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NITRIC OXIDE AND ARGININE IMPROVE DROUGHT STRESS-INDUCED OXIDATIVE STRESS TOLERANCE IN SUNFLOWER (*HELIANTHUS ANNUUS* L.) BY MODULATING SOME KEY PHYSIO-BIOCHEMICAL INDICATORS

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Abstract

A pot experiment was carried-out to evaluate the effects of exogenously sprayed nitrogenous compounds, nitric oxide (NO), arginine (Arg), and NO + Arg in mitigating the harmful impacts of drought stress applied on sunflower (*Helianthus annuus* L.). Two different cultivars of sunflower plants (FH-701 and FH-811) were subjected to control conditions [100% field capacity (F.C.)] and drought-stress (50% F.C.) regimes. Then after four weeks of water deficit conditions, foliar spray of nitric oxide (0.5 mM), arginine (0.5 mM) and combination of both (0.5 mM NO + 0.5 mM Arg) along with control (no application of either of the nitrogenous compounds) were applied to sunflower plants. Drought stress significantly suppressed the plant morphological attributes (shoot and root fresh as well as their dry weights and plant shoot-root lengths), leaf relative water contents (RWC), chlorophyll contents, ascorbic acid (AsA), proline and glycine betaine (GB) concentrations. Moreover, drought stress increased the relative membrane permeability, total soluble proteins (TSP), total phenolics and various activities of antioxidant enzymes including peroxidase (POD), catalase (CAT), and superoxide dismutase (SOD). Foliar applied nitric oxide (0.5 mM), arginine (0.5 mM), and NO + Arg triggered the increase in the lengths, fresh weights, dry weights of plants shoot-root, RWC, chlorophyll pigments (*a* & *b*), GB, total soluble proteins and proline contents under all water regimes. Of all foliar-applied treatments, the combined application of NO and Arg was the most efficient in improving the damaging impacts of drought-stress on plant biomass, chlorophyll contents, and antioxidant defense system. The sunflower cv. FH-701 was better in growth and cv. FH-811 in AsA, GB, and activities of enzymes like peroxidase and superoxide dismutase. Overall, exogenous application of NO and Arg together can be suggested to increase sunflower plants' resistance to drought under dry land conditions.

Key words: Nitrogenous compounds; Mitigation; Organic metabolites; Oxidative defense system; Water deficit conditions

Introduction

Future extreme events, like drought and high temperature/heat waves are predicted to become more severe and frequent because of global climate changes (Tian *et al*., 2014; Marx *et al*., 2021; Bolan *et al*., 2023). Future climate change makes crops difficult to grow and due to increasing temperature and water shortage (Kim *et al*., 2023). In the next thirty years, it is predicted that this scenario will become more prevalent, and by 2050 it is expected that more than half of the world's regions will face water scarcity (Gupta *et al*., 2020). It has been found that water deficiency affects growth, photosynthetic rate and other gas exchange characteristics, RWC, water potential and cell division due to turgor loss as observed in various plants including barley (Kaczmarek *et al*., 2017), sunflower (Hussain *et al*., 2009), radish (Shafiq *et al*., 2015), marigold (Asrar & Elhindi, 2011), red sage (Liu *et al*., 2011), etc. In view of a projection, the average harvest per hectare was significantly impacted during last few years, which also had a marked influence on sunflower yield (Domenco *et al*., 2022). A major response of plants to various stresses (abiotic & biotic) is the over-production of reactive oxygen species that are superoxide ($O^{2\bullet-}$), singlet oxygen, hydrogen peroxide (H_2O_2) and hydroxyl radicals (Sahu *et al*., 2022). The ROS are believed to damage cell membranes, proteins, lipids, and macromolecules (RNA and DNA), and the severity of such loss is noticeably higher in stress-sensitive plant species (Kurutas, 2015). In order to maintain plant growth under such environmental cues, the plant oxidative defense system is activated (Choudhary *et al*., 2017; Waszczak *et al*., 2018; Martin *et al*., 2022).

Free-radical molecule nitric oxide (NO) participates in different plant responses to extreme environmental factors such as heat, humidity, salt, water, heavy metal stresses and UV-B radiation (Fancy *et al*., 2017; Khan *et al*., 2023; Sharma *et al*., 2020). NO may function as an antioxidant to scavenge excess ROS in addition to triggering the production of genes of different types of antioxidant enzymes (Simontacchi *et al*., 2013; Jomova *et al*., 2023). By interacting with phytohormones including auxin, cytokinin, and abscisic acids, NO can regulate stress tolerance too because exogenous application of NO and phytohormones allay the ROS accumulation in all plants (Tossi *et al*., 2009; Xu *et al*., 2010; Xie *et al*., 2022; Khan *et al*., 2023). NO also acts as a phytohormone and serves as a signal for defensive and various hormonal reactions like closing of stomata, apoptosis, root developmental process, and multiple stress responses (Freschi *et al*., 2013; Shi *et al*., 2014; Oz *et al*., 2015; Wani *et al*., 2021). On exposure to various abiotic stressors such as drought stress, nitric oxide acts as a signaling molecule that triggers the activation of ROS-

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scavenging enzymes in plants (Kolbert *et al*., 2005; Dvorak *et al*., 2021; Lau *et al*., 2021). Moreover, NO acts as an essential component in the drought stress resistance of various species of plants by increasing their metabolism of osmolytes, proline, ROS, and antioxidant metabolism (Filippou *et al*., 2014; Pandey *et al*., 2023). Additionally, nitric oxide may function as a mediator between ABA-induced stomatal response and water loss prevention via a number of signaling pathways, including cyclic guanosine monophosphate (cGMP), Ca^{2+} and mitogen activated-protein kinase (MAPK) (Gayatri *et al*., 2013; Van Meeteren *et al*., 2020). For instance, it has been shown that foliar-applied NO increased antioxidant defense system, which in turn conferred tolerance to different stresses in plants (Xu *et al*., 2010; Hasanuzaman *et al*., 2018).

Of different amino acids known in nature, arginine (Arg) is primary precursor of amino acid, urea, agmatine, ornithine and nitric oxide (Sedik *et al*., 2023; Winter *et al*., 2015) as well as of glutamate, creatine, and polyamines (Hasanuzzaman *et al*., 2018; Tapeiro *et al*., 2002). Thus, Arg is the widely adaptable amino acid that is associated with the synthesis of molecules play role in signaling, may therefore be essential for stress recovery. Arginine has a critical role in nitrogen metabolism in both establishment of seedlings and germination of seeds (Todd *et al*., 2001; Li *et al*., 2024). For example, application of Arg induces a major effect in improving growth parameters of bean (*Phaseolus vulgaris*) plant (Nassar *et al*., 2003). Moreover, endogenous and exogenous arginine is believed to perform an essential role in plant water deficient stress response (Matysiak *et al*., 2020). Moreover, the supplementation of arginine to sunflower plants increased the number of leaves and shoot length, it also used to enhance the contents of free-amino acid, proline and total soluble sugars (Ramadan *et al*., 2019).

It further protects sunflower plants from drought stress, and as a building block for polyamines, can improve drought resistance to prevent oxidative stress of H_2O_2 on plasma membranes and maintaining the water status which significantly improved growth parameters and reduced H_2O_2 and MDA contents in drought affected plants (Hassan *et al*., 2019). L-arginine has shown promising results in increasing productivity, plant quality, and alleviating effects of environmental stress. A study on coconut fiber growing media showed higher concentration of arginine improved plant productivity and nutritional value (Freitas *et al*., 2022). Drought stress has many severe negative impacts on the growth and productivity of groundnut plants, but exogenous application of SNP and Arg alleviates such harmful influences by controlling physio-biochemical processes linked with photosynthetic and oxidative responses under drought stress (Bakhoum *et al*., 2023). It improves drought tolerance by enhancing photosynthesis and antioxidant capacity (Sun *et al*., 2023). Expression of genes linked to enzymes involved in polyamine synthesis via methionine and ornithine metabolism pathways of arginine maintain the balance of different substances involved in the osmotic regulation, significantly protecting the integrity of chloroplasts, net photosynthetic rate and photochemical efficiency resulting in high grain yields (Zhao *et al*., 2024).

As nitric oxide acts as a signaling molecule, scavenger, phytohormone and increased antioxidant defense system during multiple stress responses, similarly arginine is the most versatile amino acid involved in the biosynthesis of signaling molecules in all living cells and highly non-toxic growth regulator. It plays role in signaling and in nitrogen metabolism and may therefore be essential for stress recovery. So, the combined application of NO and Arg will prove to be beneficial in performing essential roles in the plants under water deficit conditions. Only few studies until now available emphasizing their role and mechanism in plant developmental processes and stress tolerance. It was proposed that Arg might serve a similar purpose as NO because it is a source of enzymatic NO production. Furthermore, there is a need to understand how exogenously applied Arg can induce regulation of endogenous nitric oxide and how both of these biochemicals work together to protect plants against drought stress. Thus, to improve the defensive mechanism and all physiological attributes of sunflower plants grown under drought stress circumstances, the effects of NO along with Arg were examined. So, the major objectives of the resent study were to determine the effects of foliar spray of NO and Arg individually or in combination on a pattern of growth and to regulate some key physio-biochemical attributes of sunflower plants when exposed to drought stress.

Materials and Methods

To examine the effects of drought-stress and foliar-applied plant growth regulators, i.e., nitric oxide (NO), arginine (Arg), and combined application of both NO + Arg on sunflower plant (*Helianthus annuus* L.), a pot experiment was carried out at the New Botanical Garden, located at Government College University Faisalabad, Pakistan during the winter (January-May, 2023). During this period average atmospheric conditions including temperature (day and night), 28.2 ± 2°C and 14.62 ± 2 °C; light period, 7.72 h, rainfall, 27.2 mm and relative humidity, 72.8% were noted. Regarding this, achenes (seeds) of two cultivars (FH-701 and FH-811) of sunflower were collected from the Oilseed Section, Ayub Agricultural Research Institute, Faisalabad, Pakistan. The required achenes (seeds) were submerged for fifty minutes in water before being sown. Ten achenes of each cultivar were sown in each plastic pot (20 cm diameter and 24 cm depth) filled with 8 kg soil, having a sandy-loam texture. The soil chemical characteristics including phosphorus (6.4 mg/L), saturation percentage (33%), organic matter (0.75%), EC (3.86 dS m^{-1}) and pH (8.7) were noted. The experimental units were arranged in a completely randomized design with four replicates of each treatment. Good quality tap water (pH 7.9, EC 3653 µS/cm, and TDS 1822 ppm) was used for normal irrigation. The achenes began to germinate after one week of sowing. After ten days of germination of seeds, only four plants of equal size were kept in each plastic pot for further study. The growing plants were allowed to establish for three weeks after germination before applying stress (drought). Plants were subjected to control conditions (100% F.C.) and water deficit stress (50% F.C.) after three weeks of seed germination. The drought stress treatment was carried-out on the basis of soil field capacity (F.C.). The soil F.C. at the rate of 50% and 100% (control) were determined by measuring moisture contents as well as saturation percentage capacity of the

soil. After maintaining different field capacities up to thirty days of drought stress treatments, different concentrations of plant growth regulators, nitric oxide (0.5 mM), arginine (0.5 mM), and combination of both (0.5 mM NO + 0.5 mM Arg) were applied as a foliar spray at evening once a time during experimentation. As a surfactant, Tween-20 was used in each concentration and 20 mL per plant of each treatment was applied to the respective pots. For this application, a hand plastic sprayer was used. Following two weeks of foliar treatment applied, only two plants were collected from each pot separately and their growth parameters were measured. The remaining two plants were used for the collection of fresh leaves after 14 days of foliar application and stored at low temperature (-20°C) for the estimation of the biochemical parameters.

The data for the following parameters were recorded as described below:

Shoot and root fresh and dry weights: With the help of a top-loading scale, immediately following plant uprooting and washing the fresh weights of the shoots and roots were measured of two plants from each treatment. The average values were computed. After it, both the roots and shoots of the all plants were kept in an oven to dry at temperature (65°C) for 48 h to constant dry weights for documenting data of dry weights of shoots and roots.

Shoot and root lengths (cm): From the stem's base to the top of the plant, the length of the shoot, and from the tip of root to the base of stem the lengths of roots were measured by a measuring tape.

Chlorophyll contents: Chlorophyll pigments [*a, b* and total chlorophyll] were estimated by using the protocol of Arnon (1949). A fresh leaf (0.25 g) was crushed and ground in 5 mL of 80% acetone, then centrifuged mixture at normal temperature (room temperature) for 10 min. The absorbance of the supernatant was read at 663 and 645 nm using a spectrophotometer. The chlorophyll *a*, *b* and total chlorophyll concentrations were worked out.

Relative water content of leaf (RWC): Following the procedure by Jones & Turner (1980), a fresh leaf was collected from each replicate and weighed its fresh weight using an electric balance. For six hours, all leaves were merged in deionized water, then a blotting paper was placed on leaf to remove extra water, and turgid-weights of each leaf were observed. All leaf samples were kept in an oven to dry for 72 h at a temperature 70°C and dry weights of all the samples were noted and finally RWC calculated.

Relative membrane permeability of leaf (RMP): The procedure introduced by Yang *et al*., (1980) was adopted to appraise RMP. For this purpose, a fully developed two or third leaf separated from the top of each plant. Then, 0.5 g of the green leafy sample was chopped into mini pieces using a scissor and placed all samples separately in test tubes containing 10 mL deionized water. Then, EC_0 of the assayed samples was measured, then stored all samples for overnight at 4°C, and EC_1 of the samples was noted. The assayed samples were used to autoclave at a high temperature of 105°C for 30 min and calculated their EC_2 after cooling them at room temperature. The given equation was used to measure the RMP of all leaf samples:

$$\text{RMP (\%)} = (EC1 - EC0 / EC2 - EC0) \times 100$$

Free proline contents: The protocol given by Bates *et al*., (1973) was followed to estimate the leaf free proline contents. For it, 0.5 g of fresh leaf was homogenized in sulfosalicylic acid (3%) using a pestle and mortar and filtered. After that, 2 mL of the leaf filtrate was dissolved in 2 mL glacial acetic acid and 2 mL acid ninhydrin. The triturate was warmed at 90°C in the water bath for one hour. The homogeneous mixture was placed in ice to cool immediately and toluene (4 mL) was added to all samples. Then all samples were vortexed and separated the colored phase and their absorbance was read at 520 nm on spectrophotometer. The following equation was used for the determination of proline contents in each sample.

$$\text{Proline (}\mu\text{mol g}^{-1}\text{ FW)} = (\text{mL of toluene})/115\text{ g} \times (\mu\text{g proline mL}^{-1})/\text{sample (g)}$$

G**lycine betaine (GB) contents:** The protocol employed by Grieve & Grattan (1983) was followed for the estimation of glycine betaine contents. The leaf sample (1.0 g) was dipped in distilled water (dH_2O) and placed overnight. All samples were centrifuged and 1.0 mL of that extract was mixed with 2 *N* sulphuric acid **(**1 mL**)**. Then, potassium iodide (KI; 0.2 mL) solution was mixed to 0.5 mL of the mixture in the test tube. After adding 2 mL of pre-chilled distilled water (dH_2O) and 20 mL of cooled dichloromethane to the mixture, it was shaken well and the lower (bottom) phase was separated. GB contents were measured to note absorbance at 365 nm by using a spectrophotometer.

Ascorbic acid (AsA): The quantification of ascorbic acid was estimated using the detailed method of Mukherjee & Choudhri (1983). Briefly, 10 mL of 6% trichloroacetic acid solution were used to homogenized 0.25 g of fresh leaf by using a set of pestle and mortar. After that, the homogenized mixture was placed for centrifugation for fifteen minutes at the room temperature. Supernatant (1 mL) was treated with 1 mL of 2% acid dinitrophenyl hydrazine. The triturate was heated in a water bath at high temperature (95°C) for 15 min after the addition of a drop of 10% thiourea (diluted) to all samples. All samples were then chilled in an ice bath before being treated with 5 mL of 9*N* H_2SO_4. After that, a spectrophotometer was used to determine the absorbance of the final mixture at 530 nm.

Total phenolics: A fresh leaf (0.1 g) was homogenized in 80% acetone (5 mL) and put it for centrifugation for 15 min at 25°C. After filtration, the samples (0.1 mL) were kept in 10 mL test tube and combined with 2.0 mL distilled water (dH_2O) and 1 mL of the Folin Ciocalteu's reagent. To the resultant solution, sodium carbonate (5.0 mL) was added and mixed properly. Then, the total volume of the sample was maintained to 10 mL with deionized water. The total phenolics were determined to follow the method of Julkenen Titto (1985) to read the absorbance of the given solution at 750 nm.

Total soluble proteins (TSP): The youngest leaf (0.5 g) was homogenized well in Na-P buffer (10 mL) with pH 7.8. Then obtained homogenate was centrifuged almost for 15 min at a temperature of 4°C at 10,000 × *g*. Then, this supernatant was used for the quantification of TSP and antioxidant enzyme activity. To quantify TSP, the protocol described by Bradford (1976) was employed. For it, leaf extract (1 mL) was dissolved well with 2 mL of Bradford reagents and their absorbance was noted at 595 nm using a spectrophotometer. A standard curve derived from pure-standards (200–1600 mg/L) made from analytical grade bovine serum albumin (BSA) was used for the measurement.

Activities of antioxidant enzymes: The primary protocol of Chance & Maehly (1955) was used to determine the activity of POD enzyme. For it, 1.0 mL of 50 mM phosphate buffer, 0.1 mL leaf filterate, 1.0 mL guiacol (20 mM), and 0.9 mL H_2O_2 (40 mM) were all mixed to prepare the reaction mixture. The reaction mixture's color changed as a result of guiacol oxidation, and the absorbance was determined by a spectrophotometer at 470 nm with a time interval of 20 second between each two readings.

The activity of the catalase (CAT) enzyme was assessed based on the basic phenomenon of H_2O_2 disappearing in the reaction mixture which was prepared from enzyme extract (0.1 mL), 5.9 mM H_2O_2 and phosphate buffer (50 mM). The absorbance change was measured in the mixture after every 20 seconds at 240 nm (Chance and Maehly, 1955).

To determine the SOD activity, the method given by Giannopolitis and Ries (1977) was followed. An enzymatic reaction mixture prepared with distilled water (400 µL), 50 mM phosphate buffer (250 µL), 1.3 µM riboflavin (50 µL), 13 mM L-methionine (100 µL), 0.1% triton-X (100 µL), 50 µM NBT (50 µL), and 50 µL enzyme extract. The resultant mixture was kept for 20 min under a fluorescent light source (20 W). The lowering in inhibition of NBT was noted at 560 nm and the activities of SOD in each sample was estimated as unit/mg proteins by using the content of TSP.

Statistical analyses

A three-way (cultivars, drought stress and foliar application) analysis of variance (ANOVA) was applied by using of statistical software Co-Stat version 6.0. The least significance difference (LSD) at 5% probability was applied to assess if there is difference among the mean values of all the treatments. A correlation was also worked-out to study the relationship between all morphological and physio-biochemical attributes.

Results

Morphological attributes: A significant adverse effect ($p \leq 0.001$) of imposition of drought stress (50% F.C.) was found on different morphological attributes including shoot and root fresh as well as their dry weights and lengths of root and shoot of both cultivars of sunflower namely FH-701 and FH-811. Exogenous application as a foliar spray of nitrogenous compound, nitric oxide (NO), arginine (Arg) and combination of both (NO + Arg) were considerably effective ($p \leq 0.001$) in upraising the root and shoot fresh and their dry weights and lengths of both (FH-701 and FH-811) sunflower cultivars in stress as well as control condition. Of all the external treatments, combination of NO + Arg was more effective in promoting all the morphological variables. The difference between the shoot fresh and dry weights of both sunflower cultivars was non-significant. However, cv. FH-701 was better than the other cultivar in root lengths and fresh weights and dry weights, particularly under the zone of water deficit environments (Table 1; Fig. 1).

Relative water contents and relative membrane permeability: Relative water contents (RWC) showed a significant ($p \leq 0.001$) declining effect on both (FH-701 and FH-811) cultivars during water limited conditions. Exogenously applied all levels of NO were effective and combination of NO + Arg was better ($p \leq 0.001$) in enhancing the RWC in both cultivars of sunflower under arid conditions. Both sunflower cultivars responses were the same under control and water shortage regimes (Table 1; Fig. 2).

Under water deficit conditions, a significant ($p \leq 0.05$) increase was observed in the relative membrane permeability (RMP) of both sunflower cultivars. Exogenous application of NO, Arg and combination of both was non-significant in both cv. FH-701 and cv. FH-811 and there was no cultivars difference in this attribute under both stress and control conditions (Table 1; Fig. 2).

Photosynthetic pigments: Imposition of drought stress (50% field capacity) significantly suppressed ($p \leq 0.001$) the chlorophyll contents including *a, b &* total chlorophyll. In contrast, chlorophyll ratio (*a/b)* remained unchanged by all exogenous treatments in both cultivars (FH-701 and FH-811) of sunflower. Foliar application of all three levels of nitrogenous compounds were significantly effective in enhancing the chlorophyll *a* content and their total chlorophyll content too, but chlorophyll *b* content remained unaffected in both cultivars. Of all exogenous treatments, Arg and combination of NO + Arg were found to be most efficient in enhancing the chlorophyll pigments, particularly in sunflower cv. FH-701 under water stress condition (Table 1; Fig. 2).

Osmoprotectants (Proline and GB contents): It was found that drought stress induced a significant ($p \leq 0.001$) increasing effect to accumulate free proline and glycine betaine (GB) contents in the sunflower (FH-701 and FH-811) cultivars (Table 1; Fig. 3). Foliar applied nitrogenous compounds were showed effectiveness in enhancing only the accumulation of GB and combination of both No + Arg was better for both sunflower cultivars under limited water conditions. Of both sunflower cultivars, FH-811 was lower in accumulation of proline contents while better in GB accumulation compared with the other sunflower cultivar under examination. Foliar applied arginine and nitric oxide found to be effective in cultivar FH-701 and FH-811 respectively under low water conditions (Table 1; Fig. 3).

Ascorbic acid, total phenolics and soluble proteins: It was noted that total phenolics and the contents of ascorbic acid (AsA) significantly showed increase ($p \leq 0.001$) in sunflower (FH-701 and FH-811) cultivars (Table 1; Fig. 3)

under limited water conditions. Foliar-applied all treatments (NO, Arg and combination of NO + Arg) were effective in enhancing the AsA contents in both sunflower cultivars. FH-701 was relatively better ($p \leq 0.001$) in total phenolic concentrations in the both (FH-701 and FH-811) sunflower cultivars and combination of both No + Arg was better for both sunflower cultivars under limited water. Overall, FH-811 was better in AsA contents particularly under water deficit conditions.

Data revealed that the total soluble proteins (TSP) contents increased ($p \leq 0.001$) considerably in all cultivars (FH-701 and FH-811) of sunflower cultivated under drought stress environment. All levels of foliar spray of NO were effective in increasing the TSP, but the combined application of NO + Arg was more effective ($p \leq 0.001$) to enhance TSP under drought stress. Both sunflower cultivars at both water regimes showed similar responses (Table 1; Fig. 4).

Table 1. Mean square values obtained from analysis of variance of data for different morpho-physiobiochemical characteristics of drought-stressed plants of two cultivars of sunflower (*Helianthus annuus* L.) treated with varying levels of foliar applied nitric oxide (NO), arginine (Arg) and combination of both (NO + Arg).

Source of variations	df	Shoot length	Root length	Shoot fresh weight	Root fresh weight
Cultivars (Cvs)	1	263.3*	3.273**	3.235ns	0.098*
Drought (D)	1	1659.5***	17.98***	47.94***	0.666***
Treatments (T)	3	617.3***	4.564***	12.68***	0.181***
Cvs x D	1	26.91ns	0.058ns	32.69***	0.030ns
Cvs x T	3	38.01ns	0.004ns	1.228ns	0.004ns
D x T	3	30.79ns	0.417ns	0.563ns	0.005ns
Cvs x D x T	3	6.248ns	0.118ns	1.049ns	0.008ns
Error	48	36.38	0.346	1.303	0.018
		Shoot dry weight	**Root dry weight**	**Relative water content**	**Relative membrane permeability**
Cultivars (Cvs)	1	0.208ns	0.006*	87.15ns	468.9*
Drought (D)	1	2.678***	0.096***	1578.5***	380.2*
Treatments (T)	3	0.389ns	0.006*	218.8***	74.82ns
Cvs x D	1	0.164ns	3.531ns	9.083ns	17.40ns
Cvs x T	3	0.097ns	1.346ns	11.18ns	200.1ns
D x T	3	0.031ns	0.001ns	34.18ns	53.57ns
Cvs x D x T	3	0.031ns	5.22ns	8.538ns	341.3**
Error	48	0.1497	0.001	33.87	74.72
		Chlorophyll *a*	**Chlorophyll *b***	**Total chlorophyll**	**Chl. *a/b* ratio**
Cultivars (Cvs)	1	1.233***	0.379*	2.983***	1.570ns
Drought (D)	1	1.823***	0.692**	4.763***	0.037ns
Treatments (T)	3	0.275***	0.150ns	0.778**	0.023ns
Cvs x D	1	0.009ns	0.033ns	0.007ns	0.175ns
Cvs x T	3	0.049ns	0.029ns	0.141ns	0.062ns
D x T	3	0.026ns	0.010ns	0.044ns	0.055ns
Cvs x D x T	3	0.034ns	0.046ns	0.082ns	0.028ns
Error	48	0.035	0.075	0.111	0.194
		Proline	**Glycine betaine**	**Total phenolics**	**Ascorbic acid**
Cultivars (Cvs)	1	1.116***	1.597***	328.2**	3.021***
Drought (D)	1	2.107***	1.822***	687.2***	7.699***
Treatments (T)	3	0.188ns	1.415***	101.4ns	1.149***
Cvs x D	1	0.325ns	0.014ns	2.259ns	0.453ns
Cvs x T	3	0.035ns	0.088ns	1.856ns	0.117ns
D x T	3	0.033ns	0.058ns	4.908ns	0.423ns
Cvs x D x T	3	0.027ns	0.124ns	15.23ns	0.002ns
Error	48	0.09	0.122	36.95	0.195
		Superoxide dismutase	**Peroxidase**	**Catalase**	**Total soluble proteins**
Cultivars (Cvs)	1	16.56ns	93.04**	0.123*	0.487ns
Drought (D)	1	123.9***	69.76*	0.561***	12.61***
Treatments (T)	3	96.94***	45.53*	0.248***	2.246***
Cvs x D	1	25.79ns	2.248ns	0.004ns	0.677ns
Cvs x T	3	5.937ns	2.571ns	0.021ns	0.188ns
D x T	3	10.73ns	0.107ns	0.017ns	0.121ns
Cvs x D x T	3	8.048ns	0.799ns	0.007ns	0.042ns
Error	48	8.752	11.68	0.021	0.312

ns = No significant; *, ** and *** = Significant at 0.05, 0.01 and 0.001 levels, respectively

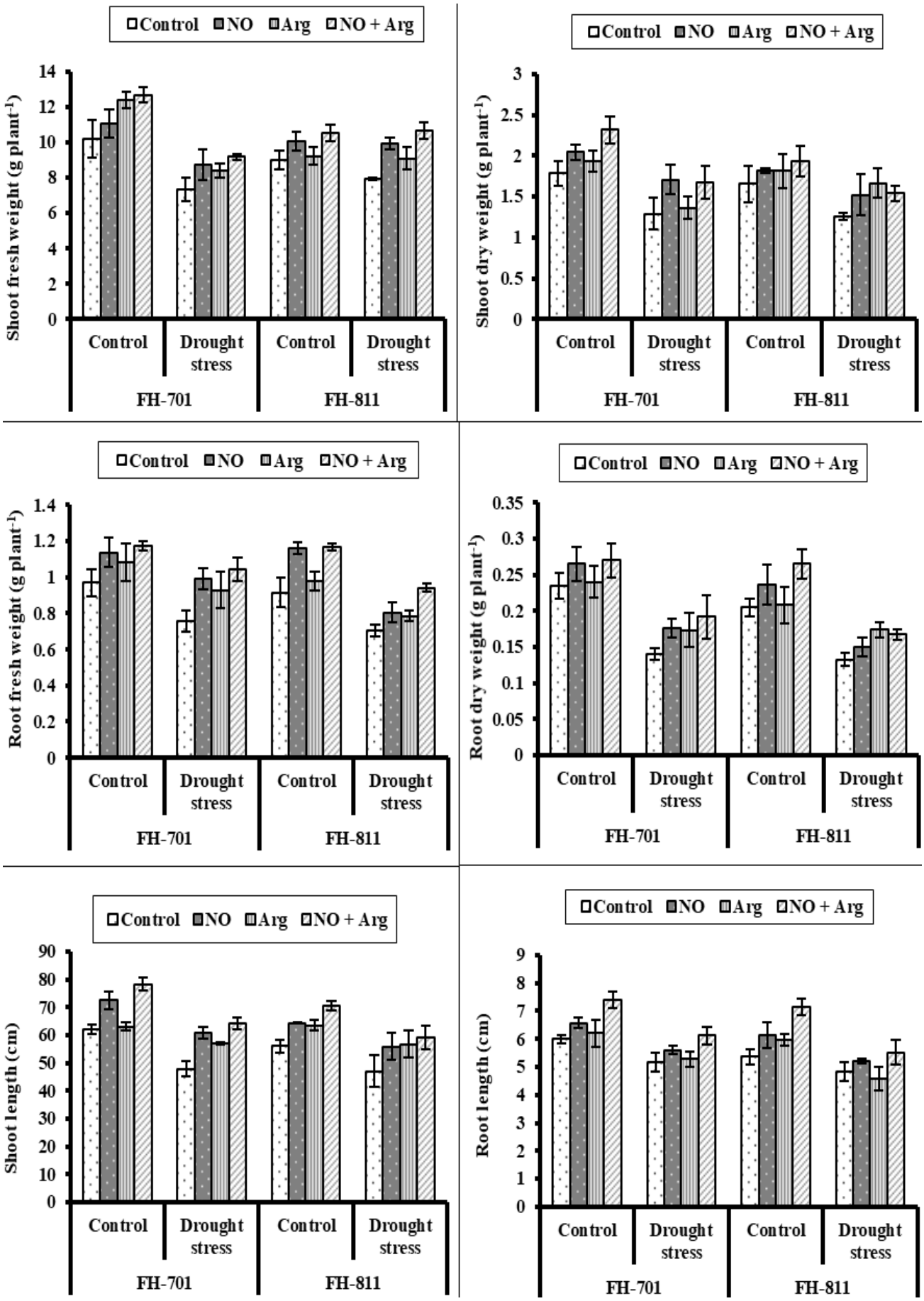

Fig. 1. Shoot fresh and dry weights, root fresh and dry weights, and shoot and root lengths of 60-day old drought-stressed plants of two cultivars of sunflower (*Helianthus annuus* L.) treated with varying levels of foliar applied nitric oxide (NO), arginine (Arg) and combination of both (NO + Arg). (Mean ± S.E.).

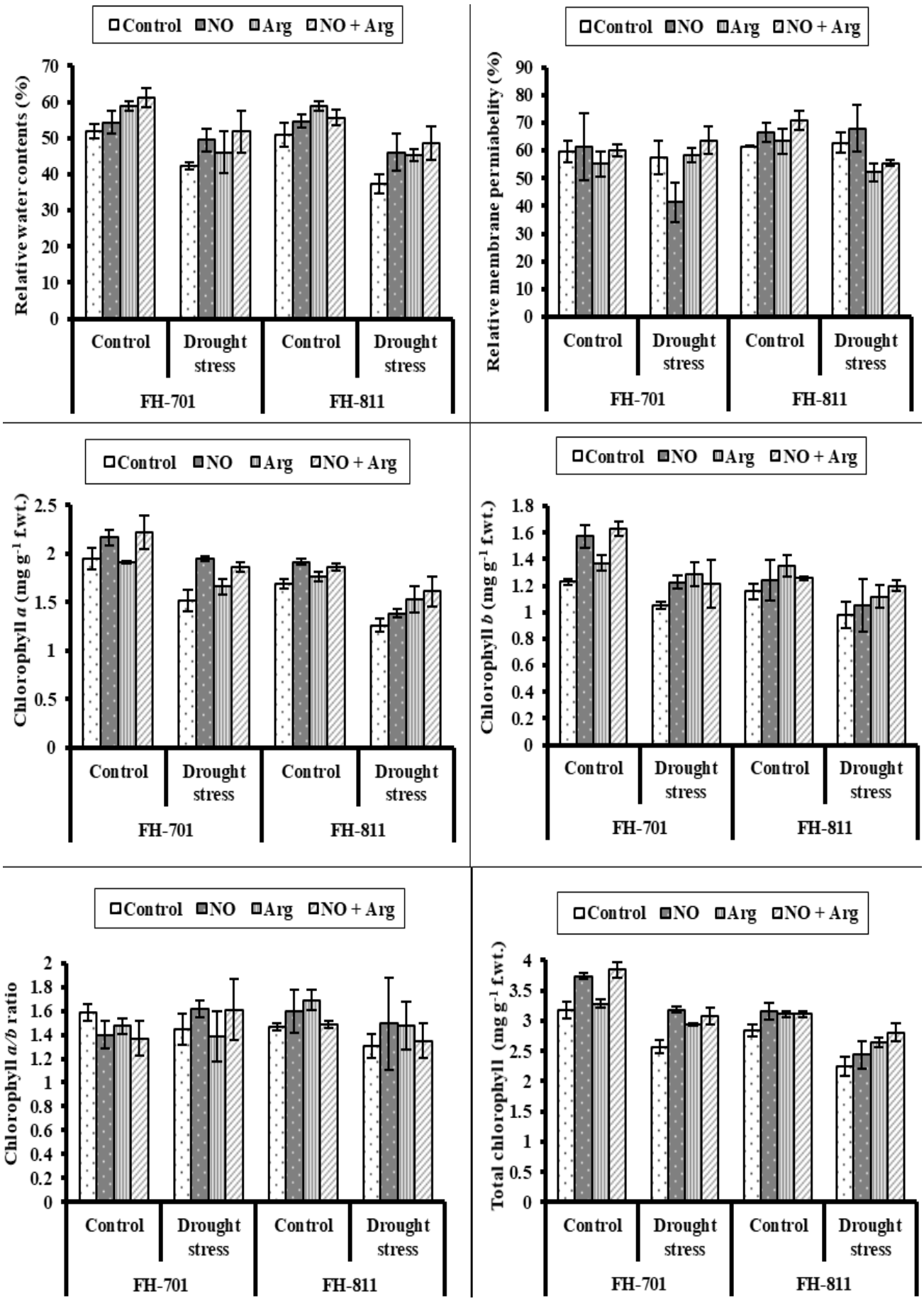

Fig. 2. Relative water contents, relative membrane permeability, chlorophyll *a, b, a/b* ratio and total chlorophyll of 60-day old drought-stressed plants of two cultivars of sunflower (*Helianthus annuus* L.) treated with varying levels of foliar applied nitric oxide (NO), arginine (Arg) and combination of both (NO + Arg). (Mean ± S.E.).

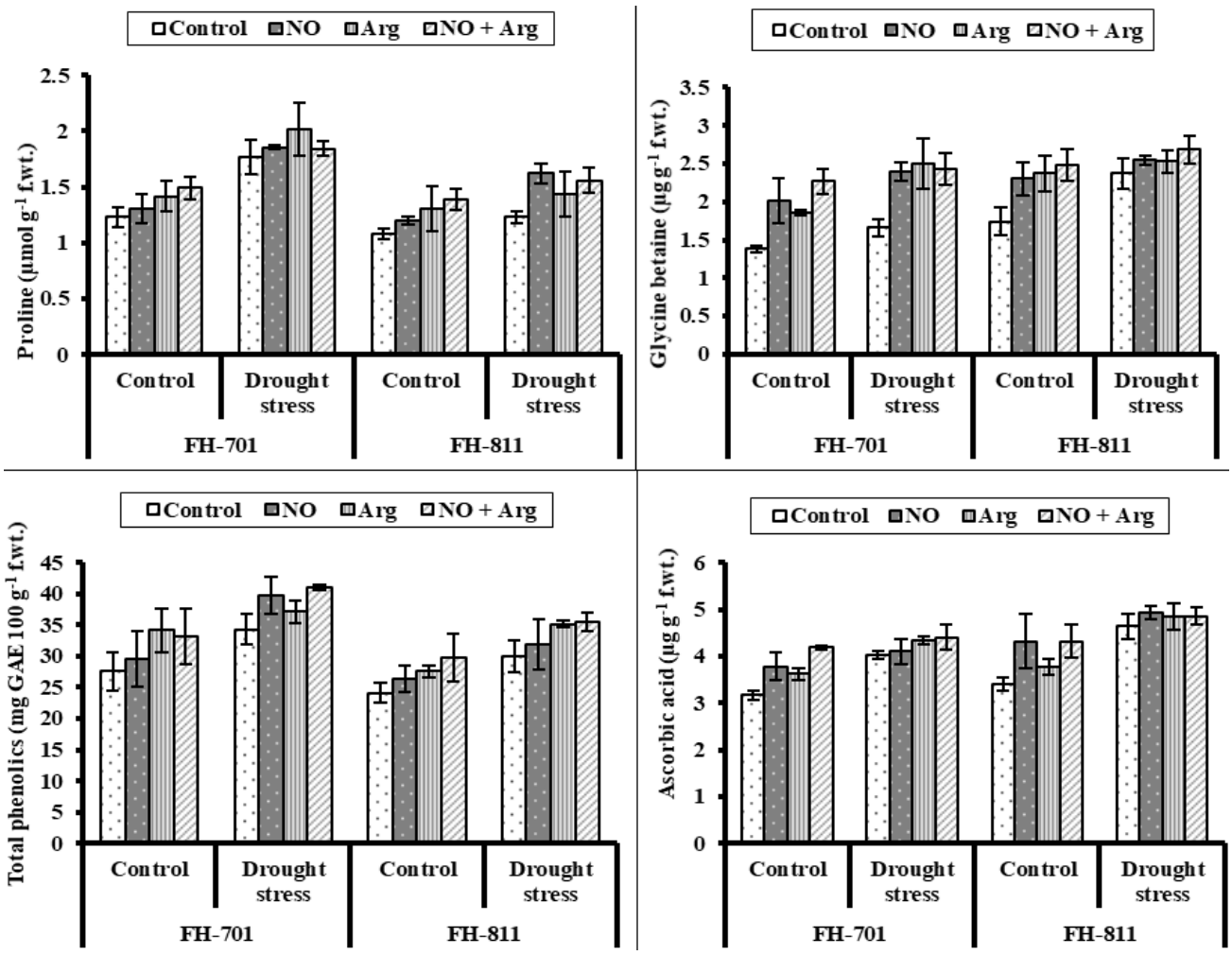

Fig. 3. Proline, glycine betaine, total phenolics, and ascorbic acid of 60-day old drought-stressed plants of two cultivars of sunflower (*Helianthus annuus* L.) treated with varying levels of foliar applied nitric oxide (NO), arginine (Arg) and combination of both (NO + Arg) (Mean ± S.E.).

Activities of antioxidant enzymes: Drought stress showed a significant increasing ($p \leq 0.001$) effect on the activities of enzymatic antioxidants including peroxidase, superoxide dismutase, and catalase [(POD), (SOD) & (CAT)] in both (FH-701 and FH-811) sunflower cultivars.

Of all exogenously applied nitrogenous compounds, arginine and combination of both NO + Arg were more effective ($p \leq 0.001$; ≤ 0.05; ≤ 0.001, respectively) in the enhancement of the enzymetic activity of POD, SOD, and CAT both under control and drought-stress. Both sunflower cultivars, FH-701 was higher in the activity of catalase and peroxidase enzymes, while the SOD enzyme activity remained consistent in both sunflower cultivars (FH-701 and FH-811) under control (non-stressed) and stressed zone (Table 1; Fig. 4).

Discussion

Drought related stress adversely impacts the regulation of different physiological as well as biochemical phenomena which ultimately result in a reduction of crop growth and productivity (Gupta *et al*., 2020; Kosar *et al*., 2021). It has been shown that despite lowering plant total dry weight, net assimilation rate and growth rate, water stress suppresses the photosynthetic rate and efficiency by reducing leaf area index as well as chlorophyll contents (Hilli & Immadi, 2021; Pekcan *et al*., 2021). In the present finding, significant adverse effects of drought-stress were observed on different morphological attributes like shoot fresh weight, root fresh weights and dry biomasses and the lengths of plants shoot-root in both cultivars (FH-701 and FH-811) of sunflower. It is believed that water deficiency at any growth stage of plants may harm physiology and biochemistry (Poudyal *et al*., 2019; Chen *et al*., 2022; Ro *et al*., 2021). Therefore, limiting plant growth and dry mass accumulation is expected during water deficit environments (Ogunkanmi *et al*., 2022; Wan *et al*., 2022).

It is known that exogenous applied plant growth regulators are a meaningful approach for enhancing plant growth and developmental mechanisms during stressful conditions (Hameed *et al*., 2021; Batool *et al*., 2022). Many previous reports have shown that foliar-applied nitric oxide (NO) had the most beneficial impacts on the developmental and growth stages of different plants during control and stressed (dryness) environments (Santisree *et al*., 2015; Kwon *et al*., 2016). Arginine (Arg) is the most diverse amino acid associated with the bio-synthesis of various signaling molecules that play vital roles in plant stress recovery. Arginine is essential for nitrogenous metabolism in seeds germination and seedlings development (Todd *et al*., 2001). For example, a significant increase in growth of bean plants was observed by applying arginine (Nassar *et al*., 2003). We observed that water stress adversely

affecting plant growth was mitigated by foliar-applied NO, Arg and combination of both (NO + Arg). Moreover, the combination of nitric oxide and arginine (0.5 mM NO + 0.5 mM Arg) was better for promoting shoot and root lengths. In an earlier study, it was reported that NO acts as a phytohormone, and it plays a role during hormonal signaling and various defensive mechanisms including root length, closing of stomata, developing roots, and apoptosis under stress conditions (Oz *et al*., 2015; Freschi *et al*., 2013). In an earlier study it has been studied that the externally applied arginine promoted length of shoot and leaves numbers of sunflower (*Helianthus annuus* L.) plants (Ramadan *et al*., 2019). Similarly, in the recent study, sunflower plants treated with 1 mM arginine exhibited better lengths of shoots and roots particularly in the combined application of both regulators (0.5 mM NO + 0.5 mM Arg). Exogenous application of these treatments could be useful in alleviating the drought-stress induced inhibition of seedling's growth of sunflower because biosynthesis of signaling molecules may occur due to exogenous application of arginine (Hamid *et al*., 2019). In a previous investigation, foliar applied arginine to maize (*Zea mays* L.) plants resulted in significantly increasing dry weights of shoots and roots (Matysiak, 2020). Moreover, pre-treatment with arginine at 0.5 mM led to a considerable increase in the plant weight of sunflower plants under the condition of stress (Nejadalimoradi, 2014). These findings are comparable to those of ours. The stimulating effect of arginine on the fresh weights and dry weights of both shoots and root of drought-stressed cultivars have been due to arginine effects as an essential amino acid that could promote plant growth (Shalaby *et al*., 2018).

In our resent findings, a significant reduction was observed in relative water contents (RWC) in both cultivars (FH-701 and FH-811) of sunflower due to drought stress. Previous results also reported that water deficit conditions can lead to lower down both water content and cell water turgor (Hernandez *et al*., 2021). It is well known that drought stress can impact a variety of plant functions, including growth, photosynthesis, gas exchange properties, relative water contents, water potential, and cell division as a result of turgor loss in various plants including barley (Kaczmarek *et al*., 2017), sunflower (Hussain *et al*., 2009), radish (Shafiq *et al*., 2015), marigold (Asrar & Elhindi, 2011) and red sage (Liu *et al*., 2011). Plants often close their stomata to reduce evaporation at the expense of photosynthesis mechanism (Yang *et al*., 2021). In our results, foliage spray to sunflower cultivars with nitric oxide, L-arginine and combination of both (NO + Arg) significantly improved the RWC. Previous findings also showed that SNP or Arg supplementation markedly raised the accumulation of proline contents in drought-stressed wheat seedlings, which may further impact the process of osmoregulation as well as water status restoration, and ultimately increase the relative water content of leaf in wheat plants. The present results also corroborate with the previous findings wherein SNP as a NO donor was used (Arasimowicz *et al*., 2009) and arginine (Yang *et al*., 2021; Nasibi *et al*., 2013). They also reported that externally applied SNP elevated the antioxidant levels during various stresses by the activation of non-enzymatic and enzyme antioxidant defensive systems, which further play crucial roles in plants resistance to stress.

In both sunflower cultivars, exogenously applied NO and Arg induced a notable rise in RMP under acute water stress. These findings clearly revealed that exogenous application may play a role in maintaining membrane integrity by lowering the generation of ROS in sunflower varieties because NO, due to its lipophilic nature, is highly diffusible through cellular membranes (Allagulova *et al*., 2023). It is also involved in multiple developmental, biochemical and physiological phenomena in plants (Krasylenko *et al*., 2010). In the present study, drought conditions reduced chlorophyll pigments (chl. *a*, chl. *b* & total chlorophyll), possibly due to impairment of chlorophyll biosynthesis mechanism. On the other hand, exogenous NO and Arg led to an increase in chlorophyll contents. Previous reports have documented an improvement in chlorophyll biosynthesis caused by nitric oxide (Hatamzadeh *et al*., 2015; Shalaby *et al*., 2018). In this study, a significant reduced in chlorophyll content was noticed because of water stress, however, it was improved by exogenous spray of L-arginine, showing that L-arginine could maintain the balance between synthesis and degradation of chlorophyll under drought stress as demonstrated in some earlier reports (Gan *et al*., 2015; Hasanuzaman *et al*., 2018).

In present results, we observed a significant enhancing effect on free proline and glycine betane contents of both sunflower cultivars under water shortage conditions. Foliar applied NO, Arg and a combination of both NO + Arg was most effective in the improvement of both proline contents and glycine betaine (GB) contents in sunflower cultivars (FH-701 and FH-811) cultivated under water deficit conditions. According to a previous report, both glycine betaine (GB) and proline may involve scavenging reactive oxygen species (ROS) and retaining water levels during adverse stress-conditions (Asghar *et al*., 2022). The application of arginine increased the contents of free-amino acids, proline, and total soluble sugars in the drought-stressed plants in comparison to those in the control sunflower plants. In a previous study with sunflower, a stressed-induced accumulation was observed in free proline and total soluble sugars, as well as in total free amino acids (Ramadan *et al*., 2019). Water stress tolerance is known to be influenced by osmotic adjustment, which can be achieved through osmoregulation, ion homeostasis and ionic compartmentalization in various crops, including sunflower (*Helianthus annuus* L.) (Kosar *et al*., 2021), canola (*Brassica napus* L.) (Akram *et al*., 2018), and maize (*Zea mays* L.) (Asghar *et al*., 2022). Furthermore, the interaction between ABA and NO under environmental stressors regulates the expression of important genes linked to the manufacture of PAs, NO, flavonoids, ABA receptors, and antioxidant enzymes. The interaction between ABA and NO works to mitigate abiotic stresses (Xu *et al*., 2024). Nitric oxide causes SlWRKY6 overexpression in tomato plants, enhancing drought resistance and antioxidant capacity by increasing photosynthetic capacity, decreasing accumulation of reactive oxygen species, and increased abscisic acid (ABA) content and also abundance of ABA synthesis and signaling of genes. These findings raise the possibility that the ABA pathway plays a role in SlWRKY6-induced drought resistance (Chen *et al*., 2024).

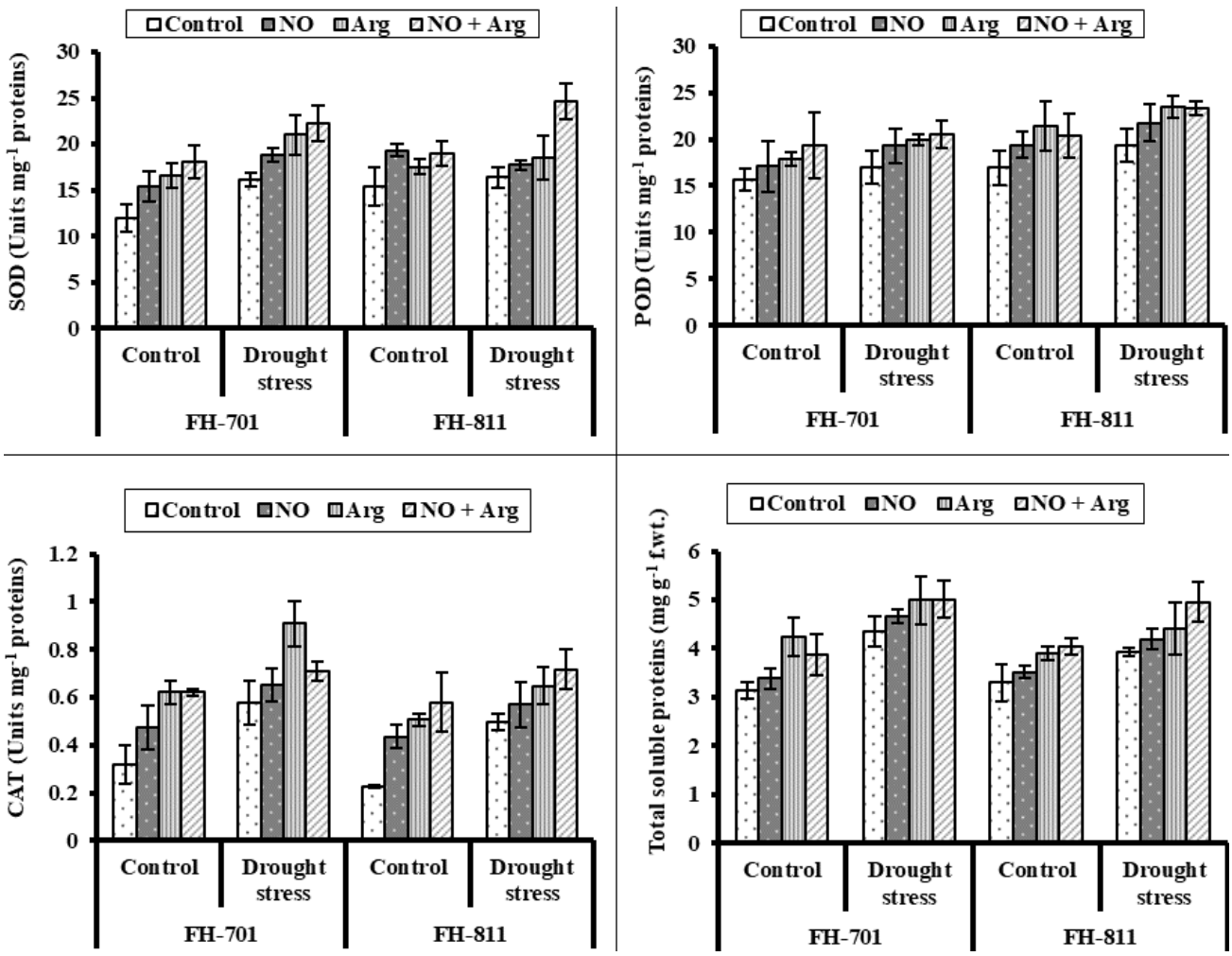

Fig. 4. Activities of enzymes (superoxide dismutase, peroxidase, catalase) and total soluble proteins of 60-day old drought-stressed plants of two cultivars of sunflower (*Helianthus annuus* L.) treated with varying levels of foliar applied nitric oxide (NO), arginine (Arg) and combination of both (NO + Arg) (Mean ± S.E.).

Under stressful conditions, high-level glycine betaine and proline contents are key indicators of osmoregulation, which is essential for plants' ability to protect themselves against harsh environments (Saadia *et al*., 2013; Hossain *et al*., 2022). Previous findings also showed that high contents of glycine betaine and proline played a useful role in promoting the various enzymatic antioxidants, activities e.g., APX, superoxide dismutase, and catalase under less water regimes (Osman *et al*., 2015). In this study, ascorbic acid (AsA) contents and total phenolics increased in both sunflower cultivars (FH-701 and FH-811) under drought conditions, which show similarity with earlier research under water deficit stress, the level of ascorbic acid, glycinebetaine, total phenolics and proline contents were significantly enhanced in both leaf and head of broccoli (Akram *et al*., 2024). Of both sunflower cultivars, cv. FH-701 was better in total phenolics and cv. FH-811 was better in AsA contents, respectively, under less water conditions. Previous findings have also shown the same roles played by SNP in increasing AsA-GSH cycle elements (Shi *et al*., 2014; Hasanuzaman *et al*., 2011; Maslennikova *et al*., 2022).

NO is considered as a vital element for drought tolerance in large numbers of plant varities by enhancing antioxidant system including ROS, proline and metabolism of osmolytes (Filippou *et al*., 2014; Corpas *et al*., 2021). Moreover, exogenously applied Arg induced regulation of endogenous NO & contents of proline in the sunflower plants during stress. In another study supplementation of NO donor and Arg had been shown to improve the antioxidant defensive mechanism and all physiological attributes of wheat (*Triticum aestivum* L.) under arid conditions (Hasanuzzamane *et al*., 2014). In the current study, NO, Arg and their combination reduced oxidative damage in drought-affected plants by lowering the amount of ROS and lipid peroxidation products, which significantly upregulated the antioxidant system. The antioxidant enzymes activities and the correlation between NO and Arg content proved the positive role of these nitrogenous compounds in protecting the membrane's stability, which decrease the inhibitory effects caused by water-limited supply on sunflower plants. It shows consistency with many previous reports (Nasibi *et al*., 2015; Zhang *et al*., 2021, Nasar *et al*., 2022). Furthermore, SOD catalyzes conversion of $O_2^{\cdot-}$ to H_2O_2 (Zhang *et al*., 2011), and drought stressed sunflower plants also showed increased SOD activity, correlating with the results of many previous reports (Zhang *et al*., 2011). However, drought-applied seedlings sprayed with NO, Arg and combination of both did not show a further increase in the activity of SOD enzyme. Catalase activity

reduction may have been because of increased generation of H_2O_2 beyond the scavenging capacity, which markedly increased the H_2O_2 contents as in salt stressed mung bean seedlings (Nahar *et al*., 2015). In our results, CAT and POD activities increased in water deficient sunflower plants treated with NO (0.5 mM) and Arg (0.5 mM) along with their combination (NO + Arg 0.5 mM). These results are analogous to previous findings wherein exogenous NO conferred stress tolerance due to increasing antioxidant defensive mechanisms (Xu *et al*., 2010; Hasanuzzaman *et al*., 2010; 2018).

In conclusion, drought stress markedly affected the plant morphology, RWC, chlorophyll contents, proline, GB, and AsA in sunflower plants. However, drought stress improved RMP, total soluble protein, total phenolic, and all activities of antioxidant enzymes. Foliar applied NO, Arg and NO + Arg triggered an increase in fresh weights, plant lengths, RWC, dry weights, chlorophyll pigments, proline, GB and total soluble protein under varying water regimes. Overall, sunflower cv. FH-701 showed better resistance than cv. FH-811 and exogenous application of NO and Arg together is suggested for achieving enhanced drought tolerance of sunflower plants under dryland conditions.

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