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Nitric oxide-mediated drought stress tolerance via improvement crop yield, antioxidants, membrane integrity and reducing the oxidative stress of two faba bean cultivars

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Abstract. Nitric oxide (NO) is a prevalent signaling molecule that regulates plant responses to potential plant stresses like drought. Growth and seed yield/plant improvement under drought stress resulted from the regulatory role of NO-priming on different physiological pathways of two faba bean plants differing in their drought tolerance. NO efficiently enhanced the machinery of photosynthesis via increasing chlorophyll a, b, and carotenoids associated with lowering hydrogen peroxide, superoxide anion, and hydroxyl radical. Thus, the tested dose of NO had an efficient free radical quenching system as witnessed from activating phenolics, ASA, SOD, APX, and CAT. These up-regulations reflected maintaining higher membrane stability via lowered lipid peroxidation and electrolyte leakage under control and drought-treated plants. The promoter role of NO on metabolic and antioxidative activities could serve as an essential component of the defense mechanism against oxidative burst induced by drought stress.

***Keywords**: antioxidants, crop yield, drought, faba bean, nitric oxide.*

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1. Introduction

Plants in a natural environment exposed to pathogens attacks such as bacteria, fungi, and viruses as well as abiotic environmental factors as drought, salt, floods, heat, cold and heavy metals that can affect the average growth, development, and production, hence affect the global food security [1]. Drought is a prevalent plant stressor affecting various morphological, physiological, biochemical, and molecular aspects in plants. Dawood et al. [2] reported that drought stress adversely affected plant growth and development. The negative impacts of water deficit included oxidative damage to the plant cell due to the imbalance between ROS production and the antioxidant defenses [3]. Plants refined ROS by increasing the activity of antioxidant enzymes, and this strategy is one of the mechanisms followed by plants to alleviate stress [4]. The induction of proline via stress is a cytosolic compatible solute that functions as osmotic buffers. However, apart from osmotic adjustment, proline seems to play a role in maintaining the functional state of macromolecules, probably by scavenging reactive oxygen species [3].

The amelioration of oxidative burst and enhanced resistance to environmental stresses is often associated with the equilibrium of the antioxidative system and ROS production. These responses may be activated in plants via application protecting substances to produce vigor plants that can withstand harsh conditions. Such responses may be prompted by applying protecting molecules such as nitric oxide (NO). Nitric oxide is a highly reactive nitrogen species (RNS) produced in cells under biotic and abiotic stress [5]. As ROS concentration is elevated to a noxious level by stress, NO may function as a detoxifier to abolish any deleterious effects. From the function of NO in plants, such as germination, development, flowering, senescence, and abiotic stress. Moreover, NO act as signaling messenger under biotic and abiotic stress and act as an active molecule in response to free radical, small size redox molecules, neutral, and easily diffusible through a cell membrane [6]. Moreover, NO also plays a role in respiratory function as mitochondrial electron transport pathways by mediating ROS and enhancing

antioxidant production for stressed plants [5]. For instance, the activation of antioxidant enzymes as superoxide dismutase of plants supplied with NO restricts superoxide anion and organic radicals that attack lipids [7]. Recent research demonstrated that exogenous NO could improve water-deficit mitigation in some plants as *Physalis angulata* [1], sunflower [8], and rice [4]. NO depends on species, cultivars, lines, organs, external factors, and signaling pathways under different stress.

Thus, the co-workers tended to soak plants in the selected protective substance, NO, with a proper concentration picked up from a preliminary experiment (data not shown) to elucidate the antioxidative response of drought faba bean plants to NO-donor (sodium nitroprusside), and they reflect on plant growth as well as crop yield production. As stressed microgreens to fruits increase quality, bioactive compound, and antioxidant capacities [9,10], there was not enough research on the stress of faba bean. Therefore, we have conducted this research to confirm the nitric oxide-mediated drought stress tolerance via improvement crop yield, antioxidants, membrane integrity, and reducing the oxidative stress of two faba bean cultivars.

2.Materials and methods

2.1. Experimental setup

Experiments were conducted at the greenhouse of Botany and Microbiology Department, Faculty of Science, Assiut University. The seeds of different varieties of faba bean plants (Giza 843, El-wadi 3) were soaked in 0.1 mM concentrations of NO donor sodium nitroprusside (SNP) for 8 hours which was selected based on a survey experiment of the year 2016. Soaked and neon-soaked seeds were sown at a depth of 1.5 cm in plastic pots containing 4 kg clay soil. All pots were irrigated with tap water around field capacity (FC) until the appearance of two true leaves (10 days) then each group was subdivided into four treatments; pots irrigated around field capacity (100% FC) and decreasing water availabilities applied water deficit to 70% and 50% and 30% FC. Four pots/treatments were conducted. The pots were weighed daily and watered to restore the appropriate moisture by adding the calculated amount of water. All groups were allowed to grow for a further five weeks when they were harvested, and the following measurements have been done.

2.2. Plant growth parameters

Root and shoot lengths were measured and expressed in cm. Subsequently, the fresh weight of seedlings was recorded then dried at 80 °C for 48 °C for dry weight determination.

2.3. Biochemical Analysis

2.3.1. Photosynthetic pigments

Chlorophyll a, b and carotenoids were elicited from fresh leaves suspended at 5 ml ethyl alcohol (95%) and heated in the water bath (60-70 °C). Absorbance readings were followed with a spectrophotometer (Unico UV-2100 spectrophotometer) at wavelengths 663, 644, and 452 nm using equations.

2.3.2. Reactive oxygen species

Hydrogen peroxide (H_2O_2) of leaves was calculated based on the published methods [11]. Superoxide anion ($O_2^{\cdot-}$): $O_2^{\cdot-}$ content in leaves was done applying the method of Elstner and Heupel [12] by monitoring the nitrite formation from hydroxylamine at wavelength 530 nm. Hydroxyl radical ($^{\cdot}OH$): The protocol of Halliwell et al. [13] was used to calculate $^{\cdot}OH$ in plant leaves suspended in a mixture of phosphate buffer and 2-deoxy-d-ribose at 37 °C for 2 hr. The absorbance of malondialdehyde was recorded at 532 nm, and the concentration was calculated using the extinction coefficient 155,000 mM^{-1} cm^{-1} and expressed as µmol g FW^{-1}.

2.3.3. Oxidative stress markers

Electrolyte leakage (EL) was estimated as given by Premachuandra et al. [14] with some modifications on fresh leaf discs and the electrical conductivity measured using the conductometer (YSI Model 35 Yellow Springs, OH, USA). Lipid peroxidation was detected in leaves, applying Madhava and Sresty [15] with some modifications.

2.3.4. Non- enzymatic antioxidants

Ascorbic acid (ASA) and reduced glutathione (GSH): 0.5 g fresh leaves were mixed in 5% trichloroacetic acid, centrifuged at 11,500 × *g* for 15 min at 4 °C, and the supernatant was utilized for quantification of ASA and GSH by protocols of Jagota and Dani [16], respectively. Phenolic compounds were calculated on the methanolic extract using Folin-Ciocalteu reagent, and the data expressed as mg/gm FW using Gallic acid as a standard curve [10]. Total flavonoid was measured with methanolic extract of fresh leaves using $AlCl_3.6H_2O$, and the absorbance was measured at 510 nm [10]. Quercetin was used as a standard curve, and the data expressed as mg/g FW. Anthocyanin content was determined according to Islam et al. [10].

2.3.5. Metabolites

Proline content was elicited according to Bates et al. [17] with a spectrophotometer (Unico UV-2100 spectrophotometer) at wavelengths 520 nm. The proline concentration was determined from a standard curve and calculated on a fresh weight basis as follows: [(µg proline/ml × ml toluene) / 115.5 µg/µmole] / [(g sample)/5] = moles proline / g of fresh weight material.

2.3.6. Enzymatic antioxidants

Leaves were homogenized in potassium phosphate buffer included ethylene diamine tetra acetic acid and polyvinylpyrrolidone, centrifuged at 11,500 × g for 30 min. The supernatant was screened as an enzyme extract of superoxide dismutase, catalase, ascorbate peroxidase, and guaiacol peroxidase. The protein content of the supernatant was evaluated by Lowry et al. [18] method. Superoxide dismutase (SOD/EC 1.15.1.1) activity was quantified by following the autoxidation of epinephrine as mentioned by Misra and Fridovich [19] in a medium containing sodium carbonate buffer (pH 10.2), EDTA, enzyme extract, and epinephrine. The change in absorbance was monitored at 480 nm for 1 min. Catalase (CAT/EC 1.11.1.6) activity was detected by monitoring the consumption of H_2O_2 for 1 min [20], and the decrease in absorbance was determined at 240 nm. Ascorbate peroxidase (APX/EC 1.11.1.11) activity was screened by monitoring the ascorbate oxidation at 290 nm using an extinction coefficient of 2.8 mM^{-1} cm^{-1} in the presence of ascorbate as substrate [21]. Guaiacol peroxidase (POD/ EC 1.11.1.7) activity was measured spectrophotometrically by following the method of [22].

2.4. Statistical analysis

Means of three replicates of each trait were compared by Duncan's multiple range tests using one-way ANOVA (SPSS 18.0 software program), where the statistical significance was estimated at a 5% level.

3. Results and discussion

Nitric oxide has been soundly reported for the multiple physiological roles in plants [23]. Our investigation declared that the priming of faba bean with NO elicited the cultivar's tolerance for the non-stressed plants or water-deficit plants on growth characteristics in terms of lengths, fresh and dry mass accumulation of both organs shoot and root (Figure 1A, a-E, e) for Giza 843 and El-wadi 3, respectively. Interestingly, the effect of the priming agent was conducted up to crop yield. NO curtailed the reduction of crop yield of the drought-stressed plant (seeds yield/plant, (Figure 1F, f) and enhanced the yield of well-watered plants of Giza 843. Surprisingly, the seeds yield/plant of Elwadi 3 was not induced by NO-priming under deficit irrigation, while the interactive effect of NO and well-watered plants recorded a highly significant increment of seeds yield/plant. The drought tolerance of both cultivars was documented via yield rather than growth (dry and fresh weight), where Elwadi 3 was more tolerant than Giza 843. The percent reductions of crop yield of both cultivars were 74 and 44% from control at 30% FC for Giza 843 and Elwadi 3, respectively, while the total dry weight at the same level recorded reduction percentages of 49 and 47%, respectively. Root lengths reflected the status drought tolerance of both cultivars that Elwadi 3 preferred increasing the root length under drought stress higher than the control, but Giza 843 reduced the root lengths in responses to drought stress. This reduced root volume in dry soils reduced the availability of the nutrients, hence growth and yield reduction. Thus, NO increased root length and weight to the better mineral and water absorptivity, hence developing both plants. The differential intrinsic concentration of NO or its application led to improved resistance against abiotic stress, enhancing crop production under stress conditions [24,25].

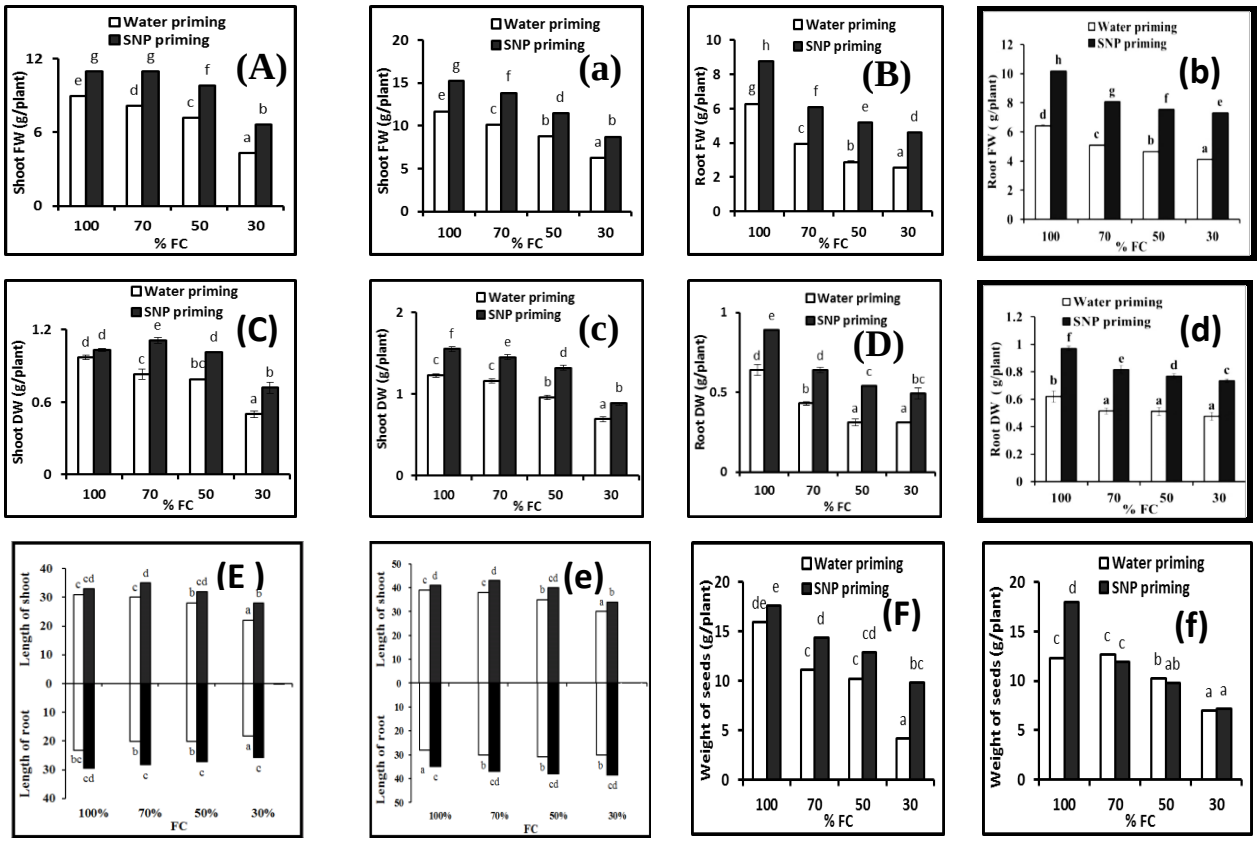

Figure 1. Shoot (A, a) as well as root (B, b) FW, DW of the shoot (C, c) as well as root (D, d), length (E, e), and seeds weight/plant (F, f) of water- and NO-primed plants (cv. Giza 843 and cv. El-wadi 3) grown under different levels of water stress. Each value represented the mean of three replicates. Different letters above each value were significantly different at $p<0.05$.

Several studies have established that NO is produced in various plant tissues, which has a crucial role in regulating critical physiological events of plants, such as photosynthesis [26]. In the present investigation, NO donor priming protected faba bean plants from the negative impact of drought on photosynthesis via enhancing Chla, Chlb, and carotenoids (Figure 2A, a-C, c) which have prime importance in plant photosynthesis system and providing the cells with the energy needed to maintain better growth under drought stress. Our results suggest that SNP could significantly increase the chlorophyll contents, similar to the findings [27]. In many plants, free proline accumulates in response to a wide range of biotic and abiotic stress. In the present investigation, water stress-induced proline biosynthesis (Figure 2D, d) was related to the susceptible cultivar and to a lesser extent for tolerant one, which only increased this metabolite at 30% FC. In this respect, the change of glutamate conversion to proline instead of chlorophyll might occur under such conditions because of a shift in plant priorities towards better survival [28]. This was concomitant with the sensitive cultivar, which reduced the chlorophyll a and b with high proline accumulation under drought stress, and vice versa was recorded for the tolerant cultivar. In this respect, Parida and Das [29] declared that although the high correlation between proline accumulation and drought-tolerance was extensively documented, such accumulation can be only a stress effect. This interpretation is recommended by the results mentioned above besides the results of both cultivars under the interactive effect of drought and NO-priming, where a reduction of proline was registered compared to the corresponding water level (Figure 2D, d).

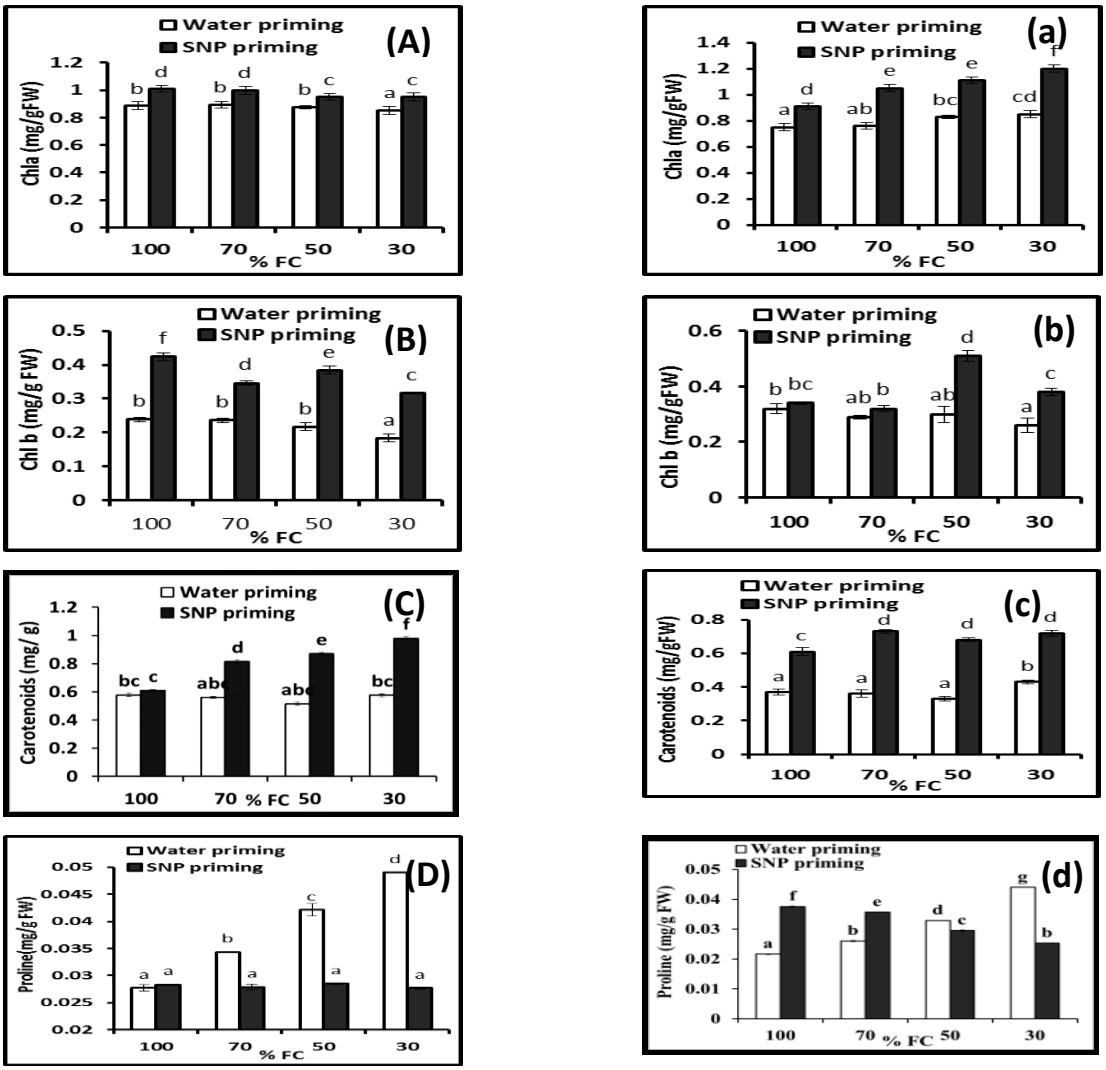

Figure 2. Chlorophyll a (A, a), chlorophyll b (B, b), carotenoids (C, c) and proline (D, d) of water- and NO-primed plants (cv. Giza 843 and cv. El-wadi 3) grown under different levels of water stress. Each value represented the mean of three replicates. Different letters above each value were significantly different at $p<0.05$.

Over-production of ROS including $O_2^{\cdot-}$, $OH^{\cdot}$ and H_2O_2 was the prime agent affecting the tested faba bean cultivars productivity and development (Figure 3, A-C, a-c) where oxidative burst was noticed for both of them, but much more so for the sensitive cultivar Giza 843 which prominently appeared at the highest water stress level. But for the plants primed with NO interact with various signaling components during ROS production to maintain steady ROS concentrations. Thus, the levels of hydrogen peroxide, superoxide anion, and hydroxyl radical were maintained lower than the corresponding level (Figure 3, A-C, a-c). NO mitigation of oxidative burst could be ascribed to promoting cell stability and elasticity by strengthening a phospholipid bilayer, improving the membrane's fluidity, and adjusting ROS [30]. Thus, in the present investigation ,the lowering of lipid peroxidation content (Figure 3E, e) was the result of NO priming to faba bean, thereby NO shields plant tissues from damage caused by the free radicles and ROS. Therefore, the increase in the lipid peroxidation and H_2O_2, $\cdot OH$ and O_2 levels lead to higher oxidative stress due to the disruption in the defense lines in faba bean plants grown under deficit irrigation. Zhang et al. [31] observed that MDA content was decreased in leaves of peanut grown on calcareous soils treated by NO. Dehydration has a significant effect on cell membrane modification, resulting in its dysfunction, which detected by increasing electrolyte leakage and can be used as an indicator of variation in drought stress tolerance besides MDA (i.e., the highest increasing values were

recorded at the level of 30% FC with an increasing percentage of 124% and 65% for lipid peroxidation as well as 30% and 76% for EC% of Giza 843 and El-wadi3, respectively). The used applicant was powerful agents in lessening EC% whatever the water levels tested in both cultivars, which was found to be lowered below that of absolute control in some cases (Figure 3D, d).

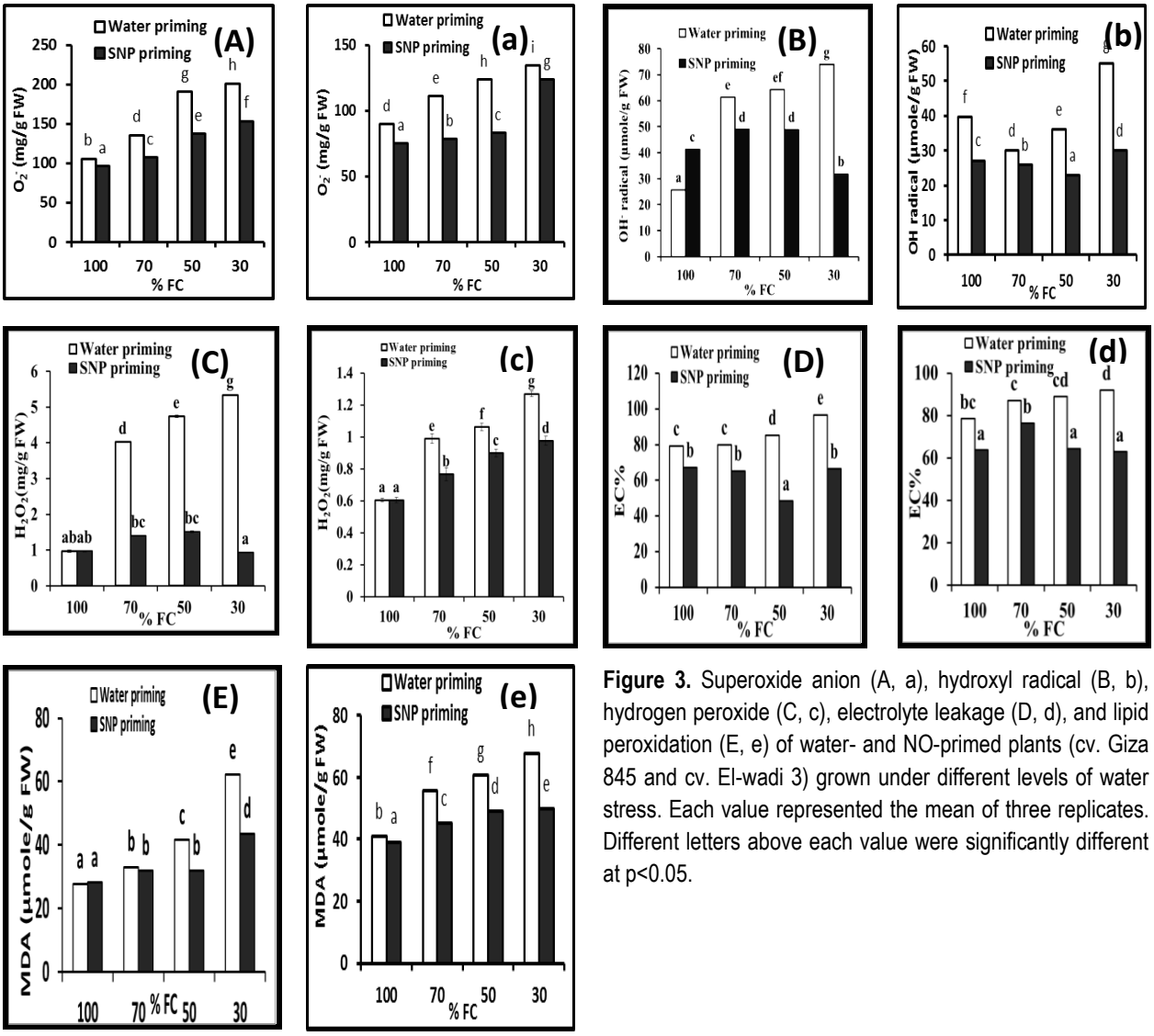

Figure 3. Superoxide anion (A, a), hydroxyl radical (B, b), hydrogen peroxide (C, c), electrolyte leakage (D, d), and lipid peroxidation (E, e) of water- and NO-primed plants (cv. Giza 845 and cv. El-wadi 3) grown under different levels of water stress. Each value represented the mean of three replicates. Different letters above each value were significantly different at $p<0.05$.

As NO could act as an effective antioxidant or potent oxidant mainly depended on its concentration [32]. In this regard, the data represented in Figure 4 have shown that NO priming can stimulate a series of antioxidant metabolites that increased the treated plants' antioxidant activity. The activation of SOD, CAT, and APX under the interactive effect of drought and NO priming was evident in the enhancement of superoxide anion- as well as hydrogen peroxide-metabolizing enzymes. Thus, the tested dose of NO had an efficient free radical quenching system, which maintained low ROS, thus higher membrane stability and lowered lipid peroxidation under both control and drought-treated plants. Conversely, guaiacol peroxidase was significantly reduced under drought stress for both cultivars, but much more so for the tolerant one with no effect of NO-priming on its activity. Thus, the criteria of guaiacol peroxidase revealed its low contribution towards ROS quenching. In this respect, we recommended the opinion of Zhang and Kirkharn [33] that the role of guaiacol peroxidase in detoxifying H_2O_2 is minor and not adequate compared to other peroxidases.

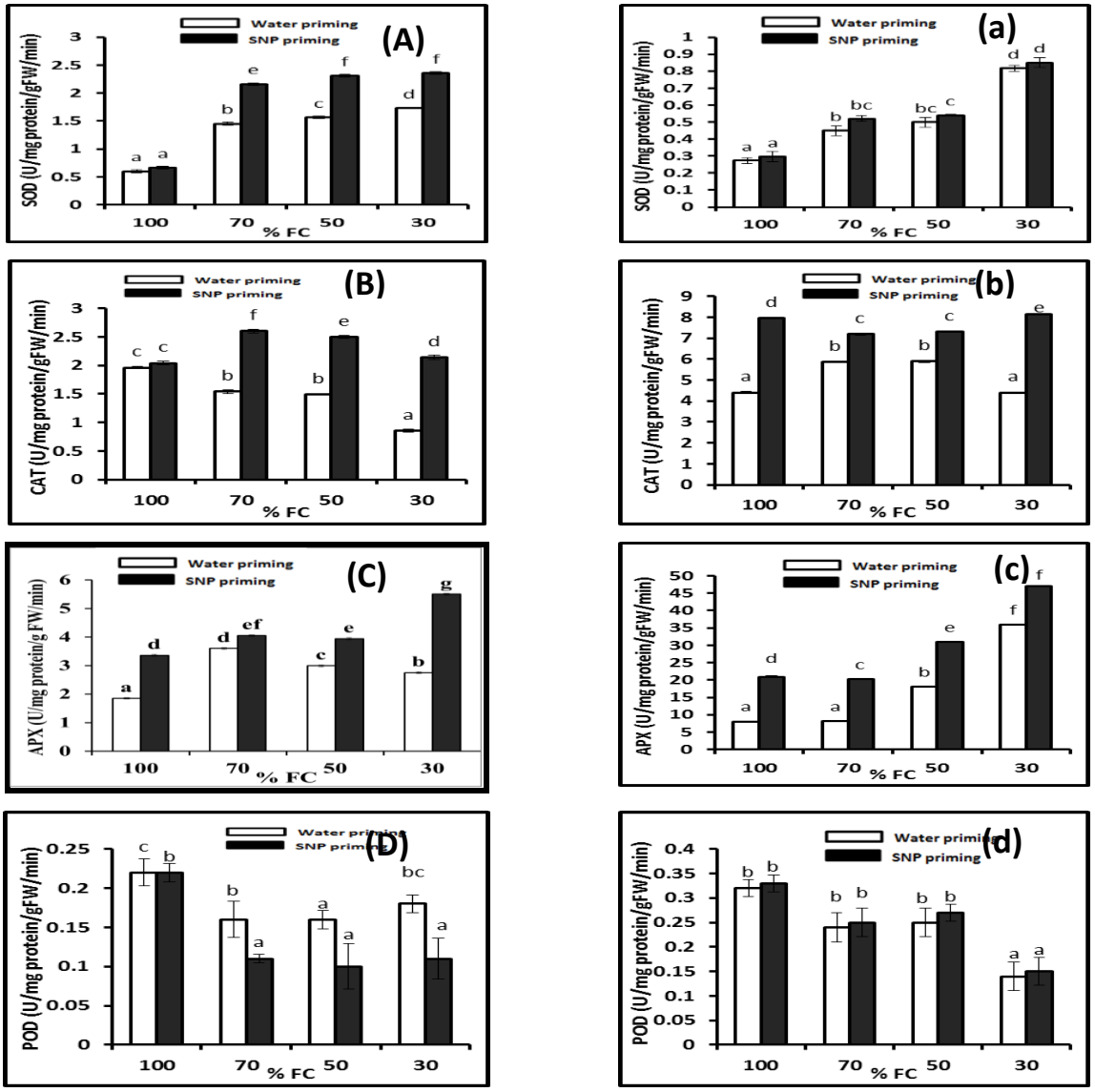

Figure 4. Superoxide dismutase, SOD, (A, a), catalase, CAT, (B, b), ascorbate peroxidase, APX, (C, c), and guiacol peroxidase, POD, (D, d) of water- and NO-primed plants (cv. Giza 845 and cv. El-wadi 3) grown under different levels of water stress. Each value represented the mean of three replicates. Different letters above each value were significantly different at $p<0.05$.

The phenylpropanoid pathway includes various structural and defense compounds, so it is an excellent plant secondary metabolite production as phenolic acid, anthocyanin, and flavonoids [34]. Anthocyanins increased by both cultivars under drought stress, and NO priming reported further exacerbation. The tolerant cultivar mainly increased phenolics and flavonoids under drought stress. At the same time, the sensitive one reduced both secondary defending metabolites at the same levels, which could be due to leaf senescence and the degradation of photosynthetic pigments under drought stress similar to that reported by Ali et al. [35]. NO priming mediated drought tolerance of both cultivars by further producing phenolics that could positively reduce ROS, hence membrane integrity (Figure 5). Phenolic compounds having antioxidant properties can prevent a series of oxidative stresses [36,37].

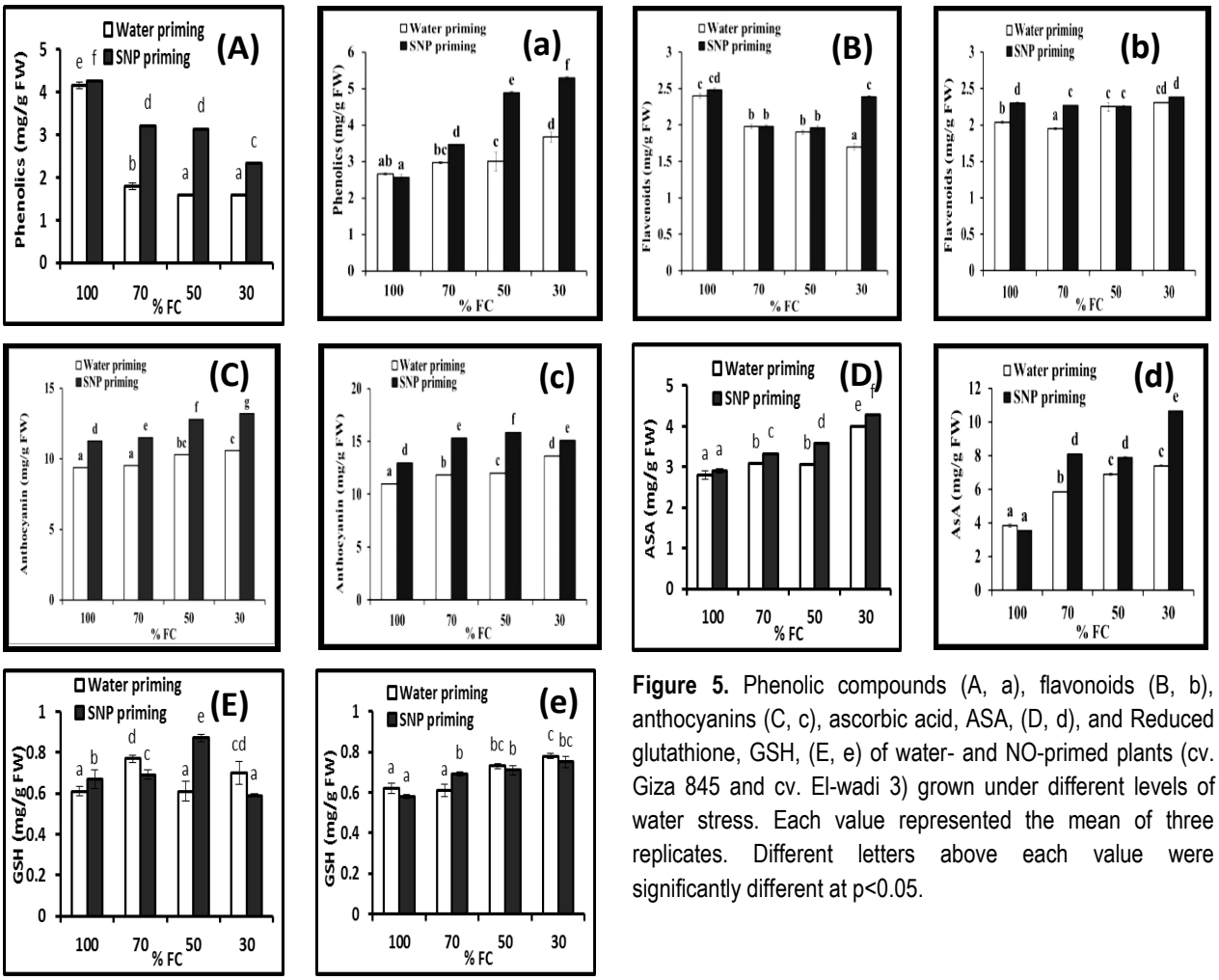

Figure 5. Phenolic compounds (A, a), flavonoids (B, b), anthocyanins (C, c), ascorbic acid, ASA, (D, d), and Reduced glutathione, GSH, (E, e) of water- and NO-primed plants (cv. Giza 845 and cv. El-wadi 3) grown under different levels of water stress. Each value represented the mean of three replicates. Different letters above each value were significantly different at $p<0.05$.

The reduction of oxidative stress by NO priming was linked with the exacerbation of ASA, which protects protein and lipids in plants, and NO priming to sensitive plants have higher ascorbic acid levels and ascorbic acid utilizing antioxidant enzyme activity, especially for the tolerant cultivar. This could be given an account of the high promotion capacity of NO on reducing drought tolerance to the sensitive cultivar. The accumulation of ascorbic acid as a non-enzymatic antioxidant conserves protein and lipids against oxidative stress in plants. The tolerant varieties have higher ascorbic acid levels ascorbic acid utilizing antioxidant enzyme activity than stress-sensitive ones [38]. The increasing trend of the ascorbic acid content of wheat seedlings under drought stress was reported by [39]. Exogenous application of SNP conferred tolerance to drought-stressed hull-less barley seedlings by activating antioxidants and ROS scavenging enzyme activity [30]. NO also interacts with plant hormones and various other signaling molecules and regulates osmoprotectants that protect against drought stress. Hasanuzzaman and Fujita [40] demonstrated that exogenous NO was increased the RWC, Chl and Pro, ASA, and GSH contents and increased antioxidant enzyme levels in wheat seedling under arsenic-induced oxidative stress. NO-responsive drought-related genes include transcription factors, promoters, and antioxidant-related genes [41].

The sensitive cultivar exhibited limited oxidative damage capacity where reduced glutathione kept around the control values, while the tolerant one enhanced reduced glutathione by drought stress (Figure 5). These collective data revealed that El-wadi 3 possessed efficient non-enzymatic antioxidants to limit the production of H_2O_2 actively, $\cdot OH$ and $O_2^{\cdot-}$ compared to the poorly induced non-enzymatic system of Giza 843. The antioxidant characteristics of the NO-regulatory mechanism appeared via exacerbates the accumulation of the reduced glutathione. ASA and GSH aid mitigate the toxicity of peroxynitrite in plants during their synthesis by triggering the reaction of NO with $O_2^{\cdot-}$. Both antioxidants preserve the reduced state by providing an electron to

free radicals to neutralize them. Accordingly, ASA and GSH confer tolerance and promote cellular protection against the water deficit-induced ROS and lipid peroxidation. NO can react with the reduced glutathione forming S-nitrosylated glutathione, which is considered a reservoir for NO that provides NO signal for the nitrosylation of proteins. GSNO reductase metabolizes GSNO to glutathione disulfide and ammonia [42]. Thus, the NO-regulatory mechanism was documented for the studied cultivars via conferring tolerance against drought stress, especially for Giza 843, where the induced resistance mechanism reflected increment of the yield of plants compared to El-wadi 3. Thus, the NO-regulatory mechanism of El-wadi 3 was reflected in the growth of plants rather than yield production.

Conclusion

Antioxidant properties of NO priming ascertained from their ability to exacerbate enzymatic antioxidants like SOD, APX, and CAT in addition to non-enzymatic ones as GSH and ASA as well as secondary metabolites, include phenolic acid, anthocyanin, and flavonoids. These up-regulatory mechanisms enhanced the quenching capacity of different recorded ROS, thus maintained membrane systems better than stressed plants. All these potential roles of NO priming greatly reflected on alleviation drought consequences of the two tested faba bean plants differed in their drought tolerance.

Conflicts of interest. There is no conflict of interest.

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