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ARTICLE

Appraisal of Improvement in Physiological and Metabolic Processes by Exogenously Applied Natural and Synthetic Ascorbic Acid in Okra (*Abelmoschus esculentus* L.) Fruit Subjected to Water Deficit Stress

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ABSTRACT

To counteract the effects of drought stress, scientists have adopted several approaches including the use of different chemicals both inorganic and organic, which is contemplated as a highly efficient and cost-effective shot-gun approach. Ascorbic acid (AsA) is a potential organic substance, which widely occurs in plants, and is considered to be an effective antioxidant to counteract reactive oxygen species (ROS). Thus, a pot experiment was performed to assess the relative mitigating impacts of synthetic AsA and naturally occurring AsA in the form of lemon juice (LJ) and orange juice (OJ) on two cultivars of okra (*Abelmoschus esculentus* L.) namely Sabz Pari and Bhindi Sanwali under varying water deficit conditions. After 30 days of seed germination, okra seedlings were subjected to different irrigation regimes, i.e., water deficit stress [(65% and 50% F.C.) and control conditions (100% F.C.)]. Different levels of AsA [control (no spray), 14 mg L^{-1} LJ, 24 mg L^{-1} OJ and 150 mg L^{-1} AsA] obtained from different sources were applied as a foliar spray to control and water-stressed plants. Drought stress prominently reduced plant growth and yield attributes of the okra cultivars. Water-deficit conditions (65% and 50% F.C.) substantially decreased the fruit chlorophyll (*a, b*) pigments and the activity of superoxide dismutase (SOD) enzyme, while an increase was observed in the contents of fruit's hydrogen peroxide (H_2O_2), malondialdehyde (MDA), total phenolics, total soluble sugars, AsA, and total soluble proteins. Drought stress also increased the activities of antioxidant enzymes like peroxidase (POD) and catalase (CAT). However, plant growth and yield attributes, fruit chlorophyll pigments, total phenolics, total soluble sugars, total free amino acids, total soluble proteins, AsA, GB, H_2O_2, and the activities of antioxidant enzymes (POD and CAT) were increased by the AsA exogenous treatment in both okra cultivars under water deficit and control conditions. Overall, LJ and OJ were more effective than the synthetic AsA in upregulating the physiological and metabolic processes of okra plants. So, cost-effective as well as multi-nutrient natural sources of AsA could be suggested for alleviating the harmful effects of water deficit stress on plants.

KEYWORDS

Okra; ascorbic acid; lemon juice; orange juice; drought tolerance

1 Introduction

Plants naturally face several environmental stresses and such constraints cause a reduction in plant growth and development, which ultimately results in yield reduction [1,2]. Water deficiency is considered as the most dreadful environmental factor, that causes a reduction in crop yield more effectively as compared to that by other environmental constraints [3,4]. According to Manivannan et al. [5], agricultural drought is the lack of sufficient quantity of irrigation water required for most crops to complete their life span. It is widely reported that the morphological and physio-biochemical characteristics of plants are adversely affected by water shortage during different growth stages [6,7]. Common visible morphological effects of drought observed in plants include a reduction in the number of leaves, plant height, decreased seed number, width and diameter, and fruit and seed weight [8,9]. Furthermore, plants upregulate drought tolerance mechanism against abiotic stress by adopting different physio-biochemical strategies including more uptake of water by a deeper root system, increased stomatal conductance, enhanced accumulation of osmoprotectants, stability of plasma membrane, scavenging of ROS and production of stress-related proteins [10,11].

Vitamin C or AsA acts as an antioxidant, and it plays an important role in the detoxification of ROS [12–15]. Previous studies have reported that AsA scavenge the free oxygen species and protect plants from oxidative damage [15,16]. The use of plant-growth biostimulants has increased in the last few years since it can improve crop quality without causing any damage to the environment [17]. It is believed that the direct use of plant extracts enriched with different biostimulants/bioregulators is more effective and environmentally safe as compared to synthetic chemicals. These biostimulants can reduce environmental pollution by eliminating or reducing the need for mineral fertilizers [18–20]. Moreover, AsA plays a role as an important signaling modulator of different physiological processes occurring in plant's cell [21,22]. AsA causes detoxification of hydrogen peroxide under drought-stress conditions [23,24]. Ascorbic acid acts as an enzyme co-factor and regulates the signaling mechanism mediated by phytohormones as well as the physiological processes of plants [15,25]. The stress tolerance mechanism of the plant can be enhanced by increasing the level of AsA, so the harmful effects of drought can be eliminated [26–28].

Moreover, the exogenous application of AsA improves plant growth and development efficiently by maintaining different processes like phytohormones' signaling, ion transport mechanism, cell expansion, and oxidative defense mechanism under both stress and non-stress environments [15,22,29–32]. In addition, exogenously applied AsA eliminates the negative role of water deficit stress on plants [33,34]. Previous research performed by different investigators on different crops, i.e., wheat [35], corn [36], soybean [37], rapeseed [38], grapes [39], canola [16] and cauliflower [7] showed that exogenously applied AsA had beneficial effect on morphological and physio-biochemical processes under drought stress, while the effects of AsA on the biochemistry as well as the quality of fruits and vegetables have been rarely investigated.

Okra (*Abelmoschus esculentus* L.) is one of the potential heat-tolerant vegetable crops and is widely grown for tender fruits and young leaves during hot seasons in worldwide tropical areas [40–44]. Calisir et al. [45] proposed that okra crop can grow well in both tropical and temperate zones and is very easy to cultivate. The fruit of okra is rich in nutritional contents like dietary fiber, amino acids, riboflavin, folic acid, thiamin, vitamins, and minerals [42,46]. Many researchers prefer to adopt an easy and cost-effective approach to mitigate the harmful effects of stressful environmental conditions including water deficiency. Many studies in the literature revealed that some potential non-enzymatic antioxidants applied externally proved better for promoting plant growth, and AsA is one of them [15,16]. The presence of AsA was mainly noticed in plant cytosol, apoplast and in actively growing parts [15,47,48]. The present study was conducted based on a potential hypothesis that natural AsA taken from the juices of fresh lemon and orange is one of the effective strategies to enhance growth and key physio-biochemical processes of okra plants and fruits as compared to that by the synthetic AsA under water deficit environment. Thus, the

experiment was conducted to evaluate the influence of externally applied natural (fresh lemon and orange juices) vitamin C (AsA) in comparison with synthetic AsA (purchased from the market) on plant growth, yield production and physio-biochemical attributes of okra fruits under drought-stress and control (non-stress) conditions. Moreover, the best source of AsA can be suggested based on improvement in growth and yield production for the crops subjected to water-deficit stress during their life cycle.

2 Materials and Methods

Influence of foliar applied synthetic AsA and natural sources of AsA such as phytoextracts of two plants [lemon (*Citrus limon* L.) and sweet orange (*Citrus sinensis* L.)] on okra (*Abelmoschus esculentus* L.) fruits were examined under water deficit conditions. For this purpose, an experiment was conducted in the village Mahar Sharif near Chishtian, Punjab Pakistan in 2019. During experimentation, the average day and night temperature 34.4°C and 19.8°C, relative humidity 58%, rainfall 22.4 mm, and sunshine duration 11.4 h, were recorded. The seeds of Cv. Sabz Pari were collected from the Vegetable Section of Ayub Agricultural Research Institute (AARI), Faisalabad, Pakistan, while those of Cv. Bhindi Sanwali were purchased from the local market of Faisalabad, Pakistan. Each pot was filled with 7.0 kg soil having the following composition: 64% sand, 28.4% silt and 7.6% clay, pH 7.9, saturation percentage 29% and ECe 1.87 dS m^{-1}. There were a total of 96 experimental units (pots) including cultivars, drought stress levels, AsA levels and four replicates of each treatment ($2 \times 3 \times 4 \times 4 = 96$). Each pot was considered as a replicate. In each pot (replicate), six seeds were planted, and thinning was done after 15 days of seed germination to retain four plantlets in every pot or replicate. After thirty days, the seedlings were subjected to 100% (control), 65% and 50% field capacities (drought stress levels). Based on the soil saturation percentage and field capacity, these drought stress levels were maintained; the drought stress levels were attained after a period of 15 days, and maintained for the next thirty days. Drought stress levels were checked daily and added water as per set value. After thirty days of maintenance of drought stress levels, 150 mg L^{-1} AsA, 14 mg L^{-1} LJ and 24 mg L^{-1} OJ besides the control (no spray) were used as foliage spray. For the LJ and OJ extraction, fresh lemons and sweet oranges were collected from the market; the peel was removed from both fruits. The juice was stored after extraction from the pulp by using an electric juicer. Before use, the content of AsA in 50% LJ (14 mg L^{-1}) and 50% OJ (24 mg L^{-1}) were determined [49]. Different solutions were prepared, and 0.1% Tween-20 was also added to each solution. After 30 days of foliar-applied treatments, a plant of uniform size from each pot was collected and air-dried after washing with distilled water, and their dry weights determined. Moreover, the following physio-biochemical traits were recorded after fruit collection.

2.1 Photosynthetic Pigments

A fresh fruit sample (0.25 g) was ground in 5 mL of 80% acetone solution; it was homogenized and kept overnight at 4°C following the procedure of Arnon [50]. The absorbance was read at 663 and 645 nm and all the concentrations of all pigments were assessed.

2.2 Free Proline Contents

According to Bates et al. [51], a fresh fruit sample (0.25 g) was ground in 5 mL of 3% sulfosalicylic acid. One mL of the extracted material was filtered and mixed with 1.0 mL of acidic ninhydrin and 1.0 mL of glacial acetic acid. The mixture was heated for 1.0 h at 100°C in a water bath. Then 2 mL of toluene were added to the cooled mixture. The absorbance was noted at 520 nm with the help of a spectrophotometer.

2.3 Ascorbic Acid Contents

Following Mukherjee et al. [49], the fresh fruit sample (0.25 g) was extracted in 5 mL solution of 6% trichloroacetic acid (TCA). Two mL of the extracted solution were filtered and mixed with 1.0 mL of 2, 4 dinitrophenylhydrazine (2%). Then, one drop of 10% thiourea was added and the solution was heated

at 100°C for 15 min on a water bath. Each sample mixture was cooled at room temperature, and mixed with 2.5 mL of 80% H_2SO_4, and the optical density of all samples were recorded at 530 nm using a spectrophotometer.

2.4 Hydrogen Peroxide Contents

Hydrogen peroxide contents were estimated following the procedure of Velikova et al. [52]. Fresh fruit sample (0.25 g) was triturated in 5 mL of 0.1% ice-cold TCA. Then, an aliquot (500 uL) of the supernatant was separated by centrifugation. Then, 1.0 mL of 1 M potassium iodide (KI) and 500 uL of 10 mM potassium phosphate buffer pH (7.0) were added and the optical density of each sample was noted at 390 nm.

2.5 Malondialdehyde (MDA) Contents

Heath et al. [53] protocol was followed to determine the MDA contents. Fresh fruit (0.25 g) was homogenized in 5 mL of TCA (5%). The supernatant (500 µL) was separated and 2 mL of 0.5% thiobarbituric acid were added to it. The solution was heated in a water bath for 50 min at 95°C and the absorbance of the cooled solution was noted at 600 and 532 nm.

2.6 Total Phenolics

A fruit sample (0.25 g) was extracted in 5 mL of 80% acetone. The supernatant (100 uL) was separated in a test tube and to it distilled water (2 mL) was added. Then 1 mL of the Folin-Ciocalteu phenol reagent was added to the mixture, and all test tubes were agitated efficiently. Then, 5 mL of 20% sodium carbonate (Na_2CO_3) solution were added to each of the test tubes followed by the addition of distilled water to make the final volume up to 10 mL. The absorbance of each treated sample was read at 750 nm following the protocol of Julkenen-Titto [54].

2.7 Glycinebetaine Contents

The Grieve et al. [55] protocol was followed to determine the GB content. A fruit sample (0.25 g) was extracted in 5 mL distilled water. Then 1.0 mL of the filtered sample was mixed with 1.0 mL of *2N* H_2SO_4 and 200 µL of potassium tri-iodide (KI_3). In the cooled mixtures, 6 mL of 1, 2 dichloroethane and 2.8 mL of distilled water were added. Resultantly, two layers appeared in the reaction mixture. The upper layer was discarded, and the lower layer was used to record absorbance at 365 nm.

2.8 Activities of Antioxidant Enzymes

For measuring antioxidant enzymes activities, 0.25 g fresh fruit sample was crushed and homogenized in cooled potassium phosphate buffer (50 mM; pH, 7.8) using a chilled pestle and mortar. Then, the extracted sample was centrifuged at 12,000 × *g* at 4°C for 20 min. The supernatant was separated and stored at −20°C to measure the following enzymatic activities and total soluble proteins.

2.9 SOD

The Van Rossum et al. [56] procedure was used, and the extracted fruit sample (50 µL) was added in a plastic cuvette. The reaction mixture was made by taking 100 µL L-methionine, 100 µL triton-X, 250 µL (50 mM) potassium phosphate buffer (pH 7.8), 50 µL nitroblue tetrazolium (NBT), 50 µL riboflavin and 400 µL distilled water. Then, the quartz cuvettes containing reaction mixture were kept under light for 5 min before recording the absorbance at 560 nm.

2.10 POD

Fresh fruit extract (100 µL) was taken in a quartz cuvette and 1.8 mL of 50 mM potassium phosphate buffer (7.8 pH) was added to it. In addition, 100 µL (40 mM) H_2O_2 and 100 µL (20 mM) guaiacol were also added. The absorbance was noted at 470 nm for 180 s following the protocol documented by Chance et al. [57].

2.11 CAT

The activity of CAT enzyme was estimated following the procedure outlined by Chance et al. [57]. For this, the reaction mixture was prepared in a quartz cuvette by adding 100 uL of the fruit extract, 1.0 mL of 5.9 mM H_2O_2 and 1.9 mL of potassium phosphate buffer (50 mM, pH 7.8). The optical density was noted after every 20 s for 2 min at 240 nm.

2.12 Total Soluble Proteins

Total soluble proteins were estimated by mixing 100 µL fruit extract with an aliquot (2 mL) of the Bradford reagent in a test tube following the method of Bradford [58]. After keeping the mixture at room temperature for 20 min the optical density was recorded at 595 nm for the determination of total soluble proteins.

2.13 Total Free Amino Acids

Total free amino acids were estimated following Hamilton et al. [59]. The fruit sample (1.0 mL) was taken in a test tube. The same volume of 10% pyridine and acidic ninhydrin were also added to the test tubes. The reaction mixture was boiled for 30 min at 100°C. Then, the mixture was cooled and distilled water added to make the final volume to 7.5 mL. Then, the absorbance was noted at 570 nm using a spectrophotometer.

2.14 Total Soluble Sugars

The Yemm et al. [60] protocol was followed and 0.25 g of the fresh fruit sample was extracted in 5 mL (80%) ethanol. A sample extract (100 µL) was taken in a test tube and 3.0 mL of the anthrone reagent (prepared in 72% H_2SO_4) were added to it. The mixture was boiled for 10 min at 100°C. The reaction mixture was maintained at room temperature for 20 min and the absorbance of all treated samples was noted at 625 nm.

2.15 Statistical Analysis

A completely randomized experiment with a three-factor factorial arrangement (cultivars, drought stress and different AsA levels) was performed in pots with four replicates. The data of all the parameters were subjected to a three-way analysis of variance (ANOVA) (Table 1) and a one-way ANOVA (Tables 2a–6b). The significant differences among the mean values and the significant effect of AsA on each okra cultivar were determined by the least significant difference test at the probability level of 0.05% and different letters were presented on each bar of all figures.

Table 1: Three-way ANOVA (mean squares values) for growth and different metabolites of okra (*A. esculentus* L.) fruits subjected to natural and synthetic AsA under different water deficit conditions

Source of variations	df	Shoot dry weight	Root dry weight	Fruit fresh weight	Fruit dry weight
Cultivars (Cvs)	1	12.08***	0.70***	53.4***	3.735***
Drought (D)	2	313.2***	16.94***	553.9***	42.59***
AsA	3	5.467***	0.17*	21.15***	0.948***
Cvs × D	2	5.63***	0.125ns	22.42***	0.431*
Cvs × AsA	3	2.392**	0.023ns	4.256*	0.464**
D × AsA	6	3.374***	0.043ns	3.213*	0.129ns

(Continued)

Table 1 (continued)

Source of variations	df	Shoot dry weight	Root dry weight	Fruit fresh weight	Fruit dry weight
Cvs × D × AsA	6	1.057ns	0.023ns	2.975ns	0.144ns
Error	72	0.552	0.042	1.397	0.090
		No. of fruits	**Chlorophyll *a***	**Chlorophyll *b***	**Total chlorophyll**
Cultivars (Cvs)	1	2.041*	0.154ns	0.208ns	0.722*
Drought (D)	2	25.96***	1.102***	1.291***	4.761***
AsA	3	0.25ns	0.392*	0.142ns	0.88***
Cvs × D	2	0.197ns	0.257*	0.058ns	0.54*
Cvs × AsA	3	0.069ns	0.074ns	0.023ns	0.136ns
D × AsA	6	0,218ns	0.08ns	0.019ns	0.176ns
Cvs × D × AsA	6	0.225ns	0.093ns	0.055ns	0.263ns
Error	72	0.388	0.081	0.055	0.134
		Chl. *a/b* ratio	**Proline**	**Glycinebetaine**	**Hydrogenperoxide**
Cultivars (Cvs)	1	0.796ns	0.508***	1.279***	1.279***
Drought (D)	2	3.254 ***	0.889***	1.405***	1.405***
AsA	3	0.134ns	0.179***	0.100ns	1.006ns
Cvs × D	2	0.067ns	0.006ns	0.758***	7.584***
Cvs × AsA	3	0.082ns	0.024***	0.249ns	2.492ns
D × AsA	6	0.081ns	0.013***	0.331ns	3.310ns
Cvs × D × AsA	6	0.165ns	0.029***	0.147ns	1.472ns
Error	72	0.259	0.003	0.097	9.783
		Malondialdehyde	**Total phenolics**	**Ascorbic acid**	**Total soluble sugars**
Cultivars (Cvs)	1	0.006*	10.90***	0.079***	0.665***
Drought (D)	2	0.051***	21.81***	3.605***	0.834***
AsA	3	0.003*	2.014***	0.922***	1.598***
Cvs × D	2	0.007***	1.098***	0.018ns	1.587***
Cvs × AsA	3	0.002ns	0.469**	0.030ns	0.271***
D × AsA	6	6.884ns	0.062ns	0.041ns	0.276***
Cvs × D × AsA	6	0.001ns	0.279**	0.065ns	0.201***
Error	72	9.625	0.082	0.075	0.034
		Total aminoacids	**Total soluble proteins**	**Catalase**	**Peroxidase**
Cultivars (Cvs)	1	1.132***	0.064**	6.343ns	350.8***
Drought (D)	2	0.057ns	3.894***	13.24*	129. 3***
AsA	3	0.260***	0.132***	8.957*	97.50***

(Continued)

Table 1 (continued)

Source of variations	df	Shoot dry weight	Root dry weight	Fruit fresh weight	Fruit dry weight
Cvs × D	2	0.139**	0.041**	19.11**	1.828ns
Cvs × AsA	3	0.010ns	0.043***	1.774ns	2.413ns
D × AsA	6	0.023ns	0.007ns	0.953ns	18.46ns
Cvs × D × AsA	6	0.017ns	0.019*	0.197ns	7.984ns
Error	72	0.026	0.006	2.793	9.327
		Superoxide dismutase			
Cultivars (Cvs)	1	2.865**			
Drought (D)	2	76.48***			
AsA	3	0.710ns			
Cvs × D	2	2.055**			
Cvs × AsA	3	1.010ns			
D × AsA	6	0.187ns			
Cvs × D × AsA	6	0.178ns			
Error	72	0.388			

Note: ns = no-significant; *, ** and *** = significant at 0.05, 0.01 and 0.001 levels, respectively.

3 Results

Shoot and root dry weights declined constantly as water deficit levels increased from 65% to 50% F.C. (Table 1). However, foliar-applied 150 mg L^{-1} ascorbic acid and the phytoextracts (14 and 24 mg L^{-1}) showed significant promoting effects on the shoot and root dry weights of both okra cultivars. For both cultivars, Sabz Pari and Bhindi Sanwali, 14 mg L^{-1} natural AsA was the most effective in enhancing the shoot and root dry weights, specifically at 50% F.C. (Tables 2a–3b). However, at 100% F.C. (control), root dry weight was promoted more by 24 mg L^{-1} OJ application in okra Cv. Sabz Pari (Table 1; Fig. 1). While both okra cultivars showed consistent responses to water stress and exogenous application of AsA in terms of these growth attributes which significantly deacreased in both okra cultivars at 65% and 50% F.C. (Tables 3a and 3b).

Different water deficit levels considerably caused a reduction in fruit fresh and dry weights as well as the number of fruits per plant (Fig. 1). While fruit fresh and dry weights were recorded to be improved ($p \leq 0.001$) by foliar applied AsA, LJ and OJ under all water deficit regimes. However, the number of fruits per plant showed no significant response to exogenously applied AsA (Table 1). The maximum fruit fresh and dry weights were observed under control conditions by foliar applied LJ in Cv. Sabz Pari and by 150 mg L^{-1} AsA in Cv. Bhindi Sanwali (Tables 4a–5c).

Water stress regimes (65% and 50% F.C.) adversely ($p \leq 0.001$) affected chlorophyll *a, b* and total chlorophyll contents in both okra cultivars (Tables 1–3b). Moreover, the ratio of chlorophyll *a/b* was increased under water stress, particularly at 65% F.C. in both okra cultivars (Fig. 2). Exogenously applied varying sources of AsA significantly improved ($p \leq 0.001$) chlorophyll *a, b* and total chlorophyll contents, while the ratio of chlorophyll *a/b* showed no significant change under both stress and control conditions. No significant difference was observed among all AsA sources under varying water regimes. Similarly, the difference between both okra cultivars was non-significant under water stress and AsA treatments.

Table 2a: ANOVA (mean square values) for Cv. Sabz Pari subjected to 65% field capacity

Source of variations	df	Shoot dry weight	Fruit fresh weight	Fruit dry weight	Chlorophyll *a*
Drought (D)	1	16.24*	50.0***	3.38**	0.162ns
Error	6	1.868	0.666	0.105	0.047
		Total chlorophyll	**Glycinebetaine**	**Hydrogen peroxide**	**Malondialdehyde**
Drought (D)	1	0.834*	2.12*	0.664ns	140.4*
Error	6	0.105	0.183	0.172	14.64
		Total phenolics	**Total soluble sugars**	**Total aminoacids**	**Total soluble proteins**
Drought (D)	1	1.423*	0.045ns	0.01ns	0.174***
Error	6	0.229	0.016	0.046	0.001
		Catalase	Superoxide dismutase		
Drought (D)	1	22.12***	5.88ns		
Error	6	0.435	1.306		

Table 2b: ANOVA (mean square values) for Cv. Sabz Pari subjected to 50% field capacity

Source of variations	df	Shoot dry weight	Fruit fresh weight	Fruit dry weight	Chlorophyll a
Drought (D)	1	28.88**	61.05***	8.79***	0.001ns
Error	6	1.466	0.571	0.018	0.027
		Total chlorophyll	**Glycinebetaine**	**Hydrogen peroxide**	**Malondialdehyde**
Drought (D)	1	0.167ns	2.06**	0.05ns	119.8*
Error	6	0.039	0.117	0.19	17.49
		Total phenolics	**Total soluble sugars**	**Total aminoacids**	**Total soluble proteins**
Drought (D)	1	5.36***	0.014ns	0.039ns	0.796***
Error	6	0.102	0.040	0.039	0.001
		Catalase	**Superoxide dismutase**		
Drought (D)	1	3.303*	23.44**		
Error	6	0.387	1.424		

Table 3a: ANOVA (mean square values) for Cv. Bhindi Sanwali subjected to 65% field capacity

Source of variations	df	Shoot dry weight	Fruit fresh weight	Fruit dry weight	Chlorophyll a
Drought (D)	1	32.8***	45.12*	2.205ns	0.007ns
Error	6	0.375	4.958	0.438	0.026
		Total chlorophyll	**Glycinebetaine**	**Hydrogen peroxide**	**Malondialdehyde**
Drought (D)	1	0.13ns	0.024ns	1.474*	33.87ns
Error	6	0.063	0.092	0.158	9.236
		Total phenolics	**Total soluble sugars**	**Total aminoacids**	**Total soluble proteins**
Drought (D)	1	1.106***	0.163ns	0.0008ns	0.262***
Error	6	0.024	0.047	0.0008	0.001
		Catalase	**Superoxide dismutase**		
Drought (D)	1	0.872ns	2.83ns		
Error	6	1.220	1.392		

Table 3b: ANOVA (mean square values) for Cv. Bhindi Sanwali subjected to 50% field capacity

Source of variations	df	Shoot dry weight	Fruit fresh weight	Fruit dry weight	Chlorophyll a
Drought (D)	1	71.4***	132.0**	7.125**	0.023ns
Error	6	0.182	4.322	0.431	0.0435
		Total chlorophyll	**Glycinebetaine**	**Hydrogen peroxide**	**Malondialdehyde**
Drought (D)	1	0.163ns	0.001ns	5.108***	171.0**
Error	6	0.065	0.015	0.099	12.01
		Total phenolics	**Total soluble sugars**	**Total aminoacids**	**Total soluble proteins**
Drought (D)	1	4.72***	0.221ns	0.013ns	0.94***
Error	6	0.028	0.041	0.002	0.002
		Catalase	**Superoxide dismutase**		
Drought (D)	1	0.088ns	8.7*		
Error	6	1.358	0.976		

Note: ns = no-significant; *, ** and *** = significant at 0.05, 0.01 and 0.001 levels, respectively.

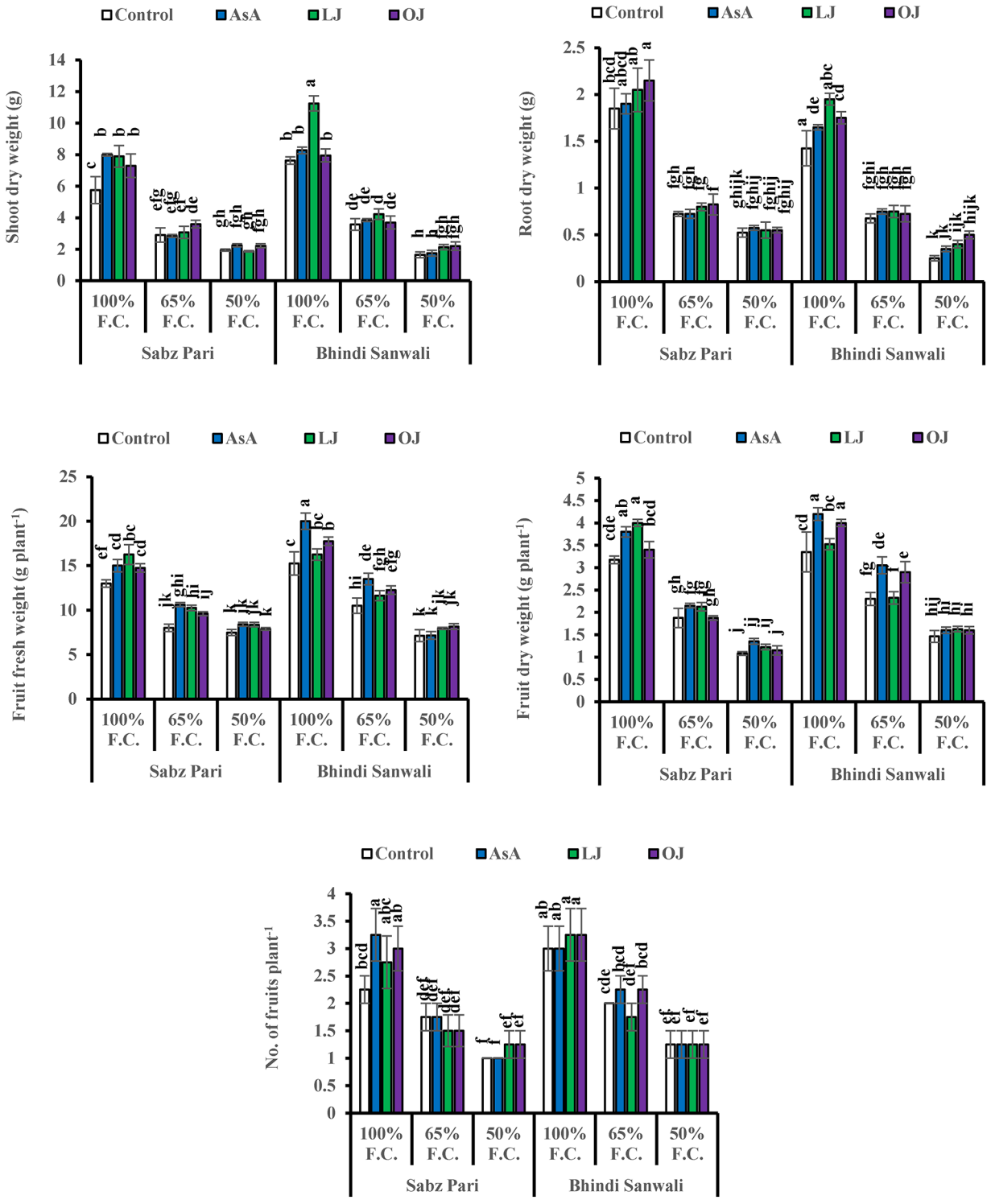

Figure 1: Shoot and root dry weights, fruit fresh and dry weights and number of fruits per plant of two cultivars of okra (*Abelmoschus esculentus* L.) subjected to varying water-deficit conditions and foliarly treated with varying sources of AsA (Mean ± S.E.); AsA, Ascorbic acid; LJ, Lemon juice; OJ, Orange juice. Different letters (a–k) showing significance of difference among different treatments

Table 4a: ANOVA for Cv. Sabz Pari subjected to 150 mg L^{-1} synthetic ascorbic acid (AsA)

Source of variations	df	Shoot dry weight	Fruit fresh weight	Fruit dry weight	Proline
AsA	1	10.12*	8.0*	0.7812**	0.002ns
Error	6	1.471	1.333	0.041	4.584
		Total phenolics	**Total soluble sugars**	**Total soluble proteins**	
AsA	1	1.828*	5.539ns	3.061ns	
Error	6	0.181	0.024	0.002	

Table 4b: ANOVA for Cv. Sabz Pari subjected to 14 mg L^{-1} lemon juice

Source of variations	df	Shoot dry weight	Fruit fresh weight	Fruit dry weight	Proline
Lemon juice (LJ)	1	9.245ns	21.12*	1.361***	0.021**
Error	6	2.398	2.791	0.027	7.051
		Total phenolics	**Total soluble sugars**	**Total soluble proteins**	
Lemon juice (LJ)	1	0.861*	0.064ns	0.001ns	
Error	6	0.134	0.017	0.003	

Table 4c: ANOVA for Cv. Sabz Pari subjected to 24 mg L^{-1} orange juice

Source of variations	df	Shoot dry weight	Fruit fresh weight	Fruit dry weight	Proline
Orange juice (OJ)	1	4.805ns	6.125*	0.101ns	0.158***
Error	6	2.588	0.791	0.081	7.816
		Total phenolics	**Total soluble sugars**	**Total soluble proteins**	
Orange juice (OJ)	1	1.531*	0.284**	0.012*	
Error	6	0.122	0.016	0.001	

Table 5a: ANOVA for Cv. Bhindi Sanwali subjected to 150 mg L^{-1} synthetic ascorbic acid (AsA)

Source of variations	df	Shoot dry weight	Fruit fresh weight	Fruit dry weight	Proline
AsA	1	0.845ns	45.12*	1.445ns	0.016**
Error	6	0.205	5.125	0.435	9.322
		Total phenolics	**Total soluble sugars**	**Total soluble proteins**	
AsA	1	0.001ns	0.092ns	0.030**	
Error	6	0.020	0.056	0.002	

Table 5b: ANOVA for Cv. Bhindi Sanwali subjected to 14 mg L^{-1} lemon juice

Source of variations	df	Shoot dry weight	Fruit fresh weight	Fruit dry weight	Proline
Lemon juice (LJ)	1	26.28***	2.0ns	0.061ns	0.047***
Error	6	0.572	4.25	0.426	5.281
		Total phenolics	**Total soluble sugars**	**Total soluble proteins**	
Lemon juice (LJ)	1	0.006ns	0.047ns	0.099***	
Error	6	0.021	0.039	0.001	

Table 5c: ANOVA for Cv. Bhindi Sanwali subjected to 24 mg L^{-1} orange juice

Source of variations	df	Shoot dry weight	Fruit fresh weight	Fruit dry weight	Proline
Orange juice (OJ)	1	0.211ns	12.5ns	0.845ns	0.036***
Error	6	0.452	3.916	0.408	4.880
		Total phenolics	**Total soluble sugars**	**Total soluble proteins**	
Orange juice (OJ)	1	0.203*	0.142ns	0.146***	
Error	6	0.015	0.048	0.001	

Note: ns = no-significant; *, ** and *** = significant at 0.05, 0.01 and 0.001 levels, respectively.

Fruit proline contents remained unaffected, while water deficit conditions significantly increased the GB contents in both okra cultivars. Exogenously applied varying treatments of AsA, LJ and OJ markedly ($p \leq 0.001$) increased proline concentrations of okra fruits, while GB content remained unchanged at different water stress levels (Table 1). Furthermore, a considerable difference was noticed in both okra cultivars for fruit proline and GB contents. The Cv. Sabz Pari was better than Cv. Bhindi Sanwali in these parameters under varying drought stress levels (Table 6a).

Accumulation of H_2O_2 augmented substantially ($p \leq 0.001$) in fruits of both okra cultivars under varying water-deficit levels. Moreover, a significant decrease in H_2O_2 contents was observed in both okra cultivars by different AsA sources at 50% field capacity (Table 1). However, of both okra cultivars, Cv. Bhindi Sanwali was higher in H_2O_2 accumulation than Cv. Sabz Pari at 50% F.C. (Fig. 3).

Fruit MDA content increased substantially under drought stress in both cultivars of okra (Table 1). While different sources of AsA significantly ($p \leq 0.05$) suppressed the MDA contents in the water-stressed plants of both okra cultivars. The MDA contents decreased more prominently in Cv. Sabz Pari than that inokra Cv. Bhindi Sanwali under varying water stress regimes (Fig. 3).

The contents of ascorbic acid and total phenolics considerably ($p \leq 0.001$) increased in fruits of both okra cultivars due to drought stress imposition. However, exogenously applied AsA, LJ and OJ considerably improved the concentrations of ascorbic acid and total phenolics in okra cultivars under both water deficit stress and non-stress conditions (Fig. 3). Of both okra cultivars, Cv. Sabz Pari showed more increase in fruit total phenolics and AsA contents at varying water deficit regimes.

Total soluble sugars increased prominently ($p \leq 0.01$) under water stress as well as on application of varying sources of AsA (Fig. 3; Table 6b). Of all sources, 14 mg L^{-1} LJ and 24 mg L^{-1} OJ notably raised the total soluble sugars, particularly at 50% F.C. Of both okra cultivars, Cv. Sabz Pari accumulated a maximum increase in the levels of total soluble sugars under water-limited environment particularly at 50% F.C.

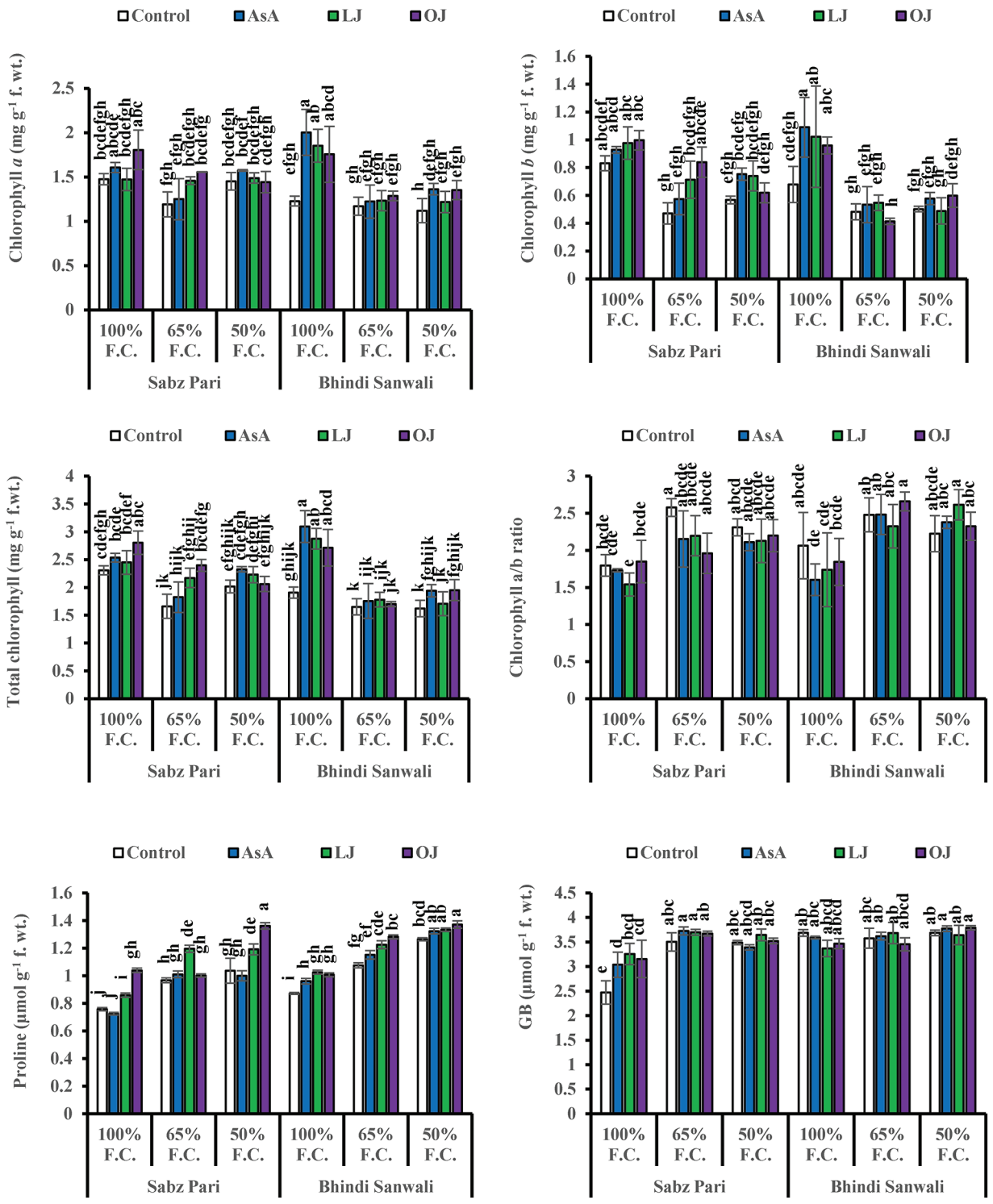

Figure 2: Chlorophyll *a, b*, total chlorophyll and chlorophyll *a/b* ratio, proline and glycinebetaine (GB) contents of fruits of two cultivars of okra (*Abelmoschus esculentus* L.) subjected to varying water-deficit conditions and foliarly treated with varying sources of AsA (Mean ± S.E.). Different letters (a–k) showing significance of difference among different treatments

Table 6a: Drought x AsA interaction of Cv. Sabz Pari

Cv. Sabz Pari subjected to synthetic AsA (150 mg L^{-1}) at 100% F.C.					
Source of variations	**df**	**Shoot dry weight**	**Fruit fresh weight**	**Proline**	**Total soluble sugars**
AsA	1	10.12*	8.0*	0.002ns	5.539ns
Error	6	1.471	1.333	4.584	0.024
Cv. Sabz Pari subjected to 14 mg L^{-1} lemon juice at 100% F.C.					
		Shoot dry weight	**Fruit fresh weight**	**Proline**	**Total soluble sugars**
Lemon juice (LJ)	1	9.245ns	21.12*	0.021**	0.064ns
Error	6	2.398	2.791	7.051	0.017
Cv. Sabz Pari subjected to 24 mg L^{-1} orange juice at 100% F.C.					
		Shoot dry weight	**Fruit fresh weight**	**Proline**	**Total soluble sugars**
Orange juice (OJ)	1	4.805ns	6.125*	0.158***	0.284**
Error	6	2.588	0.791	7.816	0.016
Cv. Sabz Pari subjected to synthetic AsA (150 mg L^{-1}) at 65% F.C.					
		Shoot dry weight	**Fruit fresh weight**	**Proline**	**Total soluble sugars**
AsA	1	0.005ns	13.78**	0.003ns	0.188**
Error	6	0.428	0.447	0.001	0.013
Cv. Sabz Pari subjected to 14 mg L^{-1} lemon juice at 65% F.C.					
		Shoot dry weight	**Fruit fresh weight**	**Proline**	**Total soluble sugars**
Lemon juice (LJ)	1	0.061ns	10.12**	0.103***	0.012ns
Error	6	0.714	0.541	0.001	0.006
Cv. Sabz Pari subjected to 24 mg L^{-1} orange juice at 65% F.C.					
		Shoot dry weight	**Fruit fresh weight**	**Proline**	**Total soluble sugars**
Orange juice (OJ)	1	0.98ns	5.12*	0.002ns	0.436ns
Error	6	0.52	0.426	0.0008	0.109
Cv. Sabz Pari subjected to synthetic AsA (150 mg L^{-1}) at 50% F.C.					
		Shoot dry weight	**Fruit fresh weight**	**Proline**	**Total soluble sugars**
AsA	1	0.151ns	0.32ns	0.210*	5.481***
Error	6	0.042	0.282	0.017	0.029
Cv. Sabz Pari subjected to 14 mg L^{-1} lemon juice at 50% F.C.					
		Shoot dry weight	**Fruit fresh weight**	**Proline**	**Total soluble sugars**
Lemon juice (LJ)	1	0.18*	1.711ns	0.002ns	0.521*
Error	6	0.026	0.331	0.018	0.040
Cv. Sabz Pari subjected to 24 mg L^{-1} orange juice at 50% F.C.					
		Shoot dry weight	**Fruit fresh weight**	**Proline**	**Total soluble sugars**
Orange juice (OJ)	1	0.011ns	1.62ns	0.047ns	1.988**
Error	6	0.012	0.352	0.019	0.088

Note: ns = no-significant; *, ** and *** = significant at 0.05, 0.01 and 0.001 levels, respectively.

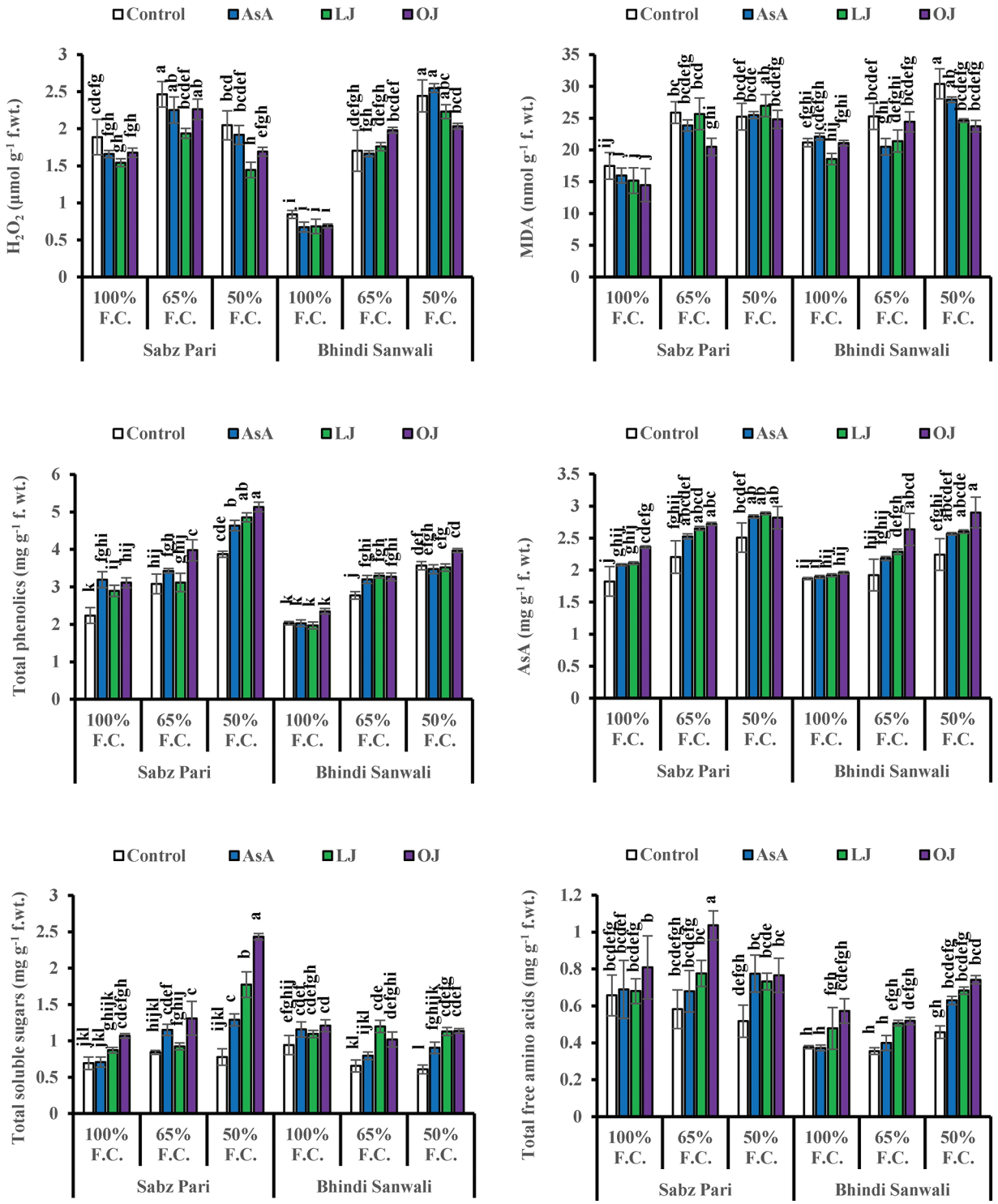

Figure 3: Hydrogen peroxide (H_2O_2), malondialdehyde (MDA), total phenolics, ascorbic acid (AsA), total soluble sugars and total free amino acids of fruits of two cultivars of okra (*Abelmoschus esculentus* L.) subjected to varying water-deficit conditions and foliarly treated with varying sources of AsA (Mean ± S.E.). Different letters (a–k) showing significance of difference among different treatments

Table 6b: Drought x AsA interaction of Cv. Bhindi Sanwali

Cv. Bhindi Sanwali subjected to synthetic AsA (150 mg L^{-1}) at 100% F.C.					
Source of variations	**df**	**Shoot dry weight**	**Fruit fresh weight**	**Proline**	**Total soluble sugars**
AsA	1	0.845ns	45.12*	0.016**	0.092ns
Error	6	0.205	5.125	9.322	0.056
Cv. Bhindi Sanwali subjected to 14 mg L^{-1} lemon juice at 100% F.C.					
		Shoot dry weight	**Fruit fresh weight**	**Proline**	**Total soluble sugars**
Lemon juice (LJ)	1	26.28***	2ns	0.047***	0.047***
Error	6	0.572	4.25	5.281	0.039
Cv. Bhindi Sanwali subjected to 24 mg L^{-1} orange juice at 100% F.C.					
		Shoot dry weight	**Fruit fresh weight**	**Proline**	**Total soluble sugars**
Orange juice (OJ)	1	0.211ns	12.5ns	0.036***	0.142ns
Error	6	0.452	3.916	4.880	0.048
Cv. Bhindi Sanwali subjected to synthetic AsA (150 mg L^{-1}) at 65% F.C.					
		Shoot dry weight	**Fruit fresh weight**	**Proline**	**Total soluble sugars**
AsA	1	0.151ns	18.0*	0.011ns	0.038ns
Error	6	0.279	2.333	0.002	0.019
Cv. Bhindi Sanwali subjected to 14 mg L^{-1} lemon juice at 65% F.C.					
		Shoot dry weight	**Fruit fresh weight**	**Proline**	**Total soluble sugars**
Lemon juice (LJ)	1	0.845ns	2.531ns	0.044**	0.586**
Error	6	0.482	2.114	0.002	0.027
Cv. Bhindi Sanwali subjected to 24 mg L^{-1} orange juice at 65% F.C.					
		Shoot dry weight	**Fruit fresh weight**	**Proline**	**Total soluble sugars**
Orange juice (OJ)	1	0.031ns	6.125ns	0.085***	0.264*
Error	6	0.601	1.958	0.001	0.034
Cv. Bhindi Sanwali subjected to synthetic AsA (150 mg L^{-1}) at 50% F.C.					
		Shoot dry weight	**Fruit fresh weight**	**Proline**	**Total soluble sugars**
AsA	1	0.02ns	0.001ns	0.006*	0.175*
Error	6	0.136	1.242	0.001	0.020
Cv. Bhindi Sanwali subjected to 14 mg L^{-1} lemon juice at 50% F.C.					
		Shoot dry weight	**Fruit fresh weight**	**Proline**	**Total soluble sugars**
Lemon juice (LJ)	1	0.480ns	1.361ns	0.009**	0.539***
Error	6	0.126	0.896	5.324	0.013
Cv. Bhindi Sanwali subjected to 24 mg L^{-1} orange juice at 50% F.C.					
		Shoot dry weight	**Fruit fresh weight**	**Proline**	**Total soluble sugars**
Orange juice (OJ)	1	0.605ns	2.101ns	0.022**	0.554***
Error	6	0.205	1.082	0.001	0.009

Note: ns = no-significant; *, ** and *** = significant at 0.05, 0.01 and 0.001 levels, respectively.

Water deficit regimes considerably ($p \leq 0.001$) increased total free amino acids in fruits of both okra cultivars (Fig. 3). Foliar application of different ascorbic acid sources improved total free amino acids in fruits of both okra cultivars and 24 mg L^{-1} OJ was the most effective for increasing this attribute under 50% F.C. Moreover, okra Cv. Sabz Pari was better in total free amino acids accumulation than that by Cv. Bhindi Sanwali at 50% F.C.

Total soluble protein content was considerably increased ($p \leq 0.01$) in okra fruits under drought stress in both cultivars (Table 1; Fig. 4). Different sources of ascorbic acid also improved total soluble proteins more prominently with the foliar-applied 14 mg L^{-1} LJ and 24 mg L^{-1} OJ at varying water-deficit levels. However, both okra cultivars were similar in response to different AsA and drought stress levels.

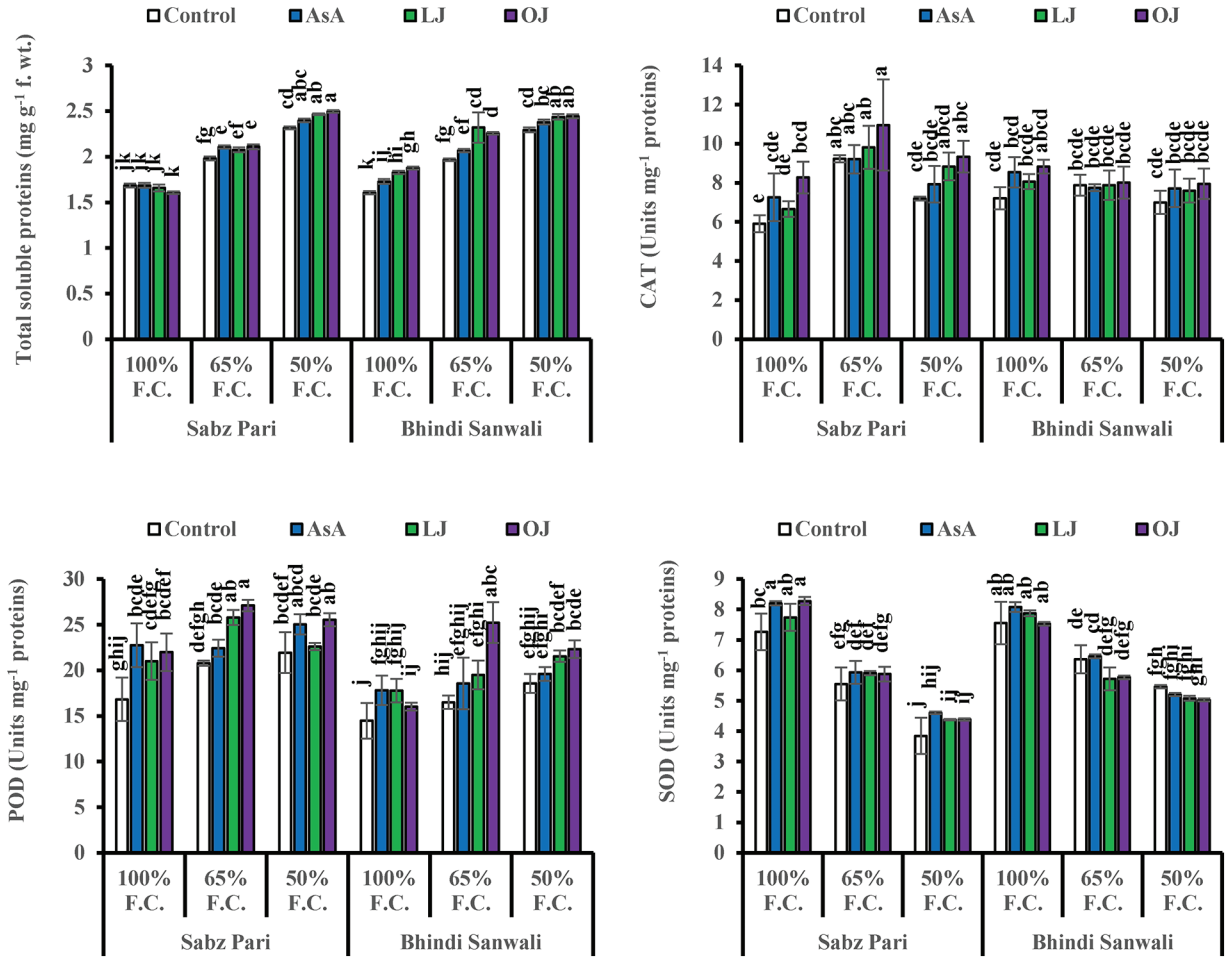

Figure 4: Total soluble proteins and activities of catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD) enzymes of fruits of two cultivars of okra (*Abelmoschus esculentus* L.) subjected to varying water-deficit conditions and foliarly treated with varying sources of AsA (Mean ± S.E.). Different letters (a–j) showing significance of difference among different treatments

The activity of SOD enzyme was decreased ($p \leq 0.001$) under drought stress conditions. However, exogenously-applied varying sources of AsA upregulated the activity of SOD enzyme only in okra Cv. Sabz Pari, while it decreased in the case of Cv. Bhindi Sanwali. Drought stress and foliar-applied AsA, LJ and OJ markedly upregulated the activities of POD ($p \leq 0.001$) and CAT ($p \leq 0.05$) enzymes in the

fruits of both okra cultivars (Table 1; Fig. 4). Moreover, a maximum improvement in the enzymatic antioxidant activities of fruit POD and CAT enzymes was observed in Cv. Sabz Pari at 65% F.C. and SOD activity at 100% F.C. in both cultivars of okra. Of all AsA sources, foliar applied 24 mg L^{-1} OJ showed a maximal improvement in the enzymatic activities of fruit POD and CAT of both okra cultivars under drought stress conditions (Fig. 4). Overall, Cv. Sabz Pari was better in POD activity and Cv. Bhindi Sanwali in the activity of SOD under water-deficit conditions.

4 Discussion

The quality and quantity of agricultural products can be enhanced by using economical approaches including the use of biostimulants, extracted from plants or their parts. Such natural chemical substances are not only profitable and effective, but are also safe environmentally and biologically [61,62]. The present experiment compared the influence of different sources of ascorbic acid used exogenously including synthetic AsA (150 mg L^{-1}) and phytoextracts [LJ (14 mg L^{-1}) and OJ (24 mg L^{-1})] naturally enriched with AsA on modulation of growth, yield and biochemical metabolites of two okra cultivars under drought stress and non-stress environments. The current study showed that the shoot and root dry weights as well as the fruit yield of water stress-induced okra plants decreased consistently. Water deficiency-induced adverse effects are believed to be usually due to the inhibition of enzyme activities, stomatal closure, impairment in photosynthesis, imbalance in nutritional and hormonal metabolism, membrane leakage as well as excessive oxidative stress [11,63–65]. We observed that both shoot and root dry weights and fruit yield of both okra cultivars were enhanced by exogenously applied AsA sources, and of all sources, 14 mg L^{-1} LJ application greatly enhanced the shoot and root dry weights, particularly at 50% field capacity. Earlier studies have shown that AsA can improve plant growth by promoting photosynthetic pigments, photosynthetic rate, water balance and oxidative defense potential under stress conditions [66,67]. It has also been reported that exogenously applied AsA protects proteins and lipids against oxidative damage [16,67], by performing the role as a substrate for ascorbate peroxidase which scavenges H_2O_2 under different stresses including water deficit conditions. Previously, Khan et al. [66] revealed the influence of foliar-applied AsA (50 and 100 mg L^{-1}) on wheat plants growing in hydroponic culture under salt stress. They reported that ascorbic acid applied as a foliar spray increased chlorophyll a content. However, in another study with okra plants, AsA application increased proline contents and decreased membrane leakage and lipid peroxidation under water deficit conditions, resulting in improved plant growth [33].

Under stress conditions, decline in chlorophyll contents and rate of photosynthesis occurs due to the inactivation of enzymes involved in the synthesis of chlorophyll pigments, resulting a reduction in crop yield and quality by altering their normal metabolic activities [41,68–70]. In the current study, increasing drought stress from 65% to 50% F.C. significantly decreased the chlorophyll *a, b* and total chlorophyll contents. Exogenously applied various sources of AsA improved the photosynthetic pigments of okra fruits (Table 1; Fig. 2). Foliar-applied AsA has been reported to improve chlorophyll pigments in different plants such as cucumber [71], and cauliflower [7]. Another study performed by Aziz et al. [28] showed that foliar-applied AsA affected positively chlorophyll *a, b* and total chlorophyll contents in quinoa plants. However, they suggested that both synthetic AsA (150 mg L^{-1}) and 25% OJ were equally effective in improving chlorophyll pigments under water-limited conditions.

Osmoprotection is one of the potential strategies adopted by plants under environmental stresses [72]. However, a variety of organic substances can play an active role in this vital process. For example, proline and GB widely occur in most plants and their contribution towards osmoprotection is significant [11,39,73]. Our results indicated that fruit proline contents remained unaffected in both okra cultivars, whereas those of GB increased prominently under water deficit conditions. However, fruit proline concentration increased significantly by foliar-applied AsA and LJ. These findings are not in agreement with those already

reported by Singh et al. [26], as they observed no considerable AsA-induced changes in the accumulation of proline contents in wheat plants by application of AsA, while GB contents increased under stress conditions. However, in several studies with different plants, an increase in proline contents was observed by exogenously applied AsA either synthetic or natural, e.g., rapeseed [38], soybean [37], and cucumber [71], etc. Both GB and proline are believed to accelerate the oxidative defense system in stressed plants [74]. So, it can be inferred from the results of the present study that exogenously applied AsA sources enhanced GB and proline accumulation, thereby resulting in enhanced osmotic adjustment.

Hydrogen peroxide and MDA contents were raised in the fruits of both okra cultivars under water deficit conditions. These results are in accordance with what has already been observed in quinoa plants by Aziz et al. [28], and in *Amaranthus* plants by Sarker et al. [75], under varying water-deficit conditions. Accumulation of H_2O_2 at a low level in plants under stress may act as a signaling molecule that can trigger tolerance to various biotic and abiotic stresses, while in higher concentrations, it is harmful to cellular metabolites [76–78]. Moreover, in the present study, foliar-applied AsA 14 mg L^{-1} LJ or 24 mg L^{-1} OJ significantly suppressed the H_2O_2 and MDA contents in both okra cultivars. Similar results were already observed in *Gossypium herbaceum* by Deeba et al. [63] and in cucumber by Nie et al. [79].

Drought stress significantly decreased a variety of metabolites such as total phenolics, total soluble sugars, AsA, free amino acids and total soluble proteins determined in okra fruits. Such results have already been observed in *Scutellaria baicalensis* [80], *Vitis vinifera* [78], and *Adonis amurensis* and *A. pseudoamurensis* [81]. Moreover, exogenous applications of AsA obtained from different sources such as 14 mg L^{-1} LJ and 24 mg L^{-1} OJ considerably improved all the above-mentioned metabolites in the fruits of both okra cultivars. These findings are in agreement with those of Khan et al. [66], and Malik et al. [82], wherein it was reported that exogenously applied AsA can mitigate the effects of drought stress. They also suggested that AsA acts as a growth regulator and it can improve plants' resistance mechanism against different environmental stresses. However, compared with synthetic AsA, exogenous applications of LJ and OJ showed better results in terms of the accumulation of these metabolites. Moreover, it is widely observed in different plants that the stress tolerance mechanism of plants could be improved by increasing endogenous levels of amino acids and proteins, e.g., canola [16], grapes [39] and wheat [83].

Plants under stress conditions protect their cells/tissues by maintaining oxidative defense system by counteracting oxidative stress mechanisms [84,85]. It is widely known that both enzymatic and non-enzymatic antioxidants could enhance the oxidative defensive mechanism of plants [15]. The present experiment also suggested that drought stress considerably enhanced the activities of antioxidant enzymes (POD and CAT) in the fruits of both okra cultivars. It is analogous to what has earlier been recorded in cotton wherein water deficit stress promoted the activities of enzymes including ascorbate peroxidase (APX), SOD, POD and CAT [86]. The current study has shown that under different water deficit regimes, foliar applied varying sources of AsA further improved the activities of antioxidant enzymes (POD and CAT) in the fruits of both okra cultivars, while that of SOD activity only in Cv. Sabz Pari. Overall, 24 mg L^{-1} OJ was the most effective in upregulating the activities of CAT, POD and SOD enzymes under drought stress conditions. These results are in agreement with those of Ejaz et al. [87] and Halimeh et al. [88]. They reported that exogenously applied AsA boosted the activities of POD and CAT enzymes under water-limited conditions. Moreover, similar results were observed in wheat by Malik et al. [83], and in quinoa by Aziz et al. [28]. They suggested that high level of endogenous AsA may be involved in improving the activities of POD and SOD enzymes in water-deficit plants by foliar-applied AsA.

In conclusion, different water-deficit regimes decreased the plant growth, fruit yield, and the activity of SOD enzyme as well as photosynthetic pigments, while an increase in fruit MDA, H_2O_2, total phenolics, total soluble sugars, AsA, free amino acids and total soluble proteins and the activities of POD and CAT enzymes in okra fruits was noticed. However, exogenously applied different AsA sources improved plant growth, fruit

yield, and key metabolites in okra fruits under both stress and non-stress environments. Overall, the foliar applied LJ and OJ were more efficient than the synthetic AsA in enhancing water deficit tolerance in okra fruits in terms of growth, yield production and key metabolites. So, the findings of the current study suggested that AsA involved in drought tolerance of plants and environment-friendly cheap and multi-nutrient sources could be more beneficial for improving stress tolerance mechanisms involved in growth and yield production.

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Availability of Data and Materials: The data can be provided on demand.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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