Saudi Journal of Biological Sciences 29 (2022) 1367–1374

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Leaf extract of neem (*Azadirachta indica*) alleviates adverse effects of drought in quinoa (*Chenopodium quinoa* Willd.) plants through alterations in biochemical attributes and antioxidants

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ARTICLE INFO

Article history:
Received 19 December 2021
Revised 14 January 2022
Accepted 16 January 2022
Available online 22 January 2022

Keywords:
Quinoa
Neem extract
Drought tolerance
Antioxidants
Osmoprotectants

ABSTRACT

The influence of varying concentrations (0, 1, 3, 4, 5, 6, 8, and 10 % v/v) of neem (*Azadirachta indica*) leaf extract on drought stressed (40 % field capacity) quinoa (*Chenopodium quinoa* Willd.) plants was assessed. During the current study two cultivars of quinoa (V7 and V9) were used. This study revealed that water stress adversely affects the fresh and dry weight of shoots and roots as well as chlorophyll pigments (*a* and *b*) of both quinoa cultivars. In contrast, drought stress enhanced glycinebetaine (GB), free proline, phenolic content, hydrogen peroxide (H_2O_2), activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) enzymes, and relative membrane permeability (RMP). However, application of neem leaf extract improved the accumulation of key osmoprotectants like proline, GB and activities of enzymatic antioxidants. Our findings showed 5 % neem leaf extract is an effective treatment in counteracting the oxidative damage caused by water stress, thereby improving overall plant growth. Of both cultivars of quinoa, the response of cv. V9 to stress as well as foliar applied neem was relatively more promising.
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1. Introduction

Growth of plants is restricted by different environmental stresses like water stress (drought and water logging), temperature and salinity. Drought stress severely impairs and affects growth and yield of plants, as availability of water is of prime importance for normal functioning of plant metabolism (Hasanuzzaman et al., 2011; Rollins et al., 2013). Due to the exposure of extended dry period, plants may adopt some physiological and biochemical changes such as restricted leaf size, and premature flowering, ultimately leading to reduced yield (Aroca, 2012; Abbas et al., 2021). Water scarcity leads to the generation of reactive oxygen species in plants that may injure cellular membranes and reduces rate of photosynthesis. Drought stress may cause damage to vital proteins, inactivating key enzymes involved in plant metabolism (Wilhelm and Selmar, 2011; Fahad et al., 2017; Mushtaq et al., 2021). Plants withstand drought stress, due to activation of various enzymatic and non–enzymatic antioxidants, which include peroxidase (POD), catalase (CAT), total phenolics, ascorbic acid etc. (Akram et al., 2016; Aziz et al., 2018; Ahmad et al., 2010, 2019; Kohli et al., 2019).

The foliar application of various natural and synthetic substances is being practiced aiming to mitigate the negative impacts of water stress (Kaya et al., 2020; Kosar et al., 2020; Jabeen et al., 2021). Natural compounds derived from plants are believed to be a cost-effective compared with such compounds produced synthetically (Ernst, 2000; Hunt et al., 2014; Zalewsk and Nogalska, 2014). *Azadirachta indica* has great importance around the world due to its multiple applications in medicine and cosmetics (Rodrigues et al., 2011). It was reported by Rodrigues et al. (2011) that neem tree contains about three hundred or more secondary metabolites, one 3rd of which are limonoids (tetratriterpenoids) having various biological applications. The most

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2022.01.038
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active compound is azadirachtin having an effective curative or healing role (Subapriya et al., 2005; Rahmani et al., 2018). Neem leaves contain various active compounds like ascorbic acid, amino acids, nimbolide and nimbin, etc. (Rodrigues et al., 2011; Sarah et al., 2019). Some polyphenolic compounds (e.g. quercetin and ß-sitosterol) are also extracted from neem leaves showing antifungal and antibacterial properties (Alzohairy, 2016).

Quinoa (*Chenopodium quinoa* Willd.) is consuming considerably substituting other important cereals including wheat (Graf et al., 2015). This is because of the reason that its grain composition is of high quality compared with that of other cereals (Angeli et al., 2020). Although quinoa is reported to be relatively more tolerant to stressful environments such as drought and salinity than the other contemporary crops, its growth and grain yield have been reported to be considerably reduced due to these stresses (Ruiz et al., 2016; Angeli et al., 2020). So, there is a need to adopt some viable approaches to mitigate the adverse effects of drought stress on growth and yield of quinoa plants. Thus, the present study tests the efficacy of neem leaf extract enriched with multiple nutrients in ameliorating the negative impact of water deficiency on quinoa plants. Moreover, it has been investigated whether or not exogenously applied neem leaf extract helps the quinoa plants to regulate their key metabolic processes such as osmoregulation, and oxidative defensive system under drought stress conditions.

2. Materials and methods

An experiment was set up from Nov. 2017 – Jan. 2018 to assess the effectiveness of neem (*A. indica*) leaf extract in improving water stress tolerance of two quinoa cultivars (V7 and V9). Four replicates of each treatment were arranged randomly. During the entire research period the average atmospheric conditions were noted as: relative humidity, 60 %; sunshine, 10.1 h; rainfall, 20.10 mm; and average day (28.0 °C) and night (18.94 °C) temperatures. Each plastic pot (30 cm in diameter) was filled with sandy-loam soil (7 kg) having electrical conductivity, 2.01 dS m^{-1}, pH 7.8, and saturation percentage, 32 %. Water stress treatment i.e., 40 % field capacity was employed in addition to control (100 % field capacity). After the maintenance of desired levels of water stress for a month, neem leaf extracts were applied as foliar application. Fresh neem leaves (100 g) were taken from an agricultural field of Punjab, Pakistan. All leaves were washed and ground in 100 ml distilled water using a pestle and mortar. After filtration, the filtrate was used to prepare varying levels of aqueous neem extract (0, 1, 3, 4, 5, 6, 8, and 10 % v/v); using deionized water and stored at 4 °C before one day of application. After 15 days of exogenous treatment of leaf neem extract, quinoa plants were uprooted, and their root and shoot lengths were measured. The root and shoot dry of plants were obtained after oven drying at 70 °C for 70 h. Thereafter, the samples were also collected for the estimation of the below mentioned biochemical characteristics and stored at ultra-low freezing temperature.

2.1. Chlorophyll contents

The contents of chlorophyll were estimated following the methodology of Arnon (1949). The absorbance of samples was recorded at 663 and 645 nm.

2.2. Relative membrane permeability (RMP)

It was appraised following the methodology of Yang et al. (1996). A fresh leaf from top was excised, chopped in distilled water (10 ml), and the original EC (EC_0) has been observed using a digital EC meter (Spectrum Technologies Inc., USA). After measuring EC_0, the samples were kept for one day at 4 °C and EC_1 has been recorded. Then the extract was autoclaved and cooled to room temperature for the measurement of EC_2. The relative membrane permeability has been calculating through Eq. (1):

$$Relative\ membrane\ permeability\ (\%) = \frac{EC_1 - EC_0}{EC_2 - EC_0} x100 \quad (1)$$

2.3. Relative water contents

After measuring fresh weights (FW) of the leaf samples, the samples were kept in water for 3.0 h. The weight of the turgid leaf (TW) was noted, and the samples were then oven dried (72 h) to record their dry weights (DW). The relative water contents (RWC) were determined by applying Eq. (2).

$$RWC(\%) = \left(\frac{FW - DW}{TW - DW}\right) x100 \quad (2)$$

2.4. Glycinebetaine contents

The Grieve and Grattan (1983) protocol was used to determine glycinebetaine contents (GB). The absorbance of the samples was noted at 365 nm.

2.5. Free proline contents

To determine the free proline content, 0.5 g of fresh leaf was homogenized in 3% sulfosalicylic acid. Glacial acetic acid (2 ml) and acid ninhydrin solution (2 ml) were added to 2 ml of each sample. All samples were placed in a water bath at 95 °C for 60 min, cooled, and toluene (4 ml) was added. After addition of toluene to the mixture, the mixture split. After discarding the top layer, the OD of the bottom layer was noted at 520 nm (Bates et al., 1973).

2.6. Hydrogen peroxide concentration

For the estimation of hydrogen peroxide (H_2O_2) concentration, the Velikova et al. (2000) protocol was followed. Fresh leaf sample (0.25 g) was mixed with an aliquot of 2.5 ml of TCA (1 %). After centrifugation at 12,000*g* for 15 min, 500 µl of the supernatant, 500 µl of the buffer solution and 100 µl of potassium iodide (KI) were added and mixed in. The absorbance of each treated sample was recorded at 390 nm.

2.7. Total phenolic contents

The total total phenolics were quantified by Julkunen-Tiitto (1985) method and the optical density was recorded at 750 nm.

2.8. Total soluble proteins

The quantification of total soluble proteins was performed by following Bradford (1976) method.

2.9. Enzyme assay

Fresh leaf (0.5 g) was extracted in 10 ml of ice cooled phosphate buffer (pH 7.8) and centrifuged at 15,000*g* for 15 min at 4 °C. After filtration, the activities of following enzymes were noted as per sections 2.10.1–2.10.3.

2.9.1. Superoxide dismutase (SOD)

Superoxide dismutase activity was determined by the van Rossum et al. (1997) methodology and OD of samples were recorded at 560 nm.

2.9.2. Catalase (CAT)

Catalase activity was assayed by Luck (1974) methodology and the OD of samples were recorded at 240 nm.

2.9.3. Peroxidase (POD)

The activity of POD was assayed by following a procedure of Chance and Maehly (1955) and the optical density of the samples was read at 400 nm.

2.10. Statistical analysis

This experiment was conducted randomly with four replicates of all treatments including water stress, foliar application, and cultivars. Data were analyzed using the Costat software version v6. 303 (Informer Technologies Inc., USA) to conduct the analysis of variance of data (ANOVA). Mean values were compared using least significant difference (LSD) test at 5 % probability level.

3. Results

Reduction in shoot dry and fresh weights was significant ($P \leq 0.001$) in both quinoa cultivars at 40 % field capacity. The data indicated that foliar-applied neem leaf extract enhanced the growth of water-stressed quinoa plants. In both lines of quinoa, 5 % leaf neem extract leaves was more efficient than the other levels used in improving growth under limited water conditions (Table 1, Fig. 1).

Shoot and root dry and fresh weights suppressed under water stress conditions, while they were recorded to be improved when neem leaf extracts were applied as foliar spray. It was noted (Table 1, Fig. 1) that 5 % and 10 % aqueous extracts were better in improving these attributes under water stress conditions.

Under water scarce conditions, shoot and root lengths decreased considerably in both cultivars ($P \leq 0.001$). Exogenously applied neem leaf extract doses increased ($P \leq 0.001$) the root and shoot lengths of both cultivars under stress. Of all levels of neem extract, 5 % and 3 % showed better results under drought conditions particularly in cv. V7, whereas in cv. V9, the performance of 5 % and 10 % neem extract was markedly better compared to all other levels of neem extract (Table 1, Fig. 1).

The water stress also caused a drastic decrease in photosynthetic pigments such as chlorophyll *a*, b and total chlorophyll content of quinoa leaves. (Table 1, Fig. 2). Varying levels of the aqueous neem leaf extract were significantly effective in increasing ($P \leq 0.001$) chlorophylls *a* and *b* and their ratio (*a/b*) in both quinoa lines under stress and non-stress conditions. However, 5 % neem extract was relatively more effective in increasing ($P \leq 0.001$) the chlorophyll contents in both quinoa cultivars.

The relative water content remained unchanged in both the cultivars of quinoa plants under the conditions of water (Table 1, Fig. 2). However, relative water content was slightly improved in

Table 1
Mean squares values for growth and physio-biochemical attributes of *C. quinoa* subjected to leaf neem extract under water stress.

Source of variations	df	Shoot fresh weight	Shoot dry weight	Root fresh weight	Root dry weight
Cultivars (Cv)	1	277.7***	59.07***	1.633***	0.057 ns
Drought stress (D)	1	6298.1***	1577.7***	36.59***	6.588***
Application of extract (Ext)	7	108.6***	28.24***	1.209***	0.228***
D × Cv	1	52.60***	16.31***	0.571***	0.036 ns
Ext × Cv	7	67.93**	18.79***	0.791***	0.233***
D × Ext	7	89.44***	20.09***	1.104***	0.216***
D × Ext × Cv	7	59.32***	16.83***	0.624***	0.204***
		Shoot length	Root length	RMP	RWC
Cultivars (Cv)	1	29.04***	50.01***	5490.1***	0.027 ns
Drought stress (D)	1	1549.7***	2.573 ns	1298.7*	1.016 ns
Application of extract (Ext)	7	25.24***	2.989**	1805.7***	0.026*
D × Cv	1	19.24***	49.43***	287.24 ns	0.003 ns
Ext × Cv	7	29.19***	2.018**	4175.7***	0.020 ns
D × Ext	7	19.79***	2.050*	4256.3***	0.018 ns
D × Ext × Cv	7	9.667***	0.9229*	2302.0***	0.077***
		Chl. *a*	Chl. *b*	Chl. *a/b*	Total Chl.
Cultivars (Cv)	1	1.555 ns	0.540***	0.269**	0.535***
Drought stress (D)	1	3.074 ns	0.034 ns	0.039 ns	0.041 ns
Application of extract (Ext)	7	9.153**	0.101***	0.070**	0.095***
D × Cv	1	0.001*	0.443***	0.157*	0.398***
Ext × Cv	7	0.001***	0.063**	0.030 ns	0.052**
D × Ext	7	0.001***	0.111***	0.073**	0.115***
D × Ext × Cv	7	0.001***	0.159***	0.057*	0.167***
		H_2O_2	Proline	GB	Total phenolics
Cultivars (Cv)	1	0.089***	138.8***	32344.1***	199.7***
Drought stress (D)	1	0.035***	321.5***	14418.0***	194.7***
Application of extract (Ext)	7	0.019***	32.70**	1574.1***	154.4***
D × Cv	1	0.045***	2.754***	19015.3***	11.76***
Ext × Cv	7	0.029***	16.33***	1863.8***	237.8***
D × Ext	7	0.020***	23.45***	1080.2***	160.3***
D × Ext × Cv	7	0.005***	15.23***	1113.3***	196.3***
		TPs	CAT	SOD	POD
Cultivars (Cv)	1	110867.3***	8.564***	1778.4***	1043.9**
Drought stress (D)	1	31824.9***	2.107**	4.653 ns	374.9**
Application of extract (Ext)	7	86923.9***	4.994***	577.4***	1347.8***
D × Cv	1	166567.7***	0.494 ns	2.015 ns	2597.6**
Ext × Cv	7	49731.7***	6.161***	504.1***	397.1*
D × Ext	7	25709.7***	1.375***	150.7***	519.0**
D × Ext × Cv	7	39101.0***	2.134***	140.8***	2101.6***

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

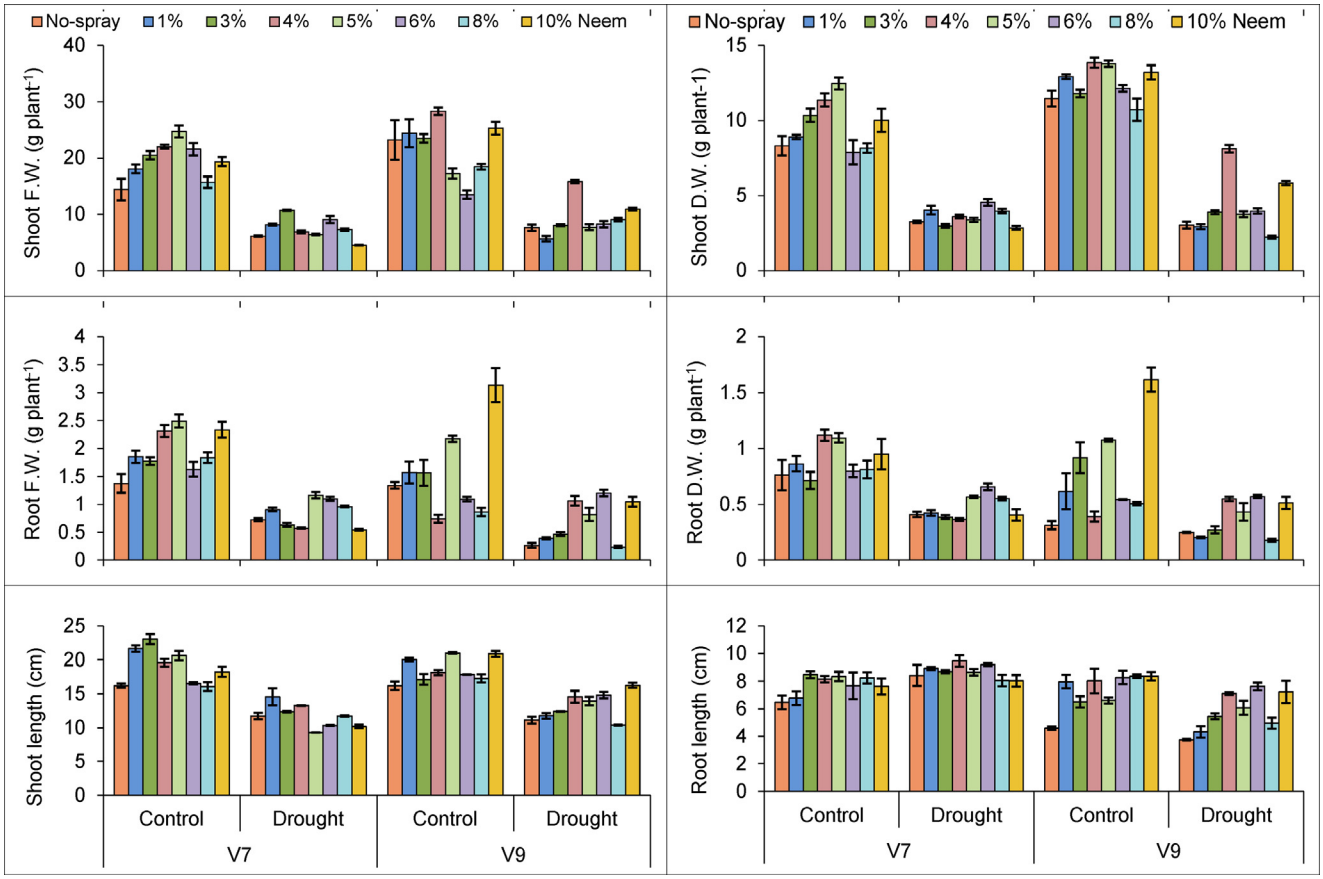

Fig. 1. Shoot and root fresh and dry weights and shoot and root lengths of two cultivars of *C. quinoa* exposed to neem extract under water stress.

both the cultivars upon exogenous foliar application of neem leaf extract. Of all selected levels of neem leaf extract, 5 % and 6 % concentrations of neem leaf extract were found to be optimum in improving relative water content in both the cultivars.

Under the shortage of water, RMP enhanced in both cultivars ($P \leq 0.05$), whereas foliar application of neem leaf extract caused a marked reduction in RMP (Table 1, Fig. 2). In reducing RMP, 5 % neem extract was more effective compared to all other levels used, particularly in cultivar V7.

Analysis of variance of data exhibited that H_2O_2 contents enhanced under drought stress conditions particularly in cv. V7, while in cv. V9 the H_2O_2 contents were not much affected (Table 1, Fig. 3). However, neem extract applied exogenously reduced the accumulation of H_2O_2 contents significantly ($P \leq 0.001$). In cultivar V7, 3 % whereas in cv. V9, 5 % neem extract was found to be effective in minimizing the accumulation of H_2O_2 contents.

Under drought conditions, free proline increased significantly in both quinoa cultivars. However, foliage spray of neem extract further enhanced proline content in drought stressed quinoa plants. Compared to all other levels of neem extract, 10 % showed good results in both quinoa cultivars (Table 1, Fig. 3). While in cv. V9, the role of 5 % extract in increasing proline was been observed prominent.

Water stress enhanced the glycinebetaine (GB) contents markedly ($P \leq 0.001$) in both quinoa cultivars. However, applications of exogenously applied neem extract significantly improved the GB contents in both quinoa cultivars (Table 1, Fig. 3). Furthermore, cultivar V9 was better in GB contents under the shortage of water due to exogenous application of neem extract.

The concentration of phenols significantly improved in both the cultivars of quinoa plants under water scarce conditions. Further, the exogenously applied leaf neem extract also enhanced the phenolic concentration of water stressed quinoa plants. (Table 1, Fig. 3).

The concentration of total soluble proteins decreased considerably in both the cultivars of quinoa plants under the conditions of water stress. On exposure to neem leaf extract at the rate of 3 %, 4 % and 5 %, a marked increase in total soluble proteins was noticed in cv. V7. However, in cv. V9, 4 % extract showed good results (Table 1, Fig. 3).

A significant ($P \leq 0.001$) increase in the activities of peroxidase (POD), superoxide dismutase (SOD) and catalase (CAT) enzymes was observed in drought stressed both quinoa cultivars. The antioxidant activities of all three enzymes were found to be considerably increased due to foliar applied neem extract (Table 1, Fig. 4).

4. Discussion

Water is one of the basic needs having direct effects on agricultural productions as well as on security of food (Garg et al., 2002; Hefft and Alberini, 2020). For scientists and farmers, it is a problem of great concern, so there is a need to introduce those plants which can cope with various environmental cues including drought stress. Generally, plant water relations become imbalanced under water deficit conditions, thereby causing osmotic stress leading to restricted cell elongation (Alam et al., 2014). In this study, weights of fresh and dry shoots reduced significantly when there was not enough supply of water. In agreement with our results Dawood and Sadak (2014) also reported that when water supply to plants is not adequate, their metabolism is adversely affected leading to reduced biomass. Fathi and Tari (2016) observed that

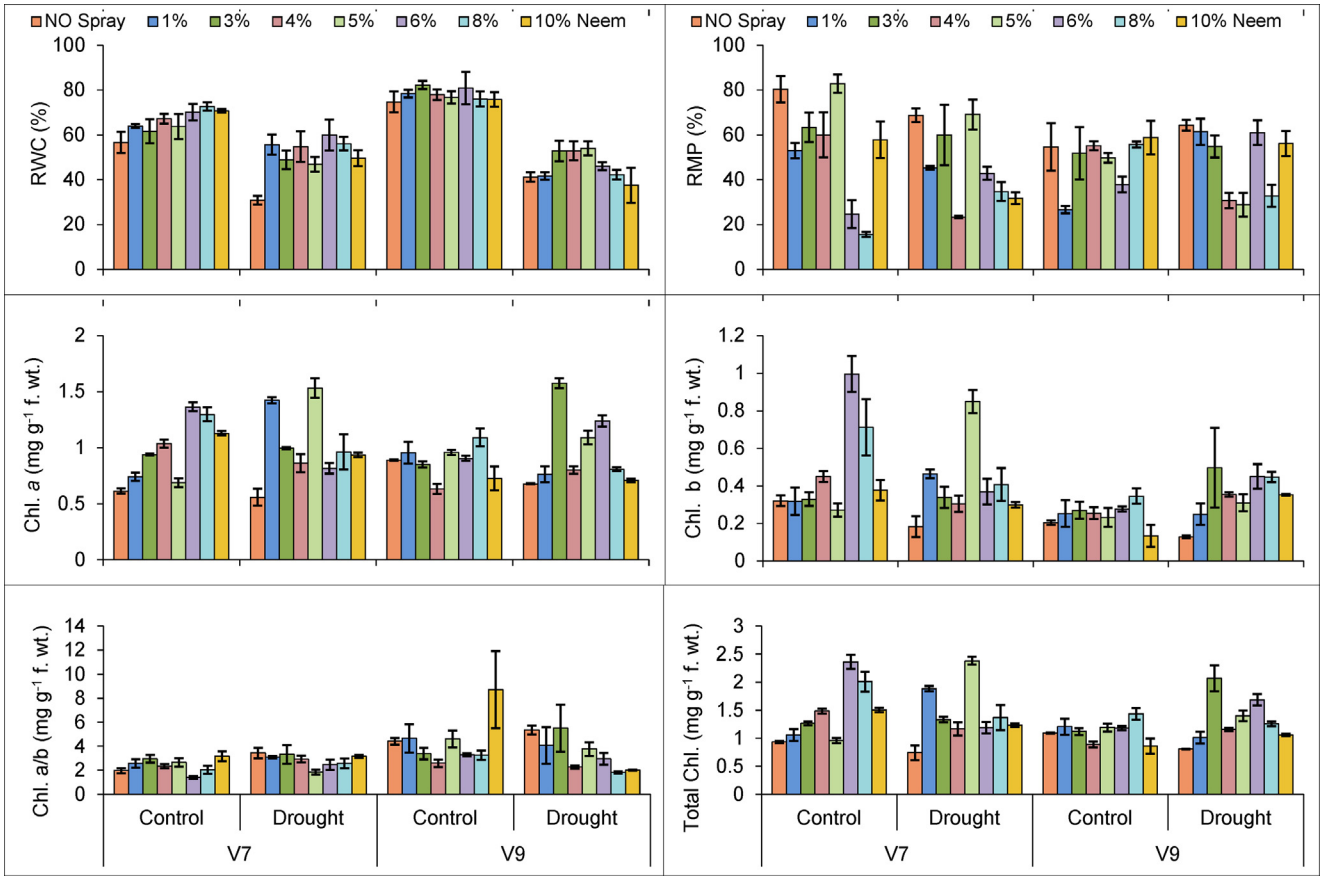

Fig. 2. Relative water contents (RWC), relative membrane permeability (RMP) and chlorophyll pigments of two cultivars of *C. quinoa* exposed to neem extract under water stress.

water deficit condition at any stage of plant life can influence the growth of most plants including millet, sorghum, cowpea, etc.

Neem leaves are supposed to be a rich source of different chemicals like amino acids and ascorbic acid, etc. so, the neem leaf extract may help the plants to cope with negative impacts of water scarcity (Alzohairy, 2016). In this study, foliar-applied neem leaf extract (a rich source of AsA) enhanced both dry as well as fresh weights of quinoa plants. Likewise, in another study with tomato, exogenously applied AsA (0.5 mM) improved plant biomass under water deficit condition induced by PEG or osmotic stress induced by salt stress (Shalata and Neumann, 2001).

Drought stress generally causes reduction in photosynthetic pigments in most plants (Aroca, 2012). This may happen due to disintegration of membranes and production of ROS which ultimately affect the overall chloroplast metabolism (Jung, 2004; Akram et al., 2018; Ahmad et al., 2019; Kohli et al., 2019). In the current study, chlorophyll contents decreased due to water shortage, but exogenous application of neem leaf extract (a rich source of AsA and various other chemicals) improved chlorophyll contents in both quinoa cultivars. Similarly, the effect of AsA was assessed on wheat plants by Amin et al. (2008). They applied four levels of AsA (0, 100, 200 and 400 mg L^{-1}) and found that exogenously applied AsA (400 mg L^{-1}) caused a marked improvement in chlorophyll contents of wheat plants.

Cell membranes are highly liable to stressful cues including drought stress (Huang et al., 2019). The stability of membrane is highly influenced due to shortage of water (ElBasyoni et al., 2017).

In the current work, it has been viewed that under limited supply of water cell membrane permeability increased as already observed by ElBasyoni et al. (2017). In the present work, neem extract applied as a foliar spray suppressed RMP which is quite consistent with the studies conducted by Zonouri et al. (2014) in in grape plants subjected to drought stress (25 % field capacity) and foliar applied AsA (1 %). AsA is one of the integral components of neem extract and confers tolerance against drought stress by augmenting the antioxidant machinery in various crop plants like canola (Shafiq et al., 2014), safflower (Farooq et al., 2020), cauliflower (Latif et al., 2016), and quinoa (Aziz et al., 2018).

Under osmotic stress, plants tend to maintain their cellular water content through osmoregulation (Ali et al., 2013; Huang et al., 2019; Punia et al., 2021). For osmoregulation, plants generally accumulate high amounts of inorganic or organic osmotica. Out of different organic osmotica known in the literature, GB and proline play an important role not only as osmoregulators but also play a significant role in various metabolic processes (Dawood and Sadak, 2014; Elewa et al., 2017) including the counteraction of reactive oxygen species (ROS) (Latif et al., 2016). In the current study, foliar-applied neem extract was found to be effective in enhancing GB and proline in the quinoa plants. Similarly, in another study with water stressed canola plants Shafiq et al. (2014) reported enhanced accumulation of GB and proline due to exogenously supplied AsA.

Total phenolics are considered as important secondary metabolites which are actively involved in ROS scavenging. Thus, most plants stressed with drought over-accumulate total phenolics as already observed in different plants such as fenugreek, tomato, and quinoa (Sgherri et al., 2003; Amin et al., 2008; Abd Elhamid et al., 2016; Elewa et al., 2017). Likewise, in the present experiment, a marked increase in total phenolics was observed in the quinoa plants experiencing water deficit conditions. Moreover,

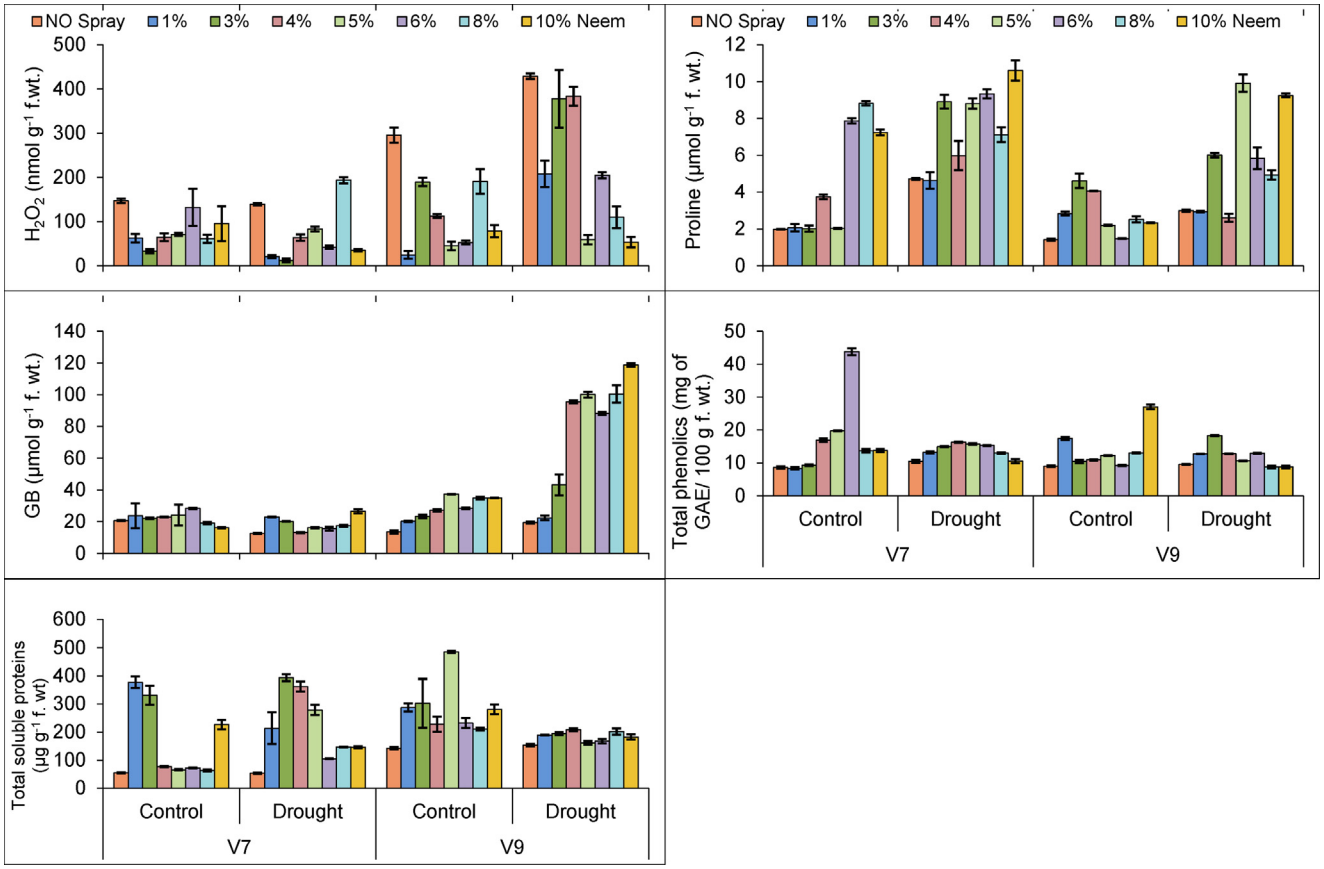

Fig 3. Hydrogen peroxide, proline and glycinebetaine contents of two cultivars of *C. quinoa* exposed to neem extract under water stress.

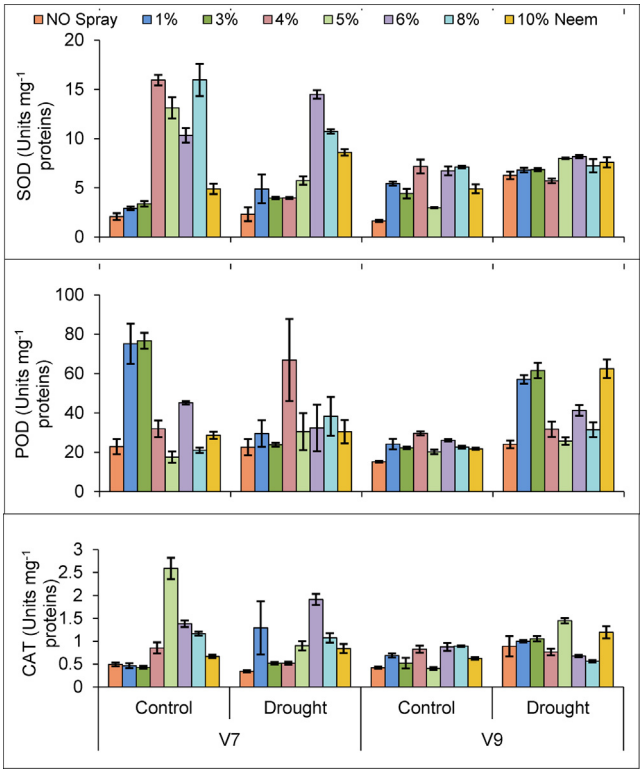

Fig 4. Enzymatic assay of water-stressed and non-stressed plants of two cultivars of *C. quinoa* exposed to neem extract under water stress.

application of neem leaf extract further enhanced the levels of total phenolics in both cultivars of quinoa plants under water stress.

It is evident that proteins accumulate in plants as a response to stressful cue (Mohammadkhani and Heidari, 2008). Proteins play a critical role in plant stress response as they are directly involved in shaping novel phenotypes by triggering specific physiological traits in response to changing environment. During the current study the concentration of total soluble proteins in quinoa plants increased under water stress as well as due to application of neem leaf extract. Similarly, Farooq et al. (2020) investigated the effect of AsA on four varying cultivars of safflower (*Carthamus tinctorious)* under stress and non-stress conditions. They suggested that total soluble proteins increased significantly under stress conditions on exposure to foliage applied ascorbic acid in safflower plants.

On exposure to stress conditions, plants activated their defensive mechanism against the stress-induced oxidative stress (Caverzan et al., 2016). Antioxidants are involved in ROS detoxification (Ahmad et al., 2010, 2019; Kohli et al., 2019). Generally, plants under adverse environmental conditions, exhibit higher activities of various enzymes like APX, CAT, SOD and POD (Amin et al., 2008; Kohli et al., 2019). These enzymatic antioxidants lessen the levels of hydrogen peroxide as well as superoxide (Kusvuran et al., 2016). In this study, in both quinoa cultivars, the activities of the antioxidant enzymes were found to be enhanced under water scarcity, and foliar applied neem leaf extract further improved antioxidant activity of these enzymes.

5. Conclusions

Overall, the growth of both quinoa cultivars was negatively affected due to the deficiency of water. While organic osmolytes

such as free proline, GB, total phenolics, H_2O_2, RMP and enzymatic activities were found to be increased due to water deficiency. Application of leaf neem extract improved the activities of enzymatic antioxidants as well as the accumulation of osmoprotectants under water stress conditions. These findings showed that 5 % neem leaf extract was more effective than the other levels used in counteracting the oxidative damage caused by water stress, thereby improving plant growth of quinoa plants.

Ethics approval

Not applicable.

Consent to participate

All authors consent to participate in the manuscript publication.

Consent for publication

All authors approved the manuscript to be published.

9. Availability of data and material

The data supporting the conclusions of this article are included within the article. Any queries regarding these data may be directed to the corresponding author.

Funding

No funding was received for conducting this study.

Author contributions

HN designed the research and NAA edited the manuscript; MA helped in Methodology and Project administration; MA, and HN conducted experiments; DIH and BLJ analyzed the data and helped in revision of the manuscript. NAA wrote the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The authors would like to extend their sincere appreciation to the Researchers Supporting Project Number (RSP-2021/168), King Saud University, Riyadh, Saudi Arabia.

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