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Ameliorating impact of exogenously applied of methanol and nano TiO_2 on antioxidant enzymes and seed oil of borage under water shortage

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Abstract

The aim of the present investigation was to determine ameliorating impact of exogenously applied of methanol and nano TiO_2 on antioxidant enzymes and seed oil of borage, under water shortage. Therefore, this study was conducted in 2012 as a split factorial on the basis complete randomized block design with four replications in the city of Shahriyar in Iran. Treatments consisted of no stress (once every 7 d) and deficit irrigation (once every 14 d) in the main plots; 0% (control; sprayed with water), 15%, 35% and 45% (v/v) concentrations of methanol aqueous solution; and 0, 0.01%, 0.03% and 0.05% titanium dioxide nanoparticle concentrations applied to the sub-plots. Deficit irrigation increased the activity of antioxidant enzymes, the oil percentage and MDA, but decreased protein content, and seed and oil yield. The binary interaction effects of deficit irrigation and methanol and the interaction of deficit irrigation and nano-TiO_2 were significant for all traits except oil percentage. Foliar application of methanol and nano TiO_2 sprays individually had the highest protein content and seed and oil yield, especially in the stress condition. The application of methanol and nano TiO_2 individually under deficit irrigation showed the highest activity for antioxidant enzymes and the least for MDA. The lowest antioxidant enzymes was achieved by 45% (v/v) methanol and no application of nano TiO_2 under no stress irrigation; however, this concentration produced the lowest seed and oil yield. The 45% v/v concentration of methanol treatment under deficit irrigation, performed best with higher oil yield, protein and antioxidant enzymes.

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Introduction

Borage (*Borago officinalis* Linn., family: Boraginaceae) is an annual herb (Tyler, 1993) contain 30-40% oil by weight, of which 23-24%, is a major commercial source of linolenic acid (GLA) (Ezzid-din and Hendawy, 2010). Drought stress limits the survival and growth of plants (Liu, 2009). It usually leads to oxidative stress from stomata closure (Ozkur *et al*., 2009), which causes a decrease in the photosynthetic electron chain (Bacelar *et al*., 2007; Ben-Ahmed *et al*., 2009) and high formation of reactive oxygen species (ROS) in the chloroplasts and mitochondria (Fu and Huang, 2001). ROS, including superoxide ($O2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical (HO^-), and singlet oxygen ($1O_2$), disrupts the normal metabolism of plants by oxidative damage to lipids, proteins, nucleic acids, photosynthetic pigments, and enzymes (Fu and Huang, 2001).

To overcome oxidative stress, plants have developed complex antioxidant systems that can include carotenoids, ascorbates, glutathione, and tocopherols. Enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), peroxidase (POD), glutathione peroxidase (GPx), and enzymes involved in the ascorbate-glutathione cycle, such as glutathione reductase, are examples of this (Baby and Jini, 2011). CAT and APX detoxify cellular hydrogen peroxide (Bowler *et al*., 1992). The acclimation of plants to drought promotes antioxidant defense systems to increased levels of ROS. This in turn causes membrane damage by lipid peroxidation, as indicated by malondialdehyde (MDA) content, which is a major parameter for evaluating membrane oxidation and is toxic to cells (Chaves *et al*., 2003; Shao *et al*., 2005).

Bailly *et al*., (2000) indicated that, increasing the CAT, SOD, and MDA content of the sunflower plant under drought stress. Jalilian *et al*., (2012) found that drought stress decreased the grain yield and total oil of the sunflower. Bannayan *et al*., (2008) found that, drought stress decreased seed yield of *Plantago ovata* and *Nigella sativa*; it significantly increased the MDA content of wheat cultivars (Wang *et al*., 2011) and cotton (Deeba *et al*., 2012). Methanol (MeOH) is a simple organic components that has recently proved to enhance biomass production of photosynthetic organisms. On the molecular level, it is smaller than the CO_2 molecule, so it is easily absorbed by plants and decomposes to CO_2, accelerating photosynthesis (Gout *et al*., 2000). It should be noted that, in plants faced with stress, foliar application of methanol, will prevent loss of biomass (Safarzadeh-Vishgahi *et al*., 2005). Only C_3 plants (those that produce ribulose 1,5-diphosphate and 3-phosphoglyceric acid during photosynthetic carboxylation) respond to methanol with increased biomass production, since CO_2 resulting from rapid oxidation of methanol can successfully compete with oxygen for rubisco (Zbieć *et al*., 2003). Plants that treated with methanol showed increased turgor, higher growth rates, and higher yields than the control plants.

Generally, methanol play a major role in preventing loss from plant-induced stress during photorespiration (Nonomura and. Benson., 1992a). In C_3 plants, periodic spraying of 10% to 50% methanol (v:v) were documented to increase biomass production up to 100% (Fall and Benson, 1996). If methanol, in fact, reduces water requirements, it may be a partial solution to increasing the water-use efficiency of crops (Nonomura and Benson., 1992b).

Paknejad *et al*., (2012) showed that oil yield, grain weight, protein percentage, and biomass decreased under drought stress, but foliar application of methanol increased these traits. Application of higher concentrations of methanol (up to 36%) decreased some traits to equal to or less than the control treatment. Positive effects of methanol have been reported by Aslani *et al*., (2011) and Li *et al*., (1995) for seed yield of mung beans and soybeans, by Mirakhori *et al*., (2009) for seed yield and 1000 seed weight of soybeans, by Jafari-Paskiabi *et al*., (2011) for seed yield and 1000 seed weight of cowpeas, and by Zbieć *et al*., (2003) for growth of oilseed rape, soybeans, small beans, cabbage and sugar beets.

Nanoparticles are atomic or molecular aggregates 1 to 100 nm in size with at least one dimension (Roco, 2003). Nano titanium dioxide (TiO_2) is used in agriculture. Titanium dioxide can increase growth and yield approximately 30%, the rate of

photosynthesis, and decrease disease in plants (Chao and Choi, 2005). Although nanoTiO_2 is non-toxic for animals and humans, its effects on plants is strongly dependent on concentration. It shows beneficial effects for plants at low doses (Jaberzadeh *et al.*, 2013).

Gao *et al.*, (2006) treated *S. oleracea,* using nanoTiO_2 and found that rubisco carboxylase activity was 2.67 times that for the control rubisco. Since 45% of rubisco content is protein, so it should be noted that TiO_2 increased protein content. TiO_2 is the most suitable photocatalyst, which, upon exposure to ultraviolet light, mineralizes organic chemicals in solution to water and carbon dioxide and has the potential to destroy microorganisms (Owolade *et al.*, 2008). NanoTiO_2 increases antioxidant stress by decreasing the accumulation of superoxide radicals, hydrogen peroxide, MDA and increasing antioxidant enzyme activity, which increases the evolution oxygen rate in spinach chloroplasts under stress (Lei *et al.*, 2008, Lu *et al.*, 2002, Hong *et al.*, 2005).

Lei *et al.,* (2007) found that nanoTiO_2 increased photosynthesis and plant growth in spinach and enhanced absorption and transmission of solar energy to electron energy and chemical active energy. They also found that nanoTiO_2 entered the Chl and was transferred in the photosynthetic electron transport chain to create $NADP^+$, was reduced to NADPH, and coupled to photophosphorylation and transformed electron energy to ATP. So, greatly increased whole chain electron transport, photoreduction in photosystem II, O_2 evolution, and photophosphorylation of spinach.

Moaveni *et al.,* (2011b) and Owolade *et al.*, (2008) found that nanoTiO_2 spraying of plants, increased the grain yield and harvest index for all treatments over the results for the control treatment. Several studies reported an increase from application of nanoTiO_2 on corn yield (Moaveni and Kheiri, 2011) and grain yield of *Hordeum vulgare* L. (Moaveni et al., 2011b). Considering the positive effects of methanol and nanoTiO_2 on aacelerate of plants and the destructive effects of deficit irrigation as oxidative stress, the present study examined foliar spraying of methanol and nanoTiO_2 to ameliorate the destructive effects of drought in borage plants. The effect of spraying methanol and nanoTiO_2 solution onto borage plants under deficit irrigation on the level of protein, seed and oil yield, oil percentage, lipid peroxidation level (MDA) and the antioxidant activity was measured for borage seeds.

Material and methods

Plant material and growth conditions

This experiment was a split-factorial completely randomized block design with four replications. It was carried out during the 2012 planting year in the city of Shahriyar in Iran. Treatments consisted of deficit irrigation (once every 14 d) and no stress irrigation (once every 7 d) in main plots. Methanol aqueous solutions in 0% (control; sprayed with water), 15%, 35%, and 45% (v/v) concentrations and nanoTiO_2 from 0% (control; sprayed with water), 0.01%, 0.03% and 0.05% concentrations were sprayed on the sub-plots. Borage (*Borago officinalis* L.) seeds were obtained from the Seed and Plant Co. (Isfahan, Iran).

Nanosized TiO_2 with a primary particle size of 4-8 nm was supplied by chemical synthesis (Plasma Chem, Germany). The size of the TiO_2 nanoparticles was determined by scanning electron microscopy (SEM) at the central laboratory of the Arts Faculty at Tarbiyat Modares University in Tehran, Iran. TiO_2 powder with >99% purity was prepared by Advanced Materials (US).

Methanol was sprayed 3 times during the growing season. The first spraying was applied after establishment of the plant, the second spraying at the appearance of stems, and the third when the plants bloomed. A back engine sprayer was used for spraying and the sprinkler was kept at 40 cm above the plants. Methanol spraying was done such that all aerial parts of the borage plants were covered.

Each plot was 3.5 × 5 m^2. The distance between rows was 50 cm and between plants in a row was 30 cm. There were two intact rows between each plot with 5 m of distance between main plots to prevent water leakage. Two multiple disks and a leveler were applied to prepare the seed bed before plugging. After

seed bed preparation and prior to cultivation, 50 kg of super phosphate triple and 60 kg urea were spread according to the results of a soil analysis. The borage seeds were antiseptic and sown at a depth of 5 cm in March 2012. All plots were harvested in June 2012.

The characteristics measured were protein, seed and oil yield, oil percentage, and antioxidant enzyme (POD, CAT, SOD, APX) and lipid peroxidation (MDA) levels. Sampling was conducted 72 h after the last spraying. Samples were 2 g in size of leaves per plant and were cut into small pieces and frozen in liquid nitrogen, then stored at -80°C.

Seed yield

To evaluate seed yield traits, they were collected at maturity of the seeds for each treatment. After harvesting, branches were dried in the shade and grain yield was measured using a Carriage scale using standard moisture at 14%.

Lipid peroxidation level

Lipoperoxidation was monitored by the spectrophotometric determination of MDA using thiobarbituric acid, according to Popham and Novacky (1991). Plant material (1 g FW) was homogenized in 2 cm^3 of trichloroacetic acid, TCA (10 %, m/v) and centrifuged at 15000×*g* for 20 min. To 250-mm^3 aliquot of crude extract 250 mm^3 of TCA (10 %, m/v) plus 1 cm^3 of thiobarbituric acid (0.2 %, m/v) in 10 % TCA was added. The mixture was boiled at 95 °C for 30 min and cooled on ice for 5 min. After centrifugation at 10000×g for 10 min, absorbance of the supernatant was determined at 532 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The concentration of MDA was calculated from its extinction coefficient (155 mM^{-1} cm^{-1}).

Protein Assay

The content of total soluble proteins was measured by the method of Bradford (1976) at 595 nm using the Bio-Rad protein assay reagent and bovine serum albumin (BSA) as a standard.

Antioxidant enzymes assay

For enzyme extracts and assays, leaves sample that were ground in solution containig 50 Mm phosphate buffer with PH 7.0, and Sodium meta bisulphite. The homogenate was centrifuged (Allegra- 64 Beckman Culterlng model) at 15000 g for 30 min, and the supernatant was collected for enzymes assay.

Catalase assay

Catalase (EC 1.11.1.6) amount was determined by method of Aebi (1983). The reaction mixture contained 20 µl of enzyme extract and 250 µl of 100 mM sodium phosphate buffer (pH 7) and the reaction was started by the addition of 250 µl of 70 mM hydrogen peroxide. Catalase activity was determined by measuring the decrease in absorbance at 240 nm. Decrease of absorbance was recorded in every 15 sec up to 3 min. Amount of catalase enzyme catalase was expressed as µmol/min/mg of protein.

Ascorbate peroxidase assay

Ascorbate peroxidase (EC 1.11.1.11) activity was determined using a method described by Nakano and Asada (1987). The assay mixture contained 100 mM potassium phosphate buffer (pH 7.0), 0. 5 mM ascorbite, and 2 mM H_2O_2. Activity was determined by following the H_2O_2 dependent decomposition of ascorbate at 290 nm. Amount of Ascorbate peroxidase enzyme catalase was expressed as µmol/ min / mg of protein.

Peroxidase assay

Peroxidase (E.C 1.15.1.1) activity was assayed by adding tissue extract (100 µl) to the reaction mixture, containing 10 mM guaicol, 70 mM H_2O_2 and 100 mM sudium phoshate buffer. Changes in the absorbance at 470 nm were read every 15s. Amount of Peroxidase enzyme catalase was expressed as µmol/min/mg of protein. (Chance and Maehley 1955).

Superoxide dismutase assay

Superoxide dismutase (EC 1.15.1.1) activity was measured based on the method of Beauchamp and Dhindsa and Matowe (1981). The reaction product was measured at 560 nm. The volume of supernatant

corresponding to 50% inhibition of the reaction was assigned a value of 1 enzyme unit.

Oil extraction

The oil extraction with conventional solvents were performed in a Soxhlet-type apparatus using *n*-hexane as solvent. For extraction, 50 g of borage seeds and 200 mL of *n*hexane were used. Extraction time was 10 hours what allowed full depletion of the grain and so maximum possible extraction yields were obtained (Kotnik *et al.*, 2006). After 10 h, borago oil was accumulated in the erlen of Soxhlet extractor, then cooling of samples, weighed by electrical scale carefully and determined oil percentage of borago. Finally, oil yield was determined by the following formula (Leal *et al.*, 2009).

Oil yield = Oil percentage × Seed yield.

Statistics analysis

After normalization test, data were subjected to analysis of variance (ANOVA) using Statistical Analysis System (SAS Institute) and followed by Duncan's multiple range tests. Terms were considered significant at $P \leq 0.05$.

Results

The results of analysis of variance demonstrated that the effects of deficit irrigation on all traits was significant at $p \leq 0.01$. The simple effects of methanol and nanoTiO_2 on POD content and MDA were significant at $p \leq 0.01$. The effect of methanol on CAT, APX and seed yield, was significant at $p \leq 0.05$, but the effect of methanol on protein, oil percentage and yield was not significant. The effect of nanoTiO_2 on protein, CAT, APX, and seed yield was significant at $p \leq 0.05$, but the effect of nanoTiO_2 on oil percentage and yield was not significant. Table 1 for analysis of variance shows that the interaction between deficit irrigation and methanol and between deficit irrigation and nanoTiO_2 were significant at $p \leq 0.01$ for all traits, exceptfor oil percentage.

Table 1. Results of variance analysis of the Borage traits under irrigation and foliar application of Methanol and nano TiO_2

		Means square								
Sources of variation	**df**	**Protein**	**CAT**	**APX**	**POD**	**SOD**	**MDA**	**Seed yield**	**Oil**	**Oil yield**
Replication	3	0.020 ns	44.9*	409.6**	1048.6*	42093.7**	0.021**	15587.8**	46.1**	1430.1**
Irrigation (a)	1	15.573**	55983.5**	10036.0**	316096.9**	5542393.9**	7.57**	1008931.4**	162.7**	78059.4**
Methanol(b)	3	0.040 ns	45.8*	41.9*	1509.3**	93666.5**	0.086**	4043.6*	2.0 ns	442.21 ns
Nanao TiO_2(c)	3	0.060*	42.2*	34.1*	1491.0**	162767.9**	0.030**	4444.8*	1.2 ns	491.5 ns
a × b	3	0.372**	130.8**	104.4**	8413.9**	240880.4**	0.336**	19151.9**	5.0 ns	3617.2**
a × c	3	0.085**	45.0*	80.5**	4705.6**	71499.1**	0.061**	5566.4**	0.7 ns	1206.7**
b × c	9	0.005 ns	8.0 ns	3.3 ns	187.9 ns	4681.5 ns	0.003 ns	930.1 ns	1.2 ns	129.4 ns
a× b×c	13	0.002 ns	20.8 ns	20.3 ns	242.2 ns	6403.5 ns	0.002 ns	329.9 ns	3.7 ns	137.8 ns
Main error	3	0.021	14.2	62.2	451.7	10658.8	0.003	3215.8	13.2	632.3
Secondary error	86	0.017	12.86	12.0	299.8	9539.3	0.002	1084.3	6.5	276.6
CV (%)		13.74	13.2	16.2	15.2	9.8	10.7	10.4	7.8	16.5

Note:* and **, Significant at 5 and 1% levels respectively

Discussion

Protein

Soluble protein content decreased because of deficit irrigation, which is similar to the results of other studies (Zhao *et al.*, 2009; Bakalova *et al.*, 2008) (Table 2). This decrease was related to the reaction of protein with free radicals, the change in amino acids, increased activity of protein decomposer enzymes, protein synthesis reduction, and the accumulation of free amino acids such as proline (Ranjan *et al.*, 2001). The results of mean comparison show that foliar applications of methanol and nanoTiO_2 had positive and additive effect on soluble protein content. A maximum value for soluble protein content was obtained after their application. The highest protein content was obtained for the 15% (v/v) concentration

of methanol under normal irrigation that placed it in the highest statistical group. The lowest protein content was found for the no-methanol application and deficit irrigation and placed in the lower statistical group (Table 3). There was no significant difference for 15% (v/v) methanol under the same condition and placed in a similar statistical group. The application of methanol is important for balancing the nutritional status of the leaves by acting as a carbon source (Mauney and Gerik, 1994). Paknejad *et al.*, (2012) found similar results; they demonstrated that foliar application of methanol on soybean increased the protein content under drought stress and higher concentrations of methanol (up to 36%) decreased the values of some traits to equal or less than the control treatment. The results showed that nanoTiO_2 spraying can increase protein content (Table 4). The nanoTiO_2 at 0.03% concentration produced the highest protein content for no stress irrigation, but showed no significant difference for the 0.01% treatment. The lowest value of this attribute was for the control treatment under deficit irrigation. This is in agreement with Jaberzadeh *et al.*. (2013) and Talebi (2009), who reported a significant effect for nanoTiO_2 on increasing protein content. The effect of the application of methanol and nanoTiO_2 under deficit irrigation was greater than for the no stress condition. It can be concluded that the effect of these substances was indirect because these materials prevented protein degradation by free radicals. It should be noted that, in the deficit irrigation condition, the use of methanol at a concentration of 45% (v/v) produced the highest protein content. This concentration for no stress irrigation produced a protein content lower than that of the control treatment, which is probably a result of the toxic effect of this high concentration. In the no stress treatment, there is no need for the plant to produce chlorophyll because the existing chlorophyll is sufficient and from the stomamats are closed (from CO_2 production and acidulation of guard cell pores). It is reasonable that, under stress caused by deficit irrigation, the dependence of the plant on higher levels of methanol for photosynthesis is greater than in the no stress condition. Under stress, the stomamats are closed, so the input CO_2 into the mesophile decreased. The free radicals of $NADPH_2$ caused by photosystems I and II required the Calvin cycle for neutralization. Sufficient amounts of CO_2 and chlorophyll are necessary for the rotation of the Calvin cycle; increased chlorophyll by methanol, is required for plant survival under stress. Nonomura and Benson (1992a) reported that plants showed rapid responses to methanol just below toxicity levels and that the toxicity levels for methanol varied according to the anatomical location of application and the variety of plant.

Table 2. Means comparison of irrigation on traits of Borag

Irrigation	Methanol % (v/v)	Protein (mg/gfw)	CAT (µmol/mg protein min)	APX (µmol/mg protein min)	POD (µmol/mg protein min)	SOD (µmol/mg protein min)	MDA (mg/gfw)	Seed yield (kg/ha)	Oil (%)	Oil yield
every 7 day	1.30a	6.10b	12.53b	63.96b	0.25b	784.7b	14.84a	403.06a	31.46b	125.33a
every 14 day	0.60b	47.92a	30.24a	163.35a	0.74a	1200.8a	10.70b	225.50b	33.72a	75.94b

Note: Means in the same columns and rows, followed by the same letter, are not significantly difference (P<0.05)

Table 3. Means comparison of irrigation and Methanol interaction effects on traits of Borage (*Borago officinalis* L.)

Irrigation	Methanol %(v/v)	Protein (mg/gfw)	CAT (µmol/g protein min	APX (µmol/g protein min	POD (µmol/g protein min)	SOD (µmol/g protein min)	MDA (mg/gfw)	Seed yield (kg/ha)	Oil Yield (lit/ha)
every 7 day	Control	1.31b	6.474c	11.86de	65.49f	745.2ef	0.25f	402.73a	126.04a
every 7 day	15	1.42a	7.025c	14.27d	82.05e	877.9d	0.16g	425.3a	135.73a
every 7 day	35	1.32b	6.237c	13.85d	64.05f	802.7e	0.24f	404.93a	127.39a
every 7 day	45	1.16c	4.669c	10.14e	44.25g	712.9f	0.36e	379.29b	112.15b
every 14 day	Control	0.50f	44.609b	27.52c	141.56d	1087.0c	0.90a	193.55e	64.11e
every 14 day	15	0.52f	46.084b	29.99b	154.37c	1136.4c	0.79b	206.49e	68.71ed
every 14 day	35	0.64e	49.434a	30.20b	171.15b	1206.3b	0.68c	233.58d	78.37d
every 14 day	45	0.76d	51.586a	33.25a	186.30a	1373.7a	0.58d	268.37c	92.56c

Note: Means in the same columns and rows, followed by the same letter, are not significantly difference (P<0.05)

Antioxidant enzyme activity

Production of reactive oxygen species are one of the most damaging elements to photosynthesis under environmental stress such as drought (Kim and Lee, 2005). Plants have defense systems to reduce the damaging effects of reactive oxygen species, including antioxidant enzymes. CAT, POD and APX are the most important enzymes that remove H_2O_2 (Shen *et al.*, 2010). POD reduces H_2O_2 to water using different electron donor substrates. APX uses ascorbate as an electron donor and reduces H_2O_2 to water; CAT converts hydrogen peroxide to water and oxygen. A comparison of means (Table 2) showed that deficit irrigation stress increased all antioxidant enzyme activity. Deficit irrigation produced the highest amount of antioxidant enzyme activity.

Results showed that methanol at concentrations of 35% and 45% (v/v) under deficit irrigation produced the highest amounts of CAT. Neither amount showed a significant difference and were in the same statistical group. The highest POD, SOD and APX enzyme activity was achieved for the 45% methanol concentration. The lowest enzyme activity was for 45% methanol (v/v) concentration under no stress irrigation, but was not significantly different from the other methanol concentrations and was in the same statistical group. The highest amounts of APX and POD enzyme activity was obtained from 45% (v/v) of methanol concentration in deficit irrigation and the lowest was achieved using 45% (v/v) methanol concentration under no stress irrigation.

This concentration under the no stress irrigation was toxic for borage, meaning that the activity of the enzymes was lower than for the control treatment. There is some evidence that non-enzymatic antioxidant content (such as anthocyanin and carotenoids) increase with the use of methanol (Ramadan and Omran, 2005; Downie *et al.*, 2004), but an increase in antioxidant enzymes from methanol foliar application has not been reported thus far, so its mechanism is unknown. Table 4 shows that nanoTiO_2 increased all antioxidant enzyme activity in this study. CAT showed the least enzyme activity at all nanoTiO_2 concentrations and in the control for no stress irrigation. All were in a similar statistical group and produced no significant differences. This level of enzyme activity was achieved at all nanoTiO_2 concentrations for deficit irrigation and were higher than for the control treatment. A comparison of means showed that foliar application of a 0.05% concentration of this nanoparticle under stress produced the highest POD, SOD and APX enzyme activity, the lowest values for these were for the control treatment in the no stress condition.

Table 4. Means comparison of irrigation and foliar application of nano TiO_2 on traits of Borage (*Borago officinalis* L.)

Irrigation	Nano $TioO_2$ (%)	Protein (mg /gfw)	CAT (µmol/ mg protein min	APX (µmol/ mg protein min	POD (µmol/mg protein min)	SOD (µmol/mg protein min)	MDA (mg/gfw)	Seed yield (kg/ha)	Oil Yield (lit/ha)
every 7 day	Control	1.24b	5.916c	9.96e	52.21e	720.3f	0.31d	392.44a	116.74b
every 7 day	0.01	1.34a	6.077c	13.75d	66.11d	808.7e	0.22f	410.44a	129.12a
every 7 day	0.03	1.36a	6.394c	14.35d	75.12d	834.5e	0.21f	414.44a	131.09a
every 7 day	0.05	1.27ab	6.018c	12.05de	62.39ed	775.2ef	0.26e	394.93a	124.36ba
every 14 day	Control	0.52e	44.829b	28.22c	145.20c	1068.6d	0.82a	203.15d	67.33e
every 14 day	0.01	0.58de	47.451a	29.43bc	153.58c	1164.0c	0.74b	211.55cd	71.41de
every 14 day	0.03	0.63dc	49.641a	30.91ba	171.05b	1243.6b	0.73b	234.65cb	79.74dc
every 14 day	0.05	0.69c	49.791a	32.40a	183.55a	1327.2a	0.66c	252.64b	85.27c

Note: Means in the same columns and rows, followed by the same letter, are not significantly difference (P<0.05)

$NanoTiO_2$ may clear a large amount of ROS using the following mechanism: (1) Ti^{4+} of $nanoTiO_2$ oxidates O_2^- to O_2 while reducing itself to Ti^{3+}; (2) Ti^{3+} reduces O_2^- to H_2O_2 and oxidates itself to Ti^{4+} and CAT and POD reduce H_2O_2 to H_2O and O_2, which repair the membranes of the chloroplasts and protect the chloroplasts from aging under stress (Hong *et al*., 2005). The increasing of these enzymes under stress was demonstrated by Hong *et al*., (2005), who showed that the production rate of free radicals with the $nanoTiO_2$ treatment was lower than for the control treatment.

Lipid peroxidation (MDA)

ROS are the main cause of lipid peroxidation (Upadhyaya *et al*., 2004). Lipid peroxidation and the production of MDA are indicators of oxidative stress damage (Jagtap and Bhargava, 1995). MDA reacts intensively with cellular components, seriously damaging enzymes and membranes. Membranous electric resistance and fluidity fall, eventually leading to the destruction of the membrane structure and physiological integrality *(*Hong *et al*., 2005*)*. The extent of lipid peroxidation was measured by MDA content. An increase in MDA content, as the end product of membrane lipid peroxidation, was observed from stress caused by deficit irrigation. Table 3 shows that means comparison indicates that the highest MDA was achieved by the control treatment under deficit irrigation and the lowest value was for 15% (v/v) concentration of methanol under no stress irrigation.

This treatment was better than the others, especially the 45% (v/v) concentration, because of the toxicity of this concentration. The 45% (v/v) condition for deficit irrigation was the best treatment for decreasing MDA production. Table 4 shows that $nanoTiO_2$ at the 0.03% and 0.01% concentrations for deficit irrigation produced the lowest amount of MDA and best stabilized the chloroplast membrane to protect them from aging. By contrast, the control treatment under deficit irrigation had the highest MDA content and the least stability for the chloroplast membrane. For the $nanoTiO_2$ foliar application, MDA was lower than for the control treatment and had the highest stability of the membrane.

In the stress condition, $nanoTiO_2$ at the 0.05% concentration had the lowest lipid peroxidation compared with the control and the other concentrations used in this study. Membrane permeability for plants sprayed with $nanoTiO_2$ was lower than for the control treatment, but slowly rose. Hong *et al*., (2005*)* showed that $nanoTiO_2$ could stabilize the integrality of chloroplast membranes by decreasing lipid peroxidation under stress and protecting chloroplasts from aging. Lipid peroxidation of the chloroplasts increased following

under drought stress, probably from an imbalance in free radical production; their removal damaged large molecules and membranes. Methanol and nanoTiO_2 increased antioxidant enzymes, eliminated free radicals, and reduced lipid peroxidation, enhancing the integrality membrane.

Oil percentage, and seed and oil yield

The results showed that deficit irrigation decreased seed and oil yield. Yield reduction under drought stress was also observed by Jalilian *et al.*, (2012). The results of the present study showed that the irrigation interval effected essential oil percentage (Table 1), but that other treatments had no effect on this trait. Table 2 shows that the highest percentage of oil was obtained for the 14 d irrigation treatment. Although oil percentage increased with drought stress, the means comparison (Table 2) indicated that the highest oil yield was produced by the no stress irrigation. The decrease in oil yield from the decrease in soil moisture may be caused by the harmful effects of drought stress on vegetative growth and seed yield.

The increase in essential oil production under drought could be a result of the reallocation of assimilated carbon as plant growth decreases for the biosynthesis of stress metabolites that protect the plant (De Abreu and Mazzafera, 2005). Singh and Ramesh (2000) reported that water deficit stress decreased the oil yield of rosemary. Simon *et al.*, (1992) reported similar results for sweet basil (*Ocimum basilicum*) and Baher *et al.*, (2002) for savory (*Satureja hortensis*). The results of means comparison (Table 3), showed that the highest seed and oil yield was obtained for the 15% (v/v) methanol in the no stress condition. No difference was shown for the 35% (v/v) concentration of methanol and the control treatments and were in one statistical group. The lowest seed and oil yield was obtained for the control treatment in under deficit irrigation.

It is interesting that, under the deficit irrigation, methanol at a 45% (v/v) concentration produced the highest seed and oil yield; this showed a significant difference with the other concentrations and the control treatment. The same concentration (45% v/v) under no stress irrigation showed the lowest seed and oil yield, even lower than the control treatment, probably because this level of methanol caused stress and chlorophyll destruction. Safarzadeh-Vishgahi *et al.*, (2005) also showed that the application of high levels of methanol decreased chlorophyll and yield.

As Table 4 shows, the highest seed yield was obtained for nanoTiO_2 at 0.03% concentration under no stress irrigation, but there was no significant difference with the other concentrations or the control treatment. The highest oil yield was achieved for nanoTio_2 at the 0.03% concentration under no stress irrigation. This was not significantly different from the other concentrations of nanoTio_2, but was significantly different from the control treatment in the no stress condition. The control treatment in deficit irrigation showed the lowest seed and oil yield. Contrary to the stress caused by deficit irrigation, no stress irrigation at lower concentrations of methanol and nanoTio_2 produced better conditions than did the other treatments.

The foliar application of methanol significantly increased seed and oil yield for both no stress and deficit irrigation and produced ameliorating effects of exogenously applied methanol for these traits. This increase in seed and oil yield was more pronounced under deficit irrigation than for no stress irrigation. Ramberg *et al.*, (2002) demonstrated that, for plants that suffer from water deficit, methanol spraying of the aerial parts increase chlorophyll concentration more than for plants in the no stress condition. The ameliorating effect of exogenously applied methanol on these traits may be a result of its role in maintaining high photosynthesis and increased allocation of assimilates to developing seeds and increasing seed yield. Nonomura and Benson (1992a) emphasized that increasing the yield may be related to the important role of methanol in facilitating the availability of mineral or organic nutrients for the plant and the utilization of methanol as a carbon source.

Heins (1980) found that molecules of methanol are smaller than those of CO_2 and are absorbed sooner by the plant, causing a delay in leaf senescence from ethylene production in the plant, increasing leaf area

duration and photosynthesis. Plants that grow in an CO_2 enriched environment are less susceptible to drought since their stomata are closed, transpiration decreases, and net photosynthesis is elevated (Besford, 1993). Similar results were reported by Zbieć *et al*., (2003), who showed that the yield of rape seeds treated with 30% or 40% methanol exceeded that of the control plants by 30%. As CO_2 increased in the ambient air as a result of methanol oxidation (Gout *et al*., 2000), the increase in photosynthetic efficiency of the leaves may be transferred to the seeds. These results are in agreement with those obtained by Nonomura and Benson (1992 b), who reported that methanol-treated C_3 plants showed high growth rates and higher yield.

The additive effect of methanol on seed and oil yield demonstrated by Jafari-Paskiabi *et al*., (2011), Aslani *et al*., (2011), Bayat *et al*., (2012), and Li *et al*., (1995) are in accordance with these results. A mean comparison of the results (Table 4) indicate that, under no stress irrigation, the 0.03% concentration of $nanoTiO_2$, produced the highest seed yield. There was no significant difference with the other concentrations or the control treatment. The lowest amount of this trait was achieved by or the control treatment under deficit irrigation. This increase is probably because nano-particles prolonged photosynthesis by transforming light energy into active electrons and chemical activity in the chloroplasts. This increases photosynthesis efficiency, motivates the rubisco activase complex and increases carbon photosynthesis. This amplification could increase dry matter and grain yield. Moaveni *et al.,* (2011b) and Owolade *et al*., (2008) confirmed the effects of TiO_2 and suggested that it could promote plant growth by increasing light absorbance, accelerating the transport and transformation of light energy (Lei *et al*., 2007).

Conclusion

Results of this study showed that drought stress led to production of free radicals, increasing the activity of protein decomposer enzymes and peroxidation of borage chloroplasts. The percentage of oil decreased, but the oil yield increased. It was found that $nanoTiO_2$ and methanol significantly increased the activity of CAT, APX, SOD and POD enzymes, decreased the accumulation of reactive oxygen free radicals and the level of MDA relative to reactive oxygen scavenging. This investigation showed that methanol and $nanoTiO_2$ overcame the deleterious effects of drought stress caused by deficit irrigation on the characteristics of borage by enhancing growth, improving cell membrane stability, and antioxidant enzymes. Based on these findings, foliar application of methanol and $nanoTiO_2$ may alleviate the negative effects of drought stress on growth and seed and oil yield of borage plants. These findings may aid commercial farmers of medicinal plants and agricultural researchers in the application of methanol and $nanoTiO_2$.

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