Stigmasterol alleviates the impacts of drought in flax and improves oil yield via modulating efficient antioxidant and ROS homeostasis

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Abstract

The present study aimed at alleviating the impacts of drought on flax growth, antioxidants, and ROS homeostasis by stigmasterol. Seeds were soaked in water or stigmasterol and sown in plastic pots. On the 24th day after sowing (DAS), water regime was applied and samples were harvested up to the 56th DAS for measuring growth parameters, free radicles, antioxidants, and POD and Rubisco quantification. At seed maturity, yield analysis measurements (capsules, seeds, oil yield, and fatty acid composition) were performed. Drought provoked significant decreases in growth parameters, ascorbic acid, and glutathione but elevated lipid peroxidation and H_2O_2 concurrently with significant inhibition in the activities of catalase, guaiacol peroxidase, ascorbic peroxidase, and glutathione reductase as well as activity and quantification of peroxidase and ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco). Yield analysis demonstrated decreases in capsule and seed numbers, the oil and fatty acid contents, and the fatty acid composition. Nevertheless, stigmasterol mitigated the drastic effects of drought on growth parameters, antioxidants, and Rubisco and rendered the contents of lipid peroxides and H_2O_2 as compared with the control. In the meantime, oil yield and fatty acid composition were improved in synchronization with the efficiency of antioxidants and ROS homeostasis. These findings conclude that drought resulted in a state of stress in flax; however, stigmasterol alleviated these drastic impacts and improved oil yield and fatty acid composition via modulating efficient antioxidant capacity and ROS homeostasis.

Keywords: fatty acid composition, free radicles, linseed, tolerance, water stress

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Introduction

Oil content and fatty acid composition are important attributes desirable in oil crops. Flax (*Linum usitatissimum* L.) is an important source of natural fibers and oil. Seeds are rich in polyunsaturated fatty acids, particularly alpha-linolenic acid, the essential omega-3 fatty acid and linoleic acid, and the essential omega-6 fatty acid. These polyunsaturated fatty acids are essential for good health of human. The oil quality is usually valued according to the content of essential fatty acids (Johnson and Bradford, 2014). These aspects

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are affected by the interactions among genetic, environmental, and agronomic factors.

Water stress is considered to be one of the most important environmental factors that limit plant growth and productivity. It affects biochemical and physiological processes in plants, causing growth inhibition and yield loss (Nayyar et al., 2006; Ghasemlou et al., 2019). Moreover, drought leads to overproduction of reactive oxygen species (ROS) and changes the activity and upregulation of antioxidant enzymes (Nemat Alla et al., 2014). ROS typically result from the excitation of O_2 to form $^1O_2$ or from the transfer of one, two, or three electrons to O_2 to form, respectively, O^{2-}, H_2O_2, or $HO^{\cdot}$ (Mittler, 2002; Nemat Alla et al., 2008). ROS cause cellular damage through oxidation of lipids, proteins, and nucleic acids.

Plants possess endogenous mechanisms to tolerate stresses; however, these mechanisms are not often enough (Perveen et al., 2018). Plants have non-enzymatic and enzymatic mechanisms to cope with the deleterious effects of ROS. The non-enzymatic antioxidants include ascorbate (AsA) and glutathione (GSH), two constituents of the antioxidative AsA–GSH cycle which detoxify H_2O_2 in the chloroplasts (Foyer et al., 2001). AsA is a major antioxidant acting as the natural substrate of peroxidases that function in the protection against oxidative damage of plant cells through the scavenging of H_2O_2 (Nemat Alla et al., 2008). GSH is a tripeptide synthesized in the cytosol and the chloroplast which scavenges $^1O_2$ and H_2O_2, and is oxidized to glutathione disulfide (GSSG) (Foyer et al., 2001). Moreover, several enzymes participate in ROS scavenging such as catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), glutathione reductase (GR), and peroxidase (POD) (Blokhina et al., 2003). CAT decomposes H_2O_2 into water and oxygen. GPX or APX oxidizes guaiacol or AsA, respectively with the degradation of H_2O_2. GR participates in the reduction of GSSG for GSH maintenance. On the other hand, Ribulose 1,5-bisphsphate carboxylase/ oxygenase (Rubisco) is a stromal protein effective in photosynthesis as carboxylase and in photorespiration as oxygenase. It is the most abundant protein on the earth (Feller et al., 2008). Stigmasterol alleviates drought impacts in flax and improves oil yield.

Water stress inhibits photochemical activities and decreases the activities of enzymes in photosynthesis causing a reduction in plant growth, development, and yield (Nayyar, et al., 2006; Hassan et al., 2008). A net degradation of Rubisco can be observed during endogenously initiated leaf senescence as well as during or after abiotic stress phases (Thoenen et al., 2007). Not only the velocity of Rubisco degradation, but also the mechanisms involved may depend on the environmental conditions (Feller et al., 2008).

Stigmasterol is a structural component of the lipid core of cell membranes and is the precursor of numerous secondary metabolites, including plant steroids. Sterols play an important role in plant development including cell expansion, vascular differentiation, etiolation, and reproductive development. It is involved in the regulatory function of plant development, gene expression involved in cell expansion and cell division, vascular differentiation, and other diverse developmental programs (Rao et al., 2002*).* So, the objective of the present work was to alleviate the impacts of drought imposed by withholding water on the growth of flax plants, antioxidants, and ROS homeostasis through soaking seeds with stigmasterol for elevation of plant tolerance to drought and improving the oil yield quality and quantity.

Materials and Methods

Plant materials and growth conditions

Flax (*Linum usitatissimum* L., cultivar Giza 10) seeds were surface sterilized by 3% sodium hypochlorite for 10 min, thoroughly washed, and divided into three groups: one was soaked in tap water to act as control, another group was soaked in tap water to be subjected to water stress, and the third group was soaked in stigmasterol solution (100 ppm) to be subjected to water stress. The seeds were spread on a filter paper overnight for air-drying and then germinated in clay/sand soil (2:1, v/v) in plastic pots (40 cm diameter x 30 cm height). The pots were kept at 12 h photoperiod with 450-500 μmol $m^{-2}s^{-1}$ photosynthetic photon flux density, 75 - 80% relative humidity, and 22/12 ± 2 ℃ day/night regime. The pots were irrigated every 4 days until

emergence; then, the seedlings were thinned on the 18th day after sowing (DAS) to 20 seedlings per pot and full strength nutrient Ashton solution was applied once (600 ml pot^{-1}). After 6 days, on the 24th DAS, water regime was applied; the control pots (water-soaked seeds) were irrigated every week while the two other groups (water- or stigmasterol-soaked seeds) were irrigated every two weeks throughout the experimental period up to seed maturity (about 5 months). Seedling samples generated from water-soaked seeds and grown normally (control), water-soaked seeds, the seeds grown under water stress (drought), and stigmasterol-soaked seeds grown under water stress (drought + stigmasterol) were harvested just before the application of regime (24 DAS) and on the 34th, 45th, and 56th DAS. At harvest, shoots and roots were separated and used for growth parameters measurements, and their fresh weights were determined before they were dried at 80 ℃ for 2 days for determination of dry weight and water content. Shoots were collected and the young leaves were frozen immediately in liquid nitrogen and stored at -80 ℃ for subsequent analyses. Samples for yield analysis were collected at the end of the experiment (on the 150th DAS).

Determination of H_2O_2 and Lipid peroxides

H_2O_2 and lipid peroxides were extracted in trichloroacetic acid (TCA, 0.1%, w/v) and centrifuged at 12,000 ×*g* for 15 min at 4 ℃. The assay of H_2O_2 was performed in potassium phosphate buffer (10 mM, pH 7.0) containing 1 M KI and the absorbance was measured at 390 nm (Alexieva et al., 2001). Lipid peroxides were assayed as malondialdehyde (MDA) (Heath and Packer, 1968). The amount of MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-l}.

Determination of ascorbic acid (AsA) and reduced glutathione (GSH)

AsA was extracted in 62.5 mM phosphoric acid and centrifuged at 12000 ×*g* for 20 min then loaded onto an ion exclusion column (300 x 7.8 mm) and eluted with 4.5 mM H_2SO_4 at a flow rate of 0.5 ml min^{-1}. The elution of AsA was detected at 245 nm (Ahn et al., 999). GSH was extracted in TCA (5%, w/v) containing 10 mM EDTA and centrifuged at 12000 ×*g* for 15 min. GSH was assayed in 100 mM phosphate buffer, pH 6.8 containing 10 mM EDTA, 1 mM 1-chloro-2,4-dinitrobenzene and 1.0 U equine Glutathione-S-transferase and incubated at 35 ℃ for 30 min. The absorbance at 340 nm was recorded before commencing the reaction and after the reaction had run to completion (Anderson and Gronwald, 1991).

Assay of antioxidant enzymes activity

Plant tissues were homogenized in sodium phosphate buffer (50 mM, pH 7.0) containing 2 mM EDTA and 5 mM β-mercaptoethanol and centrifuged at 12,000 ×g at 4 ℃ for 10 min. The activity of catalase (CAT) was measured by following the consumption of H_2O_2 at 240 nm in sodium phosphate buffer (50 mM, pH 7.0) containing 10 mM H_2O_2 and the protein extract (Aebi, 1984). Guaiacol peroxidase (GPX) activity was measured in sodium phosphate buffer (50 mM, pH 6.9) containing 3.2 mM guaiacol, 0.4 mM H_2O_2 and protein extract, and the mixture was allowed to stand for 3 min then absorbance was measured at 470 nm (Chance and Maehly, 1955). Ascorbate peroxidase (APX) activity was determined using the spectrophotometric method where the rate of decrease in the absorbance of ascorbate during its oxidation was measured at 290 nm (Nakano and Asada, 1981). Glutathione reductase (GR) activity was measured by following the oxidation of NADPH at 340 nm for 3 min using extinction coefficient of 6.22 mM^{-1} cm^{-1} (Schaedle and Basshan, 1977).

Native-PAGE quantification of peroxidase (POD) and Rubisco

The proteins of POD were resolved on 7% native acrylamide gels as described by Laemmli (1970) without sodium dodecyl sulfate in all solutions. Fifteen µg proteins were loaded onto each lane then resolved using the Bio-Rad Mini protean 3 units (BioRad Laboratories Inc, Hercules, CA). The gels were run at 80 V for 90 min at 4 ℃ and then were washed with distilled water. POD isoforms were detected by incubating the gels for 45 min in staining solution containing 2 mM diaminobenzidine, 50 mM acetate buffer (pH 5.0) and 0.03% (v/v) H_2O_2. When the bands were clearly visible, the gels were washed with distilled water (Seevers et al., 1971). Rubisco was extracted

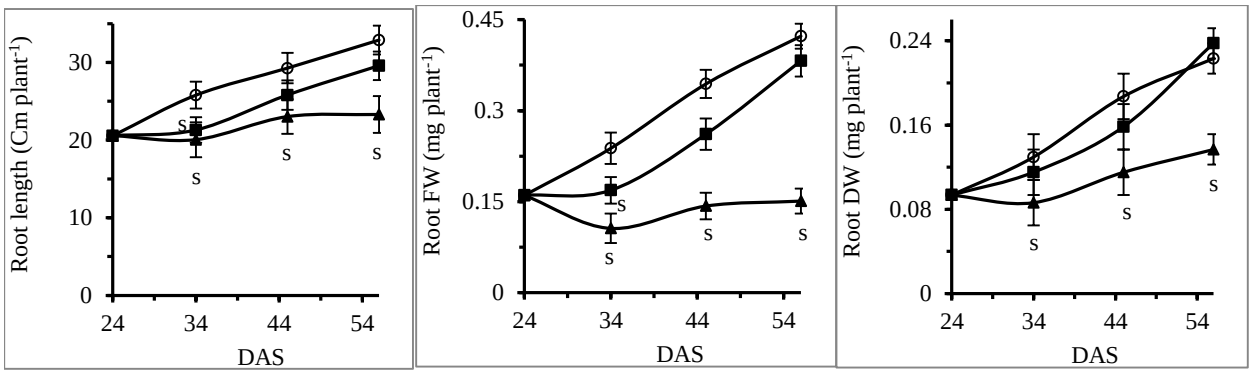

Fig. I. Influence of soaking flax seeds in 100 ppm stigmasterol on the effects of drought imposed by withholding water up to the 56th day after sowing (DAS) in terms of growth parameters of 24-day-old flax seedlings; values are means ±S D (n = 6). One-way ANOVA-LSD was performed at P ≤ 0.05. The letter 's' means significant difference from the untreated control.

in 50 mM sodium phosphate buffer containing 500 mM Polyphenylmethylsulphonylfluride, 2 mM EDTA and 5 mM β-mercaptoethanol and centrifuged at 11800 ×g for 15 min at 4 ℃. The proteins were resolved and the concentrations of acrylamide in the resolving and stacking gel were 11% and 5%, respectively. Protein (20 µg) was loaded onto each lane and resolved at 100 V for 90 min. The gels were stained with 0.25% Brilliant Blue R250 in 40% methanol and 10% acetic then de-stained with methanol and acetic acid. The gels were dried then scanned, and the band of the large subunit of Rubisco was detected at 52.7 KD. Rubisco was quantified by measuring the band volumes using Image Studio software v. 3.1.

Yield measurements

After seed maturity, on the 150th DAS, the numbers of capsules per plant and the numbers of seeds per capsule were counted. Oil of the yielded seeds was extracted by grinding flax seeds with *n*-hexane (60 - 80 ℃) as a solvent using a mortar and pestle as described in the AOAC (1990). For fatty acid composition, the seed oil was converted into fatty acid methyl ester (FAME) for gas chromatography. As described in Danish and Nizami (2019), around 0.1 g of oil was taken into 40 ml glass vial then mixed with 5 ml of 0.5 N methanolic NaOH. The mixture was heated for 3 min at 60 ℃ then was allowed to cool. Thereafter, 6 ml of 14% BF3 solution was added to the mixture and again heated for 3 mins at 60 ℃. The mixture was again cooled, and 10 ml isooctane were added and agitated well before it was kept to settle down. After settling the mixture, the upper layer was transferred to a tube containing sodium sulfate to remove the moisture. The fatty acid composition of the oil was determined by injecting an aliquot of FAME onto GC-FID. The quantity and identification of fatty acid in the oil was calculated through the comparison of standard components. Gas chromatography instrument (Device Model HP Hewlett Packard, 6890 GC) was equipped with Flame Ionization Detector, oven and injector temperatures 220 and 240 ℃, respectively, oven program initial temp 140 ℃ for 5 min with a rate of 140 ℃ min^{-1}, N carrier gas at flow of 1 ml min^{-1}, 30 m DB-23 Column (50 % Cyanopropyl methylpolysiloxane), 0.32 mm ID, 0.25 µm film thickness, 10 ml syringe size, and 1 ml injection volume.

Statistical Analysis

The experiment was repeated twice and designed as a complete randomized block consisting of 60 pots (3 set treatments) x (10 replications) x (2 repetitions). The mean values (±SD) were applied (n=6). One-way ANOVA-LSD was performed using SPSS 18.0.

Results

Flax seeds were soaked in water or Stigmasterol solution (100 ppm) for 12 h and then sown in plastic pots. Twenty-four days after sowing (DAS), water regime was applied and samples were harvested up to the 56th DAS and plants were left for yield analysis on the 150th DAS. Results showed that drought provoked significant decreases in shoot height and root length as well as fresh weight of both shoots and roots relative to control values up to the end of the experiment (Fig. I). The

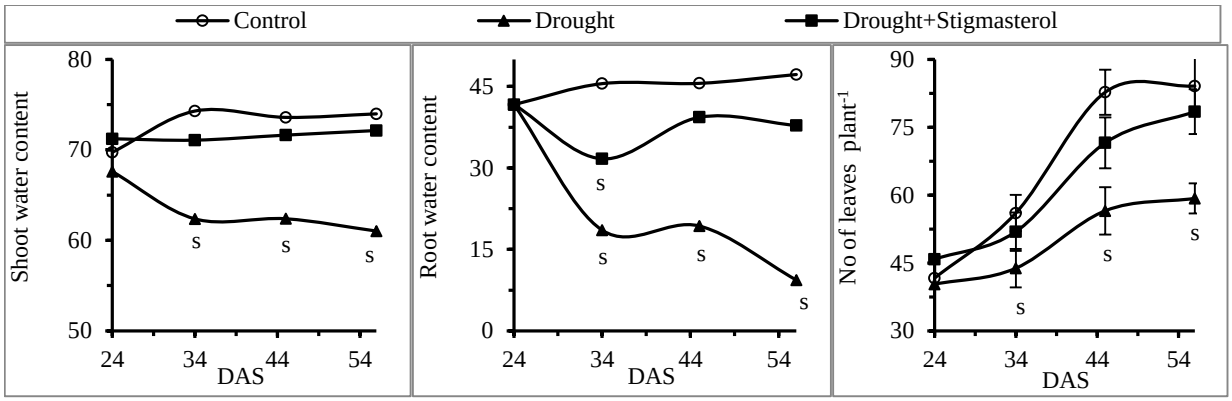

Fig. II. Influence of soaking flax seeds in 100 ppm stigmasterol on the effects of drought imposed by withholding water up to the 56th day after sowing (DAS) in terms of the number of leaves and water content of 24-day-old flax seedlings; values are means ± SD (n = 6). One-way ANOVA-LSD was performed at P≤0.05. The letter 's' means significant difference from the untreated control.

decreases augmented with the elapse of time. Meanwhile, dry weight of shoot seemed to be unaffected by drought; however, decreases were detected in root dry weight. Nonetheless, seed priming with stigmasterol greatly counterbalanced the drought-induced reductions in growth parameters, the effects of drought were highly retracted reaching mostly to control values. The retraction was more detected in shoot height and in root fresh and dry weights than in root length and shoot fresh weight.

In the same pattern, water content of shoot and root significantly dropped; meanwhile, the number of leaves was reduced by drought relative to control (Fig. II). Nonetheless, presoaking of seeds in stigmasterol counterbalanced the drop in water content and withdrew the reduction in leaf number.

On the other hand, drought significantly enhanced H_2O_2 accumulation in flax all over the experimental period as compared to control values, and the magnitude of accumulation was high on the 34th DAS and became steady thereafter (Fig. III). Also, MDA was significantly accumulated in response to drought and increased with the elapse of time. On the contrary, drought led to significant decreases in AsA and GSH contents, the decrease was higher in GSH than in AsA. However, seed pretreatment with stigmasterol highly suppressed the accumulation of H_2O_2 and MDA during the whole experimental period leading to comparable values with control. Moreover, elevations of the contents of AsA and GSH were induced by stigmasterol to reach mostly those of the control.

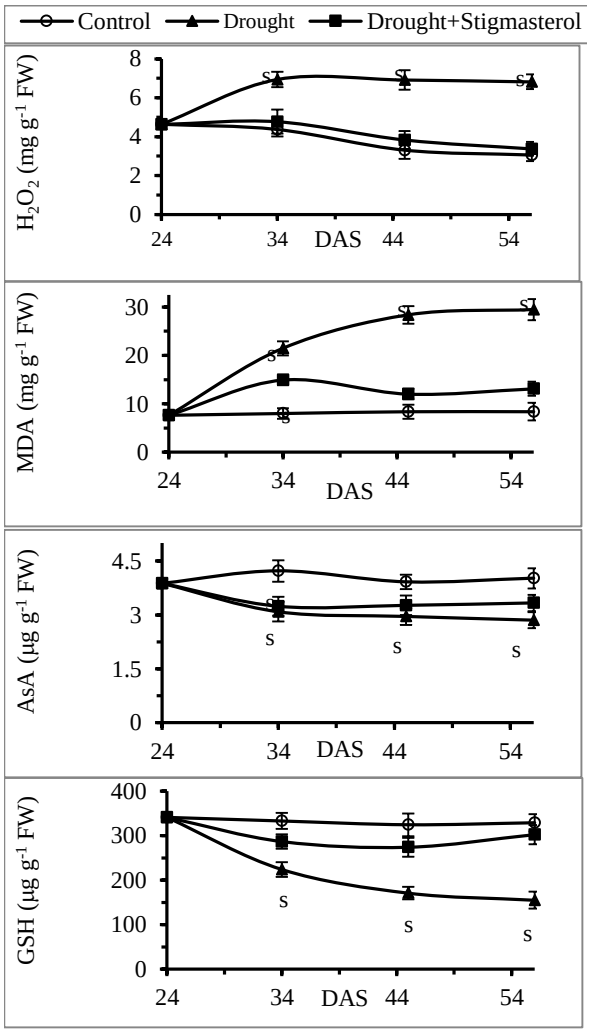

Fig. III. Influence of soaking flax seeds in 100 ppm stigmasterol on the effects of drought imposed by withholding water up to the 56th day after sowing (DAS) in terms of the contents of H_2O_2, malondialdehyde (MDA), ascorbic acid (AsA), and reduced glutathione (GSH) of shoots of 24-day-old flax seedlings; values are means ± SD (n = 6). One–way ANOVA-LSD was performed at P≤0.05. The letter 's' means significant difference from the untreated control.

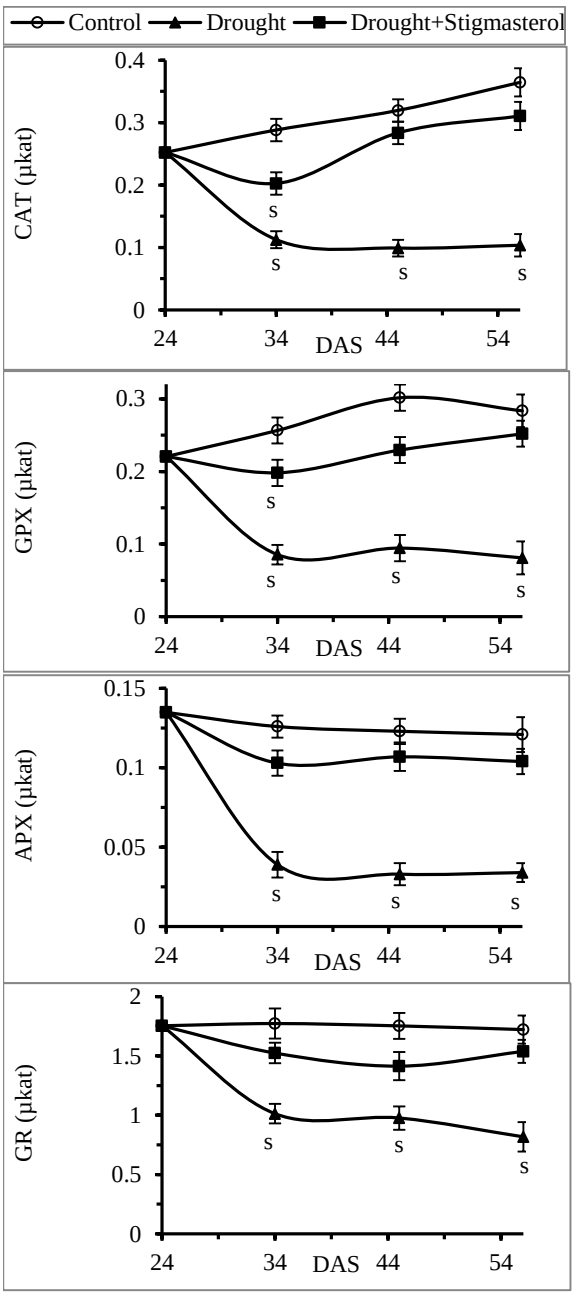

Fig. IV. Influence of soaking flax seeds in 100 ppm stigmasterol on the effects of drought imposed by withholding water up to the 56th day after sowing (DAS) in terms of the activities of catalase (CAT), guaiacol peroxidase (GPX), ascorbic peroxidase (APX), and glutathione reductase (GR) of shoots of 24-day-old flax seedlings; values are means ± SD (n = 6). One-way ANOVA-LSD was performed at P≤0.05. The letter 's' means significant difference from the untreated control.

As depicted in Fig. (IV), the activities of CAT, GPX, APX, and GR were significantly inhibited as a result of drought stress in relation to control values. The inhibitions were sharp during the first 10 days of stress and continued consistent thereafter. As a whole, soaking of seeds with stigmasterol caused

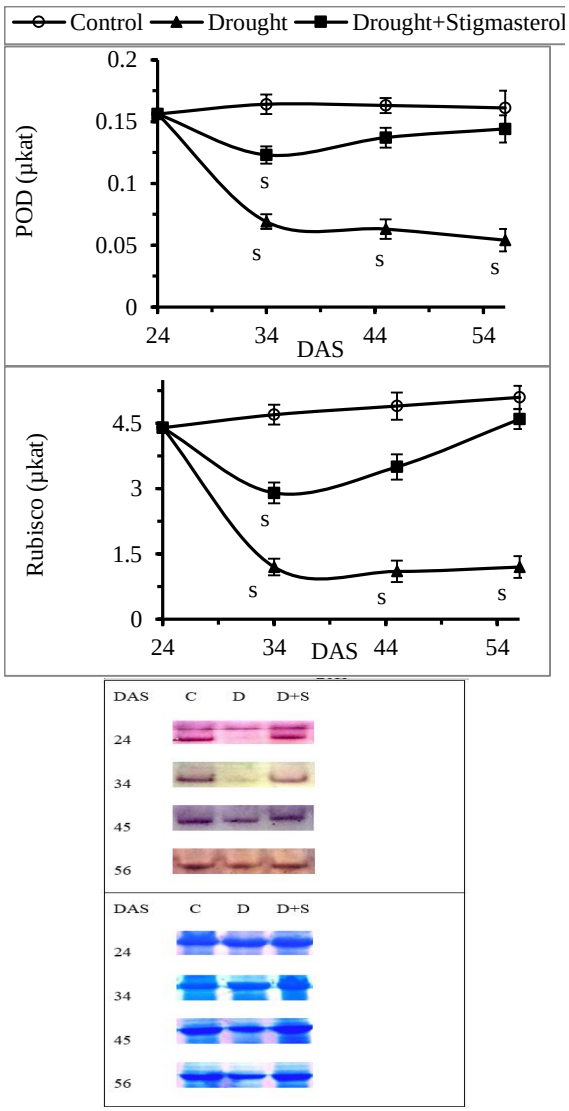

Fig. V. Influence of soaking flax seeds in 100 ppm stigmasterol on the effects of drought imposed by withholding water up to the 56th day after sowing (DAS) in terms of the activities and Native-PAGE of peroxidase (POD) and ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) of 24-day-old flax seedlings; values are means ± SD (n = 6). One-way ANOVA-LSD was performed at P≤0.05. The letter 's' means significant difference from the untreated control. Native-PAGE indicated two isozymes for POD (37 and 40 kD) and one band for Rubisco (52.7 kD).

significant increases in the enzyme activities comparing to the stressed plants. These increases rendered the activity values very close to those of the respective control on the 56th DAS.

Also, POD and Rubisco were highly inhibited in the drought-stressed samples as compared to stressed plants; the effect was great from the onset of treatment and continued consistent up to

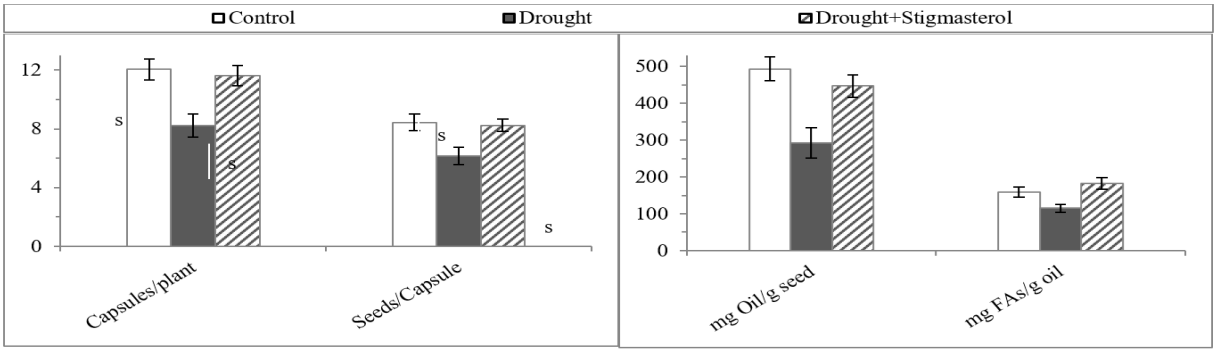

Fig. VI. Influence of soaking flax seeds in 100 ppm stigmasterol on the effects of drought imposed by withholding water in terms of yield (the numbers of capsules and seeds and also the contents of oil and fatty acids of the yielded plants); values are means ± SD (n = 6). One-way ANOVA-LSD was performed at P≤0.05. The letter 's' means significant difference from the untreated control.

the end of the experiment (Fig. V). Nonetheless, soaking of seed with stigmasterol greatly relieved the activities of POD and Rubisco. The relief was greater and faster for POD than Rubisco. Native-PAGE of POD indicates the detection of two isozymes in all samples, the density of isozyme bands was less in response to drought relative to control during all intervals of the experiment; however, stigmasterol resulted in denser bands. Similarly, a large band was detected as Rubisco, and the detected band was denser in control than in drought-stressed samples; nonetheless, the density of Rubisco band in samples derived from seeds soaked in stigmasterol became similar to that of control.

Yield analysis indicates that plants subjected to water stress produced less numbers of capsules and also less number of seeds in capsules than the yield of the normally grown control plants (Fig. VI). However, the capsule and seed numbers of plants derived from the seeds soaked in stigmasterol were very close to those of control. It is also clear that drought significantly decreased oil content of seeds as well as the percentages of fatty acids in the oil. On the other hand, the yielded seeds of plants derived from seeds soaked in stigmasterol produced more oil than the yield of the stressed plants to reach nearly the control values. In addition, the percentages of fatty acids of the yielded seeds increased and even became higher than control.

The composition of fatty acids of the yielded seeds is depicted in Table 1. As a whole, palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2), and α-linolenic acid (C18:3α) comprised the core bulk of the fatty acids in control samples, and α-linolenic acid was highest acid detected followed by oleic and linoleic acid whist palmitic and stearic acid contents were the lowest. As a result of drought treatment, α-linolenic, oleic, and linoleic acid showed some losses whereas palmitic and stearic acids increased. Moreover, drought resulted in disappearance of some fatty acids namely, heptadecenoic (17:1), eicosatrienoic (C20:3ω6), elaidic (C18:1), linolelaidic (C18:2), eicosatrienoic (C20:3), docasadienoic (C22:2), and docosahexaenoic acid (C22:6). On the contrary, behenic (C22:0) and lignoceric (C24:0) acids were generated by drought. Nonetheless, plants generated from seeds soaked in stigmasterol showed elevated levels of α-linolenic, oleic, and linoleic acid relative to the stressed plants while palmitic and stearic acids decreased, and these levels became close to control. On the other hand, fatty acid composition of these plants exerted the same pattern of disappearance of heptadecenoic, elaidic, and linolelaidic acid as induced by drought whereas eicosatrienoic, docasadienoic, and docosahexaenoic acids continued as control. As a whole, water stress decreased the sum of omega-3, omega-6, omega-9, monounsaturated, polyunsaturated, and total unsaturated fatty acids. On the contrary, drought increased only the saturated fatty acids. These alterations were highly counterbalanced in plants generated from seeds soaked in stigmasterol so that increases were detected in omega-3, omega-6, and omega-9 as well as unsaturated fatty acids concomitant with retractions in the saturated fatty acids.

Table 1
Influence of soaking flax seeds in 100 ppm stigmasterol on the effects of drought imposed by withholding water in terms of the fatty acid composition of the yielded seeds' concentration (mg g^{-1} oil) and percentage

Fatty acids	Concentration			%		
	C	D	D+S	C	D	D+S
C14:0 Myristic	0.06	3.62	0.19	0.04	3.14	0.11
C16:0 Palmitic	8.51	18.36	15.64	5.35	15.95	8.58
C16:1 Palmitoleic	0.12	2.88	1.58	0.08	2.50	0.87
C17:0 Heptadecanoic	0.10	8.11	1.75	0.06	7.05	0.96
C17:1 cis-Heptadecenoic	0.07	--	--	0.04	--	--
C18:0 Stearic acid	8.67	9.90	13.40	5.45	8.60	7.35
C18:1 trans-9-Elaidic	0.11	--	--	0.07	--	--
C18:1 (ω9) Oleic	32.20	16.62	34.08	20.22	14.44	18.70
C18:2 trans-Linolelaidic	0.32	--	--	0.20	--	--
C18:2 (ω6) Linoleic	24.16	13.64	26.99	15.18	11.85	14.81
C18:3 (ω3) a-Linolenic	78.82	35.47	79.01	49.51	30.82	43.35
C18:3 (ω6) g-Linolenic	0.38	0.92	1.47	0.24	0.80	0.81
C20:0 Arachidic	0.30	0.86	1.46	0.19	0.75	0.80
C22:0 Behenic	--	1.86	0.46	--	1.62	0.25
C24:0 Lignoceric	--	2.86	1.46	--	2.48	0.80
C20:3 cis-11,14,17-Eicosatrienoic	2.86	--	1.92	1.80	--	1.05
C22:2 cis-13,16-Docasadienoic	1.16	--	1.32	0.73	--	0.73
C22:6 (ω3) cis-4,7,10,13,16,19-Docosahexaenoic	1.35	--	1.51	0.85	--	0.83
Sum of Omega-3 (n-3)	78.82	35.47	79.01	49.51	30.82	43.35
Sum of Omega-6 (n-6)	24.16	13.64	26.99	15.18	11.85	14.81
Sum of Omega-9 (n-9)	32.20	16.62	34.08	20.22	14.44	18.70
Saturated Fatty Acids	17.34	44.71	32.90	10.89	38.84	18.05
Monounsaturated Fatty Acids	32.50	19.50	35.66	20.42	16.94	19.57
Polyunsaturated fats Fatty Acids	109.05	50.04	112.23	68.50	43.47	61.58
Total Unsaturated Fatty Acids	141.56	69.54	147.89	88.92	60.42	81.15
Total Fatty Acids	159.2	115.1	182.2			

C, control; D, drought; D+S, drought+ stigmasterol.

Discussion

The results showing that drought markedly reduced growth parameters of flax might be due to decreases in cell elongation, cell volume, and eventually cell growth. Like other abiotic stresses, drought leads to a series of morphological, physiological, biochemical, and molecular changes that adversely affect plant growth and productivity. In this respect, Shao et al. (2008) indicated that the maintenance of cell turgor is essential for survival or assisting the plant growth under severe drought conditions. A positive relationship between CO_2 fixation and fresh weight generation was observed. However, seed priming with stigmasterol greatly counterbalanced the drought-induced reductions in growth parameters, and these values became very close to the control values. In this respect, He et al. (2003) indicated that stigmasterol plays a regulatory function in plant development. Sterols play an important role in plant development including cell expansion and vascular differentiation (Rao et al., 2003). Hartmann (2009) concluded that phytosterols act as biogenic precursors of compounds involved in plant growth.

When a plant is subjected to water stress, a range of reactive oxygen species (ROS) are overproduced during photosynthesis, photorespiration, and dark respiration (Taylor et al., 2003). The production of ROS in plants is part of the normal metabolism of chloroplasts, mitochondria, and peroxisomes; however, the exposure to environmental constraints can overwhelm the systems protecting the plants and result in oxidative stress (Berni et al., 2019).

Ghasemlou et al. (2019) indicated that drought stress causes severe metabolic dysfunctions by formation of oxidative stress. ROS typically result from the excitation of O_2 to form $^1O_2$ or from the transfer of one, two, or three electrons to O_2 to form O^{2-}, H_2O_2 or $HO^{\cdot}$, respectively (Mittler, 2002; Nemat Alla et al., 2008). They are toxic to plant cells and can be combined with vital molecules, such as fats, proteins, and nucleic acids, causing lipid peroxidation, protein denaturation, DNA mutation, and damage of cellular membranes and organelles (Quiles and Lopez, 2004). In the present results, H_2O_2 and MDA were greatly accumulated in response to drought. The increases in H_2O_2 and MDA are among the biochemical changes provoked by water shortage causing oxidative damage in stressed plants (Mittler, 2002). However, stigmasterol lowered the accumulation of H_2O_2 and MDA concomitant with the alleviation of drought effect on growth parameters concluding with an improvement in plant metabolism.

When enzymatic or non-enzymatic antioxidant defense of the cell is overwhelmed with excess production of ROS, it results in disruption of lipids via oxidation, which causes production of highly reactive lipid peroxidation-derived molecules. The increased H_2O_2 and MDA confirm the existence of a status of oxidative stress due to drought; however, plants possess endogenous mechanisms to cope with these states of stress. Antioxidants, either nonenzymatic such ascorbate (AsA) and glutathione (GSH), or enzymatic such as catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), glutathione reductase (GR), and peroxidase (POD) are efficient in ROS scavenging. AsA is one of the most powerful antioxidants. The ability to donate electrons makes AsA the main ROS-detoxifying compound. AsA can directly scavenge superoxide hydroxyl radicals and singlet oxygen and reduce H_2O_2 to H_2O via APX. In addition, GSH is a key component of antioxidant defenses in most aerobic organisms. It participates in ROS scavenging through AsA-GSH cycle in which H_2O_2 is reduced to water (Aravind and Passad, 2005). Therefore, the decreased AsA and GSH by drought could indicate the impact of water stress on the antioxidant system with a consequent retardation in the scavenging of ROS. The decreased GSH content might be because of its oxidation to oxidized glutathione (GSSG) (Nakano and Asada, 1981). Nonetheless, the nonenzymatic antioxidants were elevated in plants grown from seeds soaked in stigmasterol in coincidence with retraction in H_2O_2 and MDA concluding the repair of the antioxidant system by stigmasterol. So, stigmasterol could lead to improvement in the antioxidant apparatus for increasing ROS scavenging and also in drought tolerance.

On the other hand, the decrease of CAT activity might lead to accumulation of H_2O_2 to a toxic level. Under stress conditions, inactivation of CAT is linked to H_2O_2 accumulation. Indeed, Lee et al. (2001) concluded that stress preferentially enhances H_2O_2 content but decreases CAT activity. The decrease in CAT activity may be related to either photo inactivation of the enzyme or prevention of new enzyme synthesis (Jang, 2004). Not only H_2O_2 level is decreased through catalysis of CAT but also GPX and APX can do with a consequent increase in the stability of membranes and CO_2 fixation because high levels of H_2O_2 directly inhibit CO_2 fixation (Yamazaki et al., 2003). So the inhibited GPX and APX activities by drought would leave H_2O_2 to accumulate to toxic levels. On the other hand, presoaking of seeds with stigmasterol resulted in relief of the enzyme activities to be more efficient in scavenging of H_2O_2. This relief was coincided with the elevated nonenzymatic antioxidants and was concomitant with the retracted accumulation of ROS, concluding a repair in the antioxidant system. Moreover, drought caused a significant inhibition in GR activity confirming the drop in GSH content while the increased GR activity by stigmasterol was in concomitant with an elevation in GSH level. So, the increase in the enzymatic and nonenzymatic antioxidants in response to stigmasterol could be indicative of an efficient system for ROS homeostasis and a build-up of a protective mechanism to reduce the oxidative damage triggered by stress. Moreover, the antioxidant system can participate in plant tolerance to drought stress.

In addition, POD and Rubisco were highly inhibited in drought-stressed samples. Two

isozymes of POD and only one band of Rubisco were detected in all samples; however, their densities were lesser in response to drought than in control while stigmasterol rendered them to become close to control. This would indicate the increase in the concentration of POD and Rubisco in response to stigmasterol to improve antioxidant and photosynthesis, respectively. Rubisco is a stromal protein which catalyzes two competing reactions of photosynthetic, namely CO_2 fixation and photorespiration carbon oxidation. Degradation of Rubisco can be observed during or after abiotic stress phases (Thoenen et al., 2007; Feller et al., 2008). These findings support the degradation of POD and Rubisco by drought, which might occur in the other enzymes too, the effects that were counterbalanced by stigmasterol. This degradation would decrease the enzyme concentration with a consequent delay in the catalytic efficiency by drought; nonetheless, a relief was induced by stigmasterol. As a whole, the effects of drought on growth, oxidative stress indices, and antioxidants would consequently affect the plant productivity.

Yield analysis demonstrated that the number of capsules and the number of the yielded seeds were significantly decreased by water stress by about 32 and 27%, respectively; however, these decreases were retracted by stigmasterol to 4 and 2%, respectively. Also, drought dropped oil content and the percentages of total fatty acid composition by about 41 and 28%, respectively while stigmasterol led to slight decrease in oil content by only 9% and even increases were detected in total fatty acid composition by 15%. In fact, water stress during vegetative phase might reduce yield through restricted plant size, leaf area, and root growth which subsequently reduces the dry matter accumulation, number of pods per plant, and low harvest index. Thus, yield is a result of the integration of metabolic reactions in plants; consequently, any factor that influences this metabolic activity at any period of plant growth can affect the yield. So, the observed decrease in the yield components in the present work could be attributed to the reduction in growth in addition to reduction in antioxidant system which leaves the plant suffering from stress. Nonetheless, stigmasterol improved yield as did also for growth, ROS homeostasis, antioxidant, and Rubisco.

On the other hand, the impact of drought on fatty acid composition of the yielded oil was greatly counterbalanced for the yield of plants derived from seeds soaked in stigmasterol. The percentages of the bulk fatty acids in the oil (ω9 oleic, ω6 linoleic, and ω3 linolenic) decreased by drought by 48, 44, and 55%, respectively; however, stigmasterol augmented these percentages to become very close to the control values. In this respect, Danish and Nizami (2019) reported that the core bulk fatty acids in flaxseed oil are palmitic, stearic acid, ω9 oleic, ω6 linoleic, and ω3 linolenic acid comprised about 99% of seed oil; however, these fatty acids comprised about 96% of seed oil in the present study. Nevertheless, drought augmented the saturated fatty acids particularly palmitic and stearic acids by 116 and 14% whereas stigmasterol retracted these increases. On the other hand, Razavizadeh and Karami (2018) indicated that drought stress changed the essential-oil composition in shoots and calluses of *Carum copticum*. In addition, drought decreased monounsaturated and polyunsaturated fatty acids by about 40 and 54%, respectively; nonetheless, stigmasterol counterbalanced these decreases and even induced some rises. Also, ω3, ω6, and ω9, that declined in response to drought by 55, 44, and 48%, respectively were raised by stigmasterol. In the same pattern, monounsaturated and polyunsaturated fatty acids were decreased by drought by about 40 and 50%, respectively but became very close to control by stigmasterol. So, oil content correlated positively with the unsaturated ω3, ω6, and ω9, and negatively with the saturated palmitic and stearic acids. The present findings clearly declare that drought not only decreased oil content but also lowered its characteristics. Nevertheless, the use of stigmasterol improved - to a great extent - the quantity and quality of oil yield. In general, the quality of oil is valued according to the content of essential fatty acids (Johnson and Bradford, 2014); however, some oil quality characteristics suitable for different industrial uses could also be achieved by a combination of water stress with standard or oleic typology of plant hybrids.

Conclusion

Water stress decreased flax growth and lowered enzymatic and non-enzymatic antioxidants while the oxidative stress indices significantly elevated. Moreover, yield analysis demonstrated loses in number of capsules, number of seeds per capsules, yield oil content, and percentages of fatty acids content and altered fatty acid composition. These findings conclude that drought resulted in a state of stress in flax; nevertheless, soaking of seeds in stigmasterol mitigated these drastic effects and improved the quantity and quality of the oil yield. stigmasterol raised the antioxidants and lowered the oxidative stress indices indicating efficiency in antioxidant system and ROS homeostasis. Such efficiency was synchronized with the alleviation of the deleterious effects of drought and was concomitant with improvements in the production of oil yield and composition of fatty acids, concluding that stigmasterol alleviated the deleterious impacts of drought and improved oil yield through modulating the efficiency of antioxidant and ROS homeostasis.

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