extracted in 6% TCA following the method of Mukherjee and Choudhuri (1983). An aliquot (2.0 mL) was added in a test tube along with 1.0 mL of 2% dinitrophenyl hydrazine and 1.0 mL of thiourea. The reaction contents were heated at 60 °C for 60 min. After cooling, 2.5 mL of 80% H_2SO_4 were added to the mixture and absorbance noted at 530 nm.

Total phenolics

The protocol of Julkunen-Titto (1985) was followed for the determination of total phenolics. A proportion (0.1 g) of fresh leaf was homogenized in 5 mL of acetone in an ice bath. Then in a test tube, 0.1 mL of the extract, 1 mL of Folin-Ciocalteu reagent and finally 2 mL of H_2O were added and then subjected to a vigorous shaking. After adding 5 mL of Na_2CO_3, final volume of the reaction mixture was prepared as 20 mL using distilled water and OD was measured at 750 nm.

Malondialdehyde (MDA)

The fresh leaf (3rd leaf; 0.25 g) was triturated in 2.5 mL of trichloroacetic acid (TCA) solution. The samples were centrifuged and one mL of the extract was mixed with 0.5% thiobarbituric acid (4 mL). The samples were placed in a water bath set at 95 °C and then cooled. The absorbance was measured according as documented by Cakmak and Horst (1991).

Hydrogen peroxide (H_2O_2)

A proportion (0.25 g) of fresh leaf sample was homogenized in 2.5 mL of trichloroacetic acid (TCA; 0.1%). The samples were cooled and then appropriately centrifuged. Finally, 0.5 mL of the supernatant was mixed with 0.5 mL potassium phosphate buffer and o.1 mL of potassium iodide (KI). The OD of the samples were recorded at 390 nm (Velikova et al., 2000).

Statistical analysis

For statistical analysis of the data of different plant characteristics, the COSTAT computer software (Cohort Program, 2003, Monterey, California) was used. Then, the difference among mean values was determined using the LSD test at the 5% level of probability.

Results

Imposition of water stress conditions significantly reduced ($P \leq 0.001$) shoot fresh and dry weights of both maize cultivars. The foliar treatment of neem extract showed a significant ($P \leq 0.001$) improvement in fresh and dry biomass of both cultivars under varying water regimes. Application of 1% neem extract spray exhibited better results at 75% field capacity in both cultivars. The cultivar Sadaf responded superiorly under different water regimes (**Table 1**; **Figure 1**).

Both root fresh and dry weights of maize cultivars were significantly supressed ($P \leq 0.001$) under both water regimes. The external spray of neem extract remained useful ($P \leq 0.001$) for improving root biomass of both maize cultivars. Of all different concentrations of neem extract used, 1% was found to be more effective in improving fresh biomass in both maize cultivars, but 2% neem extract was useful for cv. Sadaf at 75% field capacity (**Table 1**; **Figure 1**).

Water deficit conditions markedly decreased ($P \leq 0.001$) shoot as well as root lengths of both maize cultivars. Foliar supplementation of both levels of the neem extract showed significant results ($P \leq 0.001$) for improving shoot length, but it remained non-significant for root length. Comparatively, 1% neem spray was effective for enhancing shoot length in both maize cultivars, whereas this concentration was useful for cv. Sadaf at all field capacities.

Table 1. Analyses of variance of data for different physio-biochemical attributes of water stressed maize (*Zea mays* L.) plants subjected to varying levels of neem leaf extract.

Source of variation	df	Shoot fresh weight	Shoot dry weight	Root fresh weight	Root dry weight	Shoot length
Cultivars (Cv)	1	61.08***	1.269***	12.69***	0.257***	67.78ns
Drought Stress (D)	2	490.4***	8.689***	76.05***	0.494***	1240.0***
Neem Spray (NS)	2	32.10***	1.910***	12.66***	0.316***	188.0***
Cv x D	2	32.74***	0.865***	2.739**	0.096*	38.67ns
Cv x NS	2	17.69***	0.001ns	0.328ns	0.024ns	43.5ns
D x NS	4	4.381*	0.624***	1.569*	0.127***	22.96ns
Cv x D x NS	4	7.496***	0.167***	3.396***	0.0607*	14.66ns
Error	36	1.185	0.061	0.452	0.0198	21.24
		Root length	**Chl. *a***	**Chl. *b***	**Total soluble proteins**	**Free proline**
Cultivars (Cv)	1	10.22ns	0.0011ns	0.039ns	882.8ns	0.544***
Drought Stress (D)	2	170.5***	0.0115ns	1.140***	3372.0**	0.12*
Neem Spray (NS)	2	5.421ns	0.0314ns	0.295*	93.72ns	0.010ns
Cv x D	2	23.25*	2.681ns	0.286*	107.0***	0.025ns
Cv x NS	2	8.060ns	0.0632ns	0.071ns	905.4ns	0.029ns
D x NS	4	8.094ns	0.1266ns	0.085ns	8651.0***	0.014ns
Cv x D x NS	4	0.9837ns	0.0534ns	0.047ns	7296.0***	0.03ns
Error	36	5.060	0.0278	0.069	637.9	0.025
		Ascorbic acid	**Total phenolics**	**MDA**	**H_2O_2**	
Cultivars (Cv)	1	3.055***	13.85**	3194.0ns	83.21ns	
Drought Stress (D)	2	0.602*	5.111*	3241.0ns	2579.0***	
Neem Spray (NS)	2	0.749*	0.765ns	3230.0ns	9100.0***	
Cv x D	2	1.625***	9.569**	3229.0ns	1529.0***	
Cv x NS	2	0.188ns	5.461*	3220.0ns	2595.0**	
D x NS	4	0.161ns	2.722ns	3242.0ns	7281.0***	
Cv x D x NS	4	0.413ns	2.535ns	3244.0ns	1116.0*	
Error	36	0.167	1.501	3251.0	4156.0	

ns = non-significant; *, ** and *** = significant at 0.05, 0.01 and 0.001 levels, respectively.

Chlorophyll *a* content remained unaffected, but chlorophyll *b* was significantly reduced ($P \le 0.001$) under drought stress conditions in both maize cultivars. Chlorophyll *a* content also remained almost unaffected, but chlorophyll *b* increased significantly ($P \le 0.05$) in both cultivars upon foliage spray of neem leaf extract under soil moisture deficit conditions. Of various neem extract levels used, 2% level was effective for chlorophyll *a* in cv. Sultan, whereas 1% concentration was effective for increasing chlorophyll *b* contents in both cvs. Sultan and Sadaf under different water regimes (**Table 1**; **Figure 2**).

Under water deficit stress, total soluble proteins decreased significantly ($P \le 0.01$) in both maize cultivars. The foliage spray proved ineffective for changing this parameter. The neem spray of 2.0% promoted protein levels in cv. Sultan at 75% and 60% field capacities. However, a uniform trend was noticed of both cultivars for this attribute (**Table 1**; **Figure 3**).

Water limited conditions caused a significant ($P \le 0.05$) inhibitory impact on leaf proline in both maize cultivars. Moreover, the exogenous treatment of neem spray remained ineffective for altering this parameter. However, a significant response was noticed in cv. Sadaf under stress conditions (**Table 1**; **Figure 3**).

The levels of ascorbic acid (AsA) were found to be considerably ($P \le 0.05$) enhanced in both maize cultivars under stress field capacities. A significant ($P \le 0.05$) increase in AsA was found after foliar spray of the neem extract. Of different neem extract concentrations used, 1.0% extract showed better results at 100% and 75% water regimes in cv. Sultan (**Table 1**; **Figure 3**).

A significant increase in total phenolics ($P \le 0.05$) was detected under prevailing drought stress conditions. The externally sprayed neem leaf extract had a non-significant influence on this metabolic characteristic. The foliar-applied 1.0% extract raised the phenolic contents in cv. Sultan at 75% and 60% field capacities, whereas 2% extract was beneficial for cv. Sadaf at 100% and 75% water regimes. A significant difference between the two cultivars was observed in accumulation of these metabolites (**Table 1**; **Figure 3**).

The levels of malondialdehyde (MDA) remained unaffected under the growth medium desiccated conditions as well as upon foliage spray of neem extract. The neem extract at 2% was effective in lowering the MDA contents during water stress situations (75% and 60% F.C.) in cv. Sultan. Both maize cultivars performed differently for this parameter (**Table 1**; **Figure 3**).

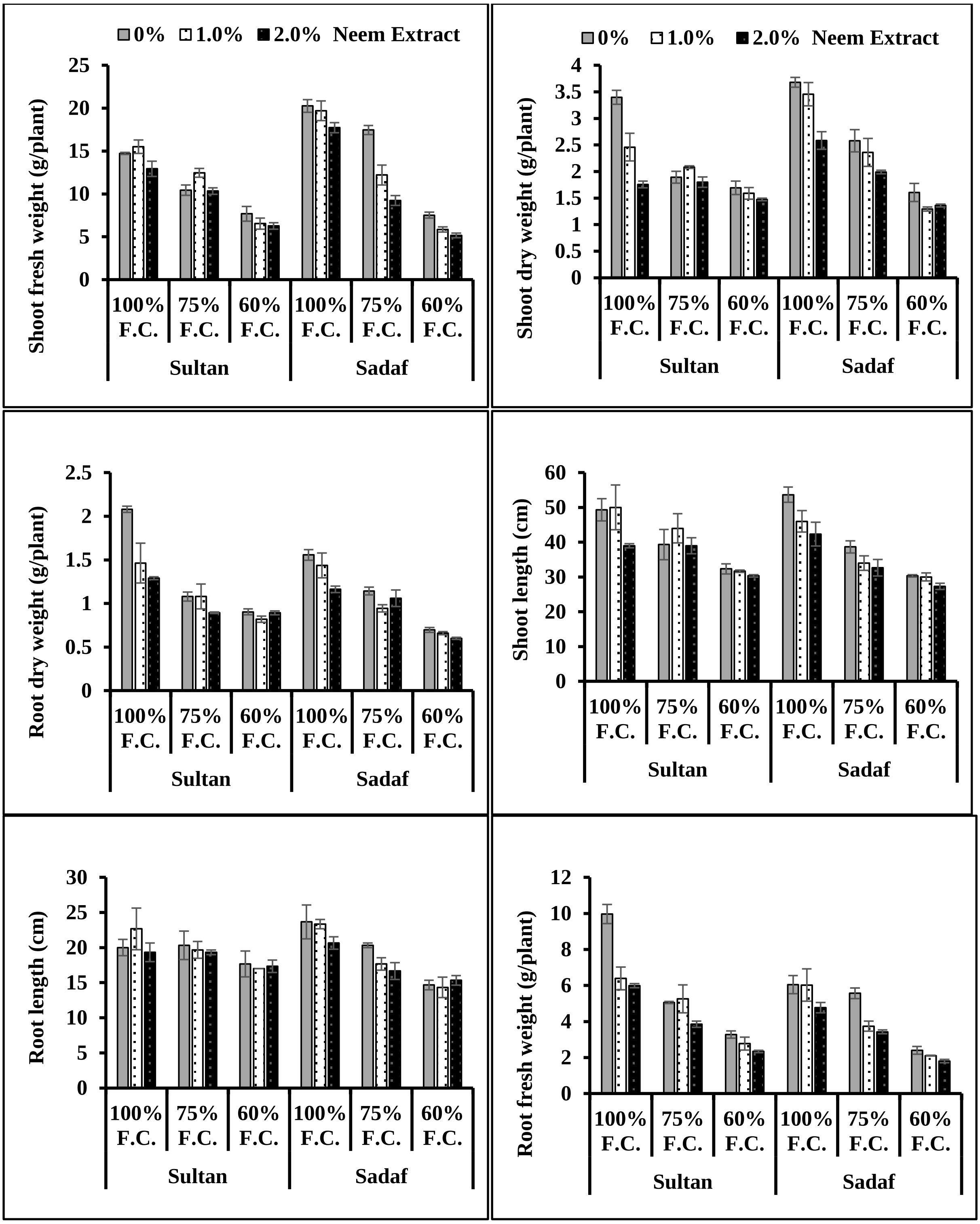

Figure 1. Shoot and root fresh and dry weights, and shoot length and root lengths of drought-stressed maize (*Zea mays* L.) plants foliar-treated with neem leaf extract. F.C., field capacity.

Water limited conditions caused a significant ($P \leq 0.001$) reduction in endogenous hydrogen peroxide (H_2O_2) contents. The neem extract application also caused a profound influence in further lowering this attribute in both maize cultivars under different field capacities. The performance of cv. Sultan was superior to the other cultivar in lowering the H_2O_2 contents under both water stress regimes (**Table 1**; **Figure 3**).

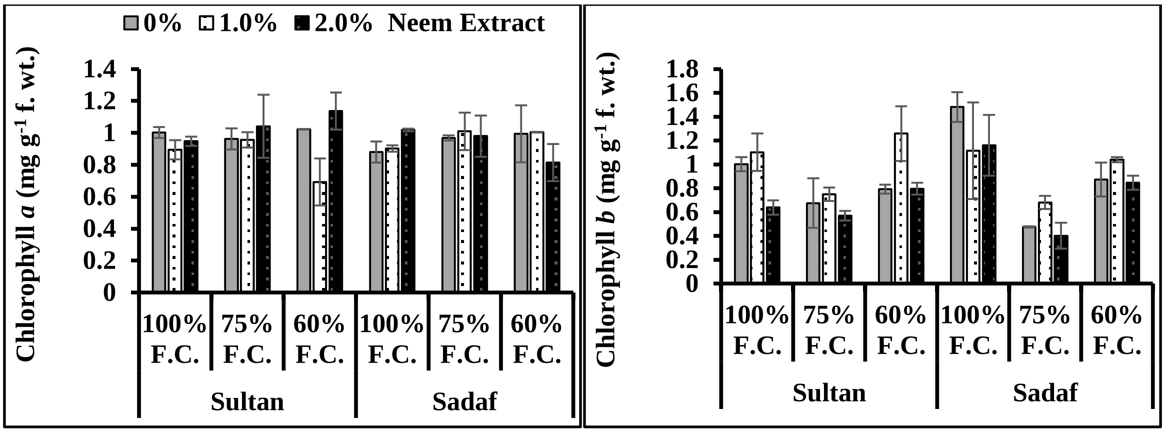

Figure 2. Chlorophyll a & b contents of drought-stressed maize (*Zea mays* L.) plants foliar-treated with neem leaf extract. F.C., field capacity.

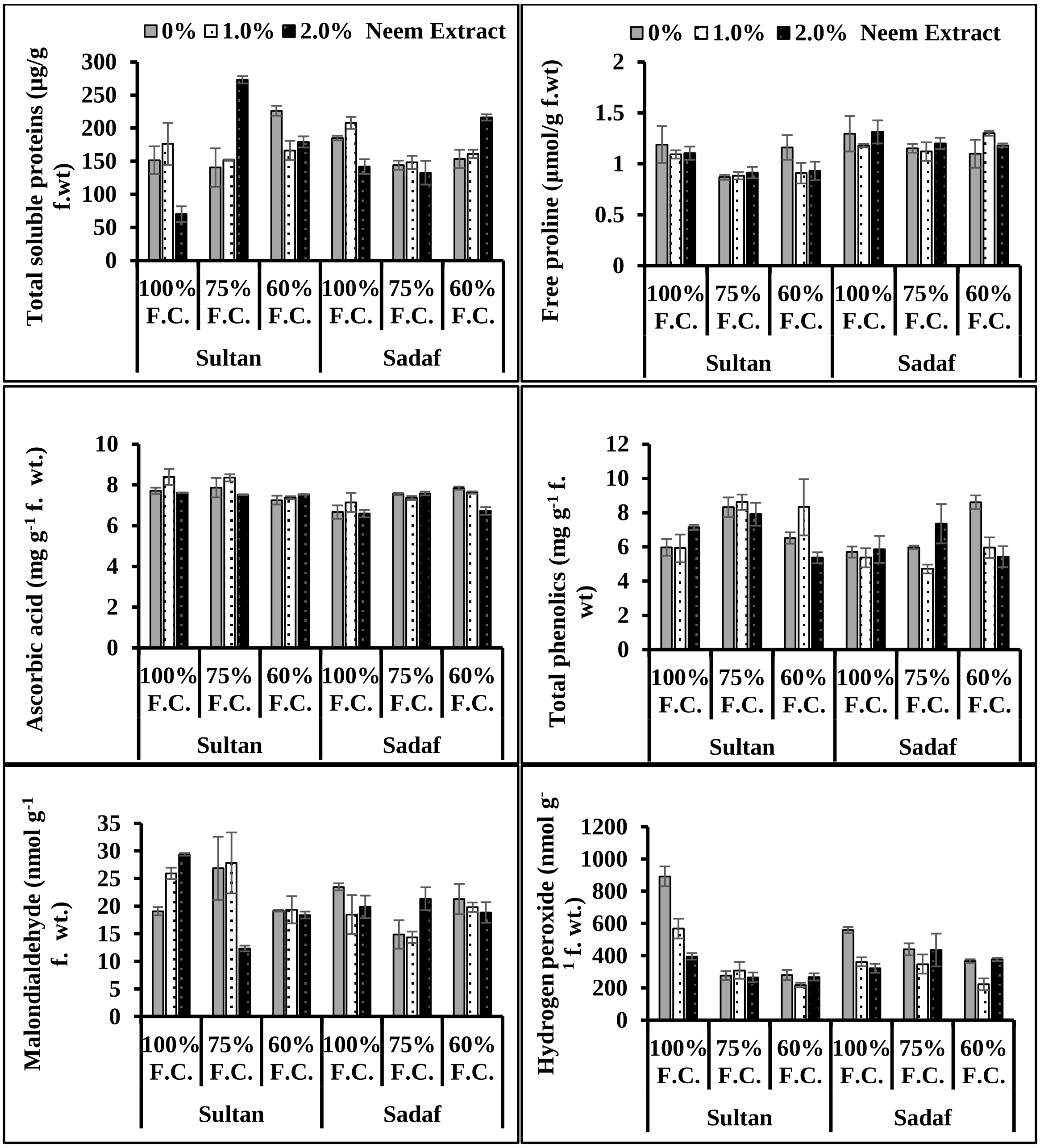

Figure 3. Total soluble proteins, free proline, ascorbic acid, total phenolics, malondialdehyde and hydrogen peroxide concentrations of drought-stressed maize (*Zea mays* L.) plants foliar-treated with neem leaf extract. F.C., field capacity.

Discussion

To examine the influence of aqueous extract of neem (*Azadirachta indica*) leaves on growth as well as physiochemical parameters of maize (*Zea mays*) under various applied water regimes (100%, 75% and 60%), a pot experiment was conducted. The water deficit conditions severely affected the development of the maize plants, when applied at the pre-flowering stage, as earlier observed elsewhere (Kosar et al., 2020). Water stress negatively affects plant metabolites such as soluble proteins, carbohydrates, minerals and antioxidants (Sadiq et al., 2018). Different mechanisms are adopted by plants to endure such stress adversaries, so that they can successfully complete their life cycle (Raja et al., 2019). Various chemicals are being frequently used as foliar spray on crop plants to minimize the deleterious effects of stress conditions they are exposed to. For example, extracts of different plants were used by Khan and Ullah (2019) on tomato plants under field conditions and the treated tomato plants showed enhanced growth. Similarly, moringa leaf extract used by Anand et al. (2020) on rice plants under salinity conditions showed a positive effect on plant growth. In the present experimentation, the external treatment of neem extract significantly improved various growth parameters (fresh and dry biomass, and shoot and root lengths) under desiccated soil conditions. These findings are similar to those reported earlier., e.g., Hassanein et al. (2010) documented growth promotion of tomato plants foliar-fed with neem extract. Similarly, the leaf extracts of tomato and soybean were effective in increasing plant height of *Peucedanum japonicum* plants (Jang et al., 2020). Likewise, height of tomato plants was reported to be increased by foliar spray of mixed plant extracts (Khan and Ullah, 2019). These findings suggest that increase in growth of different plants might have been occurred due to various vital nutrients present in leaf extracts.

Water deficit conditions inhibit photosynthesis by disrupting the structure and concentration of vital photosynthetic pigments (Anand et al., 2020). The present results showed that chlorophyll (*a* and *b*) contents were enhanced by the exogenously applied neem leaf extract in both maize cultivars under water shortage regimes. These findings support an earlier study in which chlorophyll contents were reported to be increased in groundnut upon exogenous application of a mixture of panchagya and neem extracts under natural conditions (Kumawat et al., 2009). Moreover, application of moringa leaf extract on wheat plants caused enhanced accumulation of chlorophyll contents under salinity stress. It was suggested that magnesium present in the moringa leaf extract might be useful for promoting chlorophyll in plants (Latif and Mohamed, 2016). Likewise, application of seaweed extract also elevated chlorophyll contents in celeriac plants (Shehata et al., 2011).

Sugar contents in cellular metabolism play a significant role in osmoprotection, membrane stabilization and maintenance of turgor potential under stress conditions (Singh et al., 2015). In the present work, the limited water regimes as well as neem extract caused non-significant effect on sugar content in the maize plants. These findings do not align to what has been reported in some earlier studies. For example, increased sugar contents were observed in water stressed sunflower plants (Kosar et al., 2020), and water stressed radish plants (Shafiq et al., 2015).

Under induced stressful cues, the stressed plants tend to accumulate a variety of osmoprotectants like sugars, proline and many others in plant tissues to safeguard plant metabolic processes like alleviation of ROS activities, maintenance of osmolality, buffering of oxidative reactions and acting as an energy source (Okunlola et al., 2016). In the current study, water limited conditions caused a significant inhibitory impact on leaf proline in both maize cultivars. Moreover, the exogenous treatment of neem spray remained ineffective for this parameter. These results do not agree to what has been reported earlier in rice that foliar spray of *Ocimum* reduced proline contents in water stressed plants (Pandey et al. (2016).

Accumulation of ascorbic acid in plants primarily activates the antioxidant system under drought stress to minimize reactive oxygen species and improve plant acclimatization against stress situation (Mittler, 2002). In the present study, increased AsA concentration was observed under applied water stress conditions and upon foliar application of neem extract. These results are in harmony with those of Khan and Ullah (2019) in which the treatment of moringa leaf extract caused a significant increase in endogenous ascorbic acid in salinized tomato plants. Similar findings were also reported by Nasira et al. (2016) in Kinnow, Javaheri et al. (2012) in tomato and Reuhua et al. (2008) in orange plants.

Phenolics, non-enzymatic antioxidants, are known to safeguard plant macromolecules from deterioration during abiotic stresses (Apel and Hirt, 2004). In the current study, total phenolics increased in both maize cultivars under water limited regimes. Moreover, exogenously supplemented neem leaf extract caused a marked increase in phenolic content in the maize plants. These results can be supported by some earlier studies. For example, exogenous applications of various leaf extracts and phyto-hormones were found to be effective in recovering plant growth and maintaining plant endogenous phenolics under stress adversaries (Myung-Min et al., 2010). In cabbage, the external application of seaweed extract also improved phenolic compounds (Lola-Luz et al., 2013). Hussain et al. (2020) were of the view that water stressed plants utilize phenolic compounds in mitigating the toxicity of stress metabolites.

The formation of lipid peroxidation is the first biochemical symptom of induced oxidative stress. The harsh conditions of abiotic stresses trigger an imbalance between the activities of antioxidant enzymes and generations of reactive oxygen species (Mittler, 2002). In the recent investigation, the water limited regimes caused a non-significant impact on malondialdehyde, but they significantly reduced hydrogen peroxide in both maize cultivars. Contrary to this, Latif and Mohamed (2016) reported significantly higher contents of MDA and H_2O_2 under temperature, salinity and radiation stresses in common beans, whereas the spray of moringa leaf extract considerably suppressed both MDA and H_2O_2.

Conclusion

The foliar treatment of neem extract at pre-flowering stage significantly improved shoot fresh weight, shoot dry weight, root fresh weight, root dry weight, shoot length, chlorophyll *b* and ascorbic acid, but it remained non-effective for promoting root length, chlorophyll *a*, total soluble proteins, proline, total phenolics, and MDA contents. However, neem leaf extract was effective in lowering the hydrogen peroxide. These results suggest that neem leaf extract is effective in improving the growth and physio-biochemical attributes of maize crop under water deficit conditions.

Author(s), Editor(s) and Publisher's declarations

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The authors declare no conflict of interest.

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Contribution of authors
Planning and conduction of experiment: RH, IS, Data acquisition, analysis, and interpretation: MS. Write-up of initial draft: AA. Research supervision: NAA. Review of final draft: PG.

Ethical approval
This study does not involve Human/animal subjects and no ethical approval is needed.

Handling of bio-hazardous materials
The authors certify that all experimental materials were handled with care during collection and experimental procedures. After completion of experiment, all materials were properly discorded to minimize any types of bio-contamination(s).

Availability of primary data and materials
As per editorial policy, experimental materials, primary data, or software codes are not submitted to the publisher. These are available with the corresponding author and/or with other author(s) as declared by the corresponding author of this manuscript.

Authors' consent
All contributors have critically read this manuscript and agreed for publishing in IJAaEB.

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