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Pigments apparatus and anthocyanins reactions of borage to irrigation, methylalchol and titanium dioxide

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Key words: Chlorophyll, nitrate reductase enzyme, Spad value, severe moisture, rubisco carboxylase, anthocyanin.

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Abstract

This study was done to evaluate the effect of methanol and nano TiO_2 foliar application on photosynthetic apparatus, anthocyanin and changes of nitrate reductase in borage (*Borago officinalis* L.) under deficit irrigation. Tests were done as a split factorial experiment set up as a completely randomized block design with four replications, in the year 2012, at Shahriyar City, Iran. Irrigation conditions were the factors tested in the main plots: deficit irrigation condition (irrigation every 7 days) and irrigation every 14 days. Methanol solutions 0 (control or sprayed with water), 15%, 35% and 45% (v/v) and, nanoTiO_2 treatment (0 or control and nano TiO_2 at concentrations of 0.01%, 0.03% and 0.05%) were tested in the sub plots. Plant characteristics under evaluation were those of; chlorophyll (Chl) a,b and total chlorophyll, Spad value, net photosynthetic rate, rubisco carboxylase activity, anthocyanin and nitrate reductase enzyme (NR). Also evaluated were the effects of interaction of deficit irrigation and methanol on all measured traits, and results determined the effect as significant with the exception of NR and interactions of deficit irrigation and nano TiO_2 on all measured traits. Test results showed that maximum amount of traits at deficit irrigation was achieved by spraying with 45% v/v oncentration of methanol and nano Tio_2 at the concentration of 0.05%. But the best treatments under normal irrigation were 15% v/v of methanol and 0.03% of nano Tio_2. but at the methanol concentration of 45% and non-application of nano Tio_2 under normal irrigation had the least effect. In summary, results of these tests determined that application of methanol and nano Tio_2, can promote plant resistance to drought stress (particularly borage plants).

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Introduction

Borage (*Borago officinalis L.*) is an annual or biannual plant with sky blue flowers and green leaves covered with white hairs. It is native to Europe, Asia and North Africa and has been used for centuries for both medicinal and culinary purposes (Fetrow and Avila, 1999). The seeds contain 30-40% oil by weight, of which 23-24% constitutes Gamma Linolenic Acid (GLA) (Ezz El-Din, 2010). Recently, many reports have highlighted the potential market for Gamma Linolenic Acid (GLA) from borage (Boraginaceae family), which has been the subject of increasing interest in agriculture (Mhamdi *et al.*, 2009). Agricultural production is limited in many areas by drought stress in plants, which is a major abiotic stress. Plants respond to drought stress and become acclimatized through various physiological and biochemical changes (Farooq *et al.*, 2009). Also, stress induced by water deficit can affect photosynthesis, either directly or indirectly by a decrease in CO_2 availability that is caused by conditions such as limited diffusion (Flexas, 2007); changes in photosynthetic metabolism (Lawlor and Cornic, 2002) or restrictions in the photochemical system apparatus under severe stress conditions (Souza *et al.*, 2004). Research has also revealed that components of the photosynthetic apparatus could be significantly damaged by drought stress. However, values of chlorophyll content decreased (Li *et al.*, 2006). Other research has determined that a typical symptom of stress is that of a reduction of pigments as a result of either slow synthesis or fast breakdown (Smirnoff, 1993). Rubisco is an enzyme located in chloroplast stroma and it has two important functions, so evaluation of Rubisco sensitivity to abiotic stresses is an important consideration in terms of factors effecting photosynthesis (Bock and Khan, 2004). Likewise, research by Majumdar *et al.*, (1991) reported that a loss of Rubisco activity was an indication of rapid response to drought stress in soybean. So, it must be emphasized that increasing severity and duration of drought stress does decrease Rubisco activity (Tezara and Lawlor, 1995). The research of Feng *et al.*, (2007) showed that evaluations for photosynthetic capacity, biomass of pigment content, and yield, were all reduced under drought stress. Other authors have explained this phenomenon as a photo-protection mechanism, such that the rate of light absorbance is reduced according to decreased pigment content (Elsheery and Cao, 2008). The fndings of Holaday *et al.*, (1992) demonstrate that total rubisco activity of wheat flag leaves decreased when drought stress was applied at the anthesis stage. The study reported that this decrease was accompanied by a decrease in levels of soluble protein and chlorophyll. Water stress also has an affect on nitrate reductase enzyme activity. This enzyme accelerates conversion of inorganic nitrogen (NO_3- N and NH_4 -N) to organic nitrogen (protein and chlorophyll) (Yang *et al.*, 2006). It is well documented that this enzyme in the leaves of higher plants, is very sensitive to changes in plant water status (Chen and Sung., 1983). Deficit irrigation (drought stress), as with other stress responses, produces oxidative stress. Oxidative stress is described as an in-balance between pro oxidants (or reactive oxygen species, ROS) and antioxidants in biological systems, and it may be triggered by increased production of ROS or by a reduction in antioxidant defenses (Munne-Bosch and Alegre, 2000). However, plants combat oxidative stress by developing enzymatic and non-enzymatic antioxidant defense mechanisms to scavenge ROS (Smirnoff, 1993). Non-enzymatic antioxidant agents such as anthocyanins can also quench ROS (e.g. $1O_2$) and stabilize photosynthetic complexes (Metwally *et al.*, 2003).

Also, many studies have reported that anthocyanins accumulation and an increase in the ratio of carotenoids to total chlorophylls under water deficit conditions may effect the process of photo-protection under direct drought stress (Merzlyak *et al.*, 2008). Methanol is a single carbon that is lipid-soluble and passively traverses lipid membranes affecting rapid incorporation in cells (Salisbury and Ross, 1992). It is synthesized into serin and methionine and oxidized to CO_2 in C_3 plants (Cossins, 1964). Plants treated with methanol have increased turgor and a better growth rate that results in the production of a higher yield compared to untreated plants. Only C_3 plants, that is those that produce ribulose 1,5-diphosphate and then 3-phosphoglyceric acid, during the proces of photosynthetic carboxylation, respond to methanol by producing more biomass, since carbon dioxide resulting from the rapid oxidation of methanol, can successfully compete with oxygen for Rubisco (Nonomura and Benson, 1993). In plants under stress from water deficit, application of methanol spray to the aerial parts serves to increase chlorophyll concentrations, and such treatment causes the concentration to have a slight reduction in plants with adequate water supply (Ramberg *et al.*, 2002).

Nonomura and Benson, (1992a; 1992b) proposed that the metabolism of methanol, lessened water use in plants. Metabolism of methanol to sugars, would affect the osmotic potential in leaf by increasing plant turgor and stomatal conductance. Keeping the stomata open serves to increase the assimilation rate and, subsequently has an affect on plant growth. An accelerated growth rate results in earlier maturation and thereby reduces plant irrigation requirements. Research has demonstrated that methanol has a very positive effect on photosynthesis (Li and Yi, 2004). An increase in photosynthetic rate, induced by methanol was also reported in David *et al.*, (2003). The research demonstrated that the rates of oxygen evolution and photosynthesis of *Lemna gibba* increased under application of methanol spray. More recently, methanol was found to improve photosynthesis and stomatal conductance but did not affect growth in tests on cotton (Faver and Gerik, 1996). Also, tests on alstromeria fowers showed that methanol application increased Spad evaluations (Mousavi Bazaz and Tehranifar, 2011). Other investigations have shown that productivity and photosynthetic activity were increased by methanol (Theodoridou *et al.*, 2002). The same effects were also found in higher plant species when sprayed with methanol within the concentration range of 10-50% (Li and Yi, 2004). Ramadan and Omran, (2005) reports that foliar application of methanol spray, induced significantly increased evaluations for chl. a, b; total chl. and carotenoids concentrations especially by foliar spray treatments of methanol concentrations at 30% and 40%. It was also reported that all treatments increased anthocyanin content in berry skins especially at methanol concentrations of 20 % and 30 %. Other research such as Nikolas *et al.*, (2003) and Downie *et al.*, (2004) concluded that methanol spray treatment advanced and increased evaluations for anthocyanins in berries and Arabidopsis, respectively.

Nano TiO_2 is one of the most popular among manufactured nano materials (MNMs). It is used in a variety of consumer products, such as sunscreens, cosmetics, paints and surface coatings (Kaida *et al.*, 2004). Anatase nano TiO_2, has properties such as a large specific surface area, high thermal conductivity and high photocatalytic ability. Lei *et al.*, (2007) reported that nano Tio_2 increases photosynthesis and plant growth in spinach and serves to enhance absorption and transmission of the sun's energy to electron energy and active chemical energy. Also reported, was that nano TiO_2, could greatly improve plant processes such as whole chain electron transportation, photoreduction activity of photosystem II, O_2-evolving and photophosphorylation activity of spinach Chl, not only under visible light but also energy-enriched electrons from nanoanatase TiO_2, which entered the Chl and was transferred by a photosynthetic electron transport chain to produce NADP+ reduce into NADPH, and coupled to photophosphorylation and transferred electron energy to ATP. Zheng *et al.*, (2005) reported that, 2.5% rutile nano TiO_2, promoted the germination of spinach seeds, whereas at 0.25% rutile nano TiO_2, enhanced the rate of photosynthesis in spinach by promoting cyclic and linear photophosphorylation. This promotion is closely related to Mg^{2+}-ATPase activity [38]. Furthermore, nano TiO_2, stimulates antioxidant activity that produced a positive affect on spinach by protecting the chloroplast membrane structure from production of reactive oxygen species, thus increasing antioxidant enzyme activity (Hong *et al.*, 2005a). It is well known that Rubisco catalyzes the carboxylation and oxygenation of ribulose-1, 5-bisphosphate

(RuBP), the first committed steps in the competitive metabolic pathways of photorespiration and photosynthetic CO_2 fixation in higher plants plants (Hong *et al.*, 2005c; Spreitzer, 1999). An increase in traits of net photosynthetic rate, Rubisco carboxylase activity and chlorophyll, with nano TiO_2, was determined in the experiments reported in Xuming *et al.*, (2008). The report determined that application of nano TiO_2, enhanced Rubiso activase activity in spinach by significantly promoting expression of Rubiso activase mRNA (Ma *et al.*, 2008). Another study determined that nano TiO_2, significantly promoted the genetic expression of *Arabidopsis thaliana* light-harvesting complex II b (Ze *et al.*, 2011). Results of Zhang *et al.*, (2008a) demonstrated that cucumber leaves sprayed with a nanoTiO_2 promoted chlorophyll and carotene syntheses. Therefore, spraying with nano TiO_2, after a few hours promoted photosynthesis in cucumber, which caused increased root growth (Zhang *et al.*, 2008b). TiO_2 nanomaterial can promote soybean root activity and leaf nitrate reductase activity, enhance plant water and nitrogen use and stimulate some antioxidant activities, such as, SOD, POD, and CAT (Lu *et al.*, 2002). Considering that the nitrogen content of plant leaf affects its photosynthetic capacity, it generally has a positive correlation with plant photosynthesis (Zhang *et al.*, 2008c). Spraying plants with nano TiO_2, can significantly promote nitrate reductase enzyme activity Yang *et al.*, (2006). Yang *et al.*, (2007) reported that spinach treated with nano TiO_2, can absorb N_2 directly or reduce N_2 to NH_3 in nitrogen-poor nutrient solutions under sunlight, thereby significantly increasing plant nitrogen content. The nitrogen content of plant leaf affects its photosynthetic capacity as it generally has a positive correlation with plant photosynthesis (Zhang *et al.*, 2008a). So nano TiO_2 can affect the microenvironment of PSII in spinach and increase the rate of visible-light absorption in leaf, improving a plant's energy transport capacity (Su *et al.*, 2007). It has also been found that nano TiO_2, promoted antioxidant stress by decreasing the accumulation of superoxide radicals, hydrogen peroxide, malonyldialdehyde content and enhance antioxidant enzyme activity and thereby increase the evolution oxygen rate in spinach chloroplasts under stress (Lei *et al.*, 2008). The findings of Morteza *et al.*, (2013) showed that evaluations for photosynthetic pigment contents, carotenoids and anthocyanins of maize were increased under spray treatment of nano TiO_2. So, according to the importance of *borago officinalis* as a medicinal plant and its value as a source of linolenic acid (GLA) the objective of this present work was to determine the effect of deficit irrigation and foliar application of methanol and nano TiO_2, on photosynthetic traits, anthocyanins and characteristics of borage leaves.

Materials and method

Plant characteristics measured in this study were those of total chlorophylls (a+b), chlorophyll a, chlorophyll b, anthocyanins content, Spad value, net photosynthetic rate, Rubisco carboxylase and nitrate reductase activity. Samples were obtained for evaluations from the youngest fully expanded leaves of different individual plants 72 h after the final spray treatment, frozen in liquid nitrogen and then stored at -80°C.

Chlorophyll assay

Total chlorophylls (Chl a+b), chlorophyll a (Chl a) and chlorophyll b (Chlb) and carotenoids (Car), were determined spectrophotometrically, using 80% acetone as a solvent. The pigment extract was measured against a blank of 80% (V/V) acetone at wavelengths of 647 and 663 nm for chlorophyll assays. Finally, amounts of traits, was determined by the following formula (Lichtenthaler, 1987).

$$\text{Chl a} = 12.25\ A_{Abs\ 663} - 2.79\ A_{Abs\ 647}$$

$$\text{Chl b} = 21.5\ A_{Abs\ 647} - 5.1\ A_{Abs\ 663}$$

$$\text{Chl T} = \text{Chl a} + \text{Chl b}$$

Anthocyanins content assay

Anthocyanins content was analyzed from samples according to the method cited in Wanger, (1979). For determination of anthocyanins content, frozen tissue samples (100 mg) were soaked immediately in 10 ml of acidified methanol (methanol: HCl 99:1 (v/v)). The tissue was crushed using a glass pestle and kept at 25 C° for 24 hours in the dark. The extract was then centrifuged at 4000 × g, for 5 min at room temperature (22 C°) and absorption at 550 nm of the supernatant was read by a UV- VIS spectrophotometer (model Jenway 4506). Anthocyanins content was calculated by the following formula for each sample.

$A_{Abs\ 550} = \varepsilon bc$

A= Read absorbance at 550 nm

ε = Extinction coefficient =33,000 $mol^{-1} cm^{-1}$

b= cell width=1cm

c= concentration of anthocyanin (µ $mol.g^{-1}$.fw)

Spad value

Chlorophyll readings were taken with a hand-held dual wavelength meter (SPAD 502, Chlorophyll meter, Minolta Camera Co., Ltd., Japan). The 30 youngest fully expanded leaves from each plot were used for sampling 72 h after the final spray treatment. The instrument was stored and readings were automatically averaged to generate one reading per plot.

Net photosynthetic rat

The rate of photosynthetic CO_2 assimilation was measured on attached leaves using an IR gas analyzer (model LCA4, Analytical Developmental Co., Hoddesdon, UK) (Foyer, 1998).

Rubisco carboxylase activity assay

For measurements of Rubisco activity, frozen leaf discs were ground to a fine powder in liquid nitrogen and rapidly extracted with 2·ml ice-cold extraction buffer containing 50 mM Bicine, pH 8·0, 20 mM magnesium chloride ($MgCl_2$), 2 mM phenylmethlysulfonyl fluoride, 50 mM 2-mercaptoethanol and 30 mg polyvinylpolypyrrolidone (PVPP). The extracts were clarified by centrifugation (10 000 *g* at 4 °C for 2 min) and evaluations were determined for initial total and maximum Rubisco carboxylase activity according to the method cited in Parry *et al.*, (1997). Soluble protein content was determined according to the method cited in Bradford, (1979) at 595 nm using the Bio-Rad protein assay reagent and bovine serum albumin (BSA) as a standard.

Nitrate reductase (NR) assay

NR was prepared and assayed based on the method cited in Sagi *et al.*, (1997). Shoot and root samples from the control and treated plants were frozen in liquid nitrogen immediately after harvesting. Crude extracts were obtained by maceration with acid-washed sand in an ice-cold extraction medium containing 25 mM Tris-HCl (pH 8.5), 3 mM dithiothreitol, 1 mM ethylenediaminetetraacetate, 10 µM flavine adenine dinucleotide sodium salt, 1 µM sodium molybdate, 2% (w/v) casein, 10 µM leupeptin, 5 mM reduced glutathione and 3% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 30,000 g for 15 min in a refrigerated centrifuge (Beckman, L7 Ultracentrifuge, USA) at 4°C. The resulting supernatant was assayed in a modified reaction mixture containing 15 mM K-phosphate buffer (pH 7.5), 25 mM Tris-HCl buffer (pH 7.5), 12.5 mM KNO_3 and 0.4 mM nicotenamide adenine dinucleotide (reduced) by nitrite accumulation, which was analyzed by sulphanilamide and N-(1-napthyl) ethylenediamine dihydrochloride addition and subsequent measurement of absorption at 540 nm.

Statistical analyses

The statistical analyses of data was determined by analysis of variance (ANOVA) and separation of means was done by the Duncan's new multiple range method at 99% level of probability, conducted using the SAS System (SAS institute, 1988) for Windows statistical software.

Results

The results of analysis of variance demonstrated that the effects of deficit irrigation on the traits of; chlorophyll content (a and b), total chlorophyll (a+b), Spad value, net photosynthetic rate, Rubisco carboxylase activity, anthocyanins and nitrate reductase was significant ($P \le 0.01$). The effect of methanol on chlorophyll content (a and b), total chlorophyll (a+b), net photosynthetic rate, Rubisco carboxylase activity was significant ($P \leq 0.01$) and the effect of methanol, on Spad value, was significant at the probability level $P \leq 0.05$. Also, nano TiO_2 had a significant effect at probability level $P \leq 0.01$ on chlorophyll a, total chlorophyll (a+b), net photosynthetic rate and Rubisco carboxylase activity, but the effect of nano TiO_2 on chlorophyll b and Spad value, was significant at the probability level $P \leq 0.05$. The effect of the interactions between deficit irrigation and methanol on all the measured traits was significant at $P \leq 0.01$ and interactions between deficit irrigation and nano TiO_2 on chlorophyll a, total chlorophyll (a+b), Spad value, net photosynthetic rate, Rubisco carboxylase activity and nitrate reductase was significant at $P \leq 0.01$ but the interaction between drought and nano TiO_2, on chlorophyll b and anthocyanin, was significant at $P \leq 0.05$. But, the simple effect of methanol, on traits of anthocyanin was not significant. Also interactions between methanol and nano TiO_2, was significant only on the trait of anthocyanin ($P \leq 0.01$) (Table 1).

Table 1. Results of variance analysis of the Borage (Borago officinalis L.) traits under defict irrigation and foliar application of methanol and nano TiO_2..

Means square

Sources of variation	df	Chlorophyll a	Chlorophyll b	Total chlorophyll	SPAD value	Anthocyanin	Net photosynthetic rate	Rubisco carboxylase activity	Nitrate reductase
Replication	3	0.0087**	0.0060*	0.0287**	7.525^{ns}	1154.10**	5.204^{ns}	0.064**	0.0236^{ns}
Irrigation (a)	1	9.176**	1.7440**	18.922**	1603.195**	12236.34**	2150.122**	0.019**	16.601**
Methanol(b)	1	0.0355**	0.0116**	0.085**	26.539*	38.45^{ns}	41.569**	0.923**	0.036^{ns}
Nanao TiO_2(c)	3	0.0143**	0.0054*	0.0301**	24.744*	44.87^{ns}	37.207**	0.322**	0.046^{ns}
a × b	3	0.0983**	0.0256**	0.221**	76.592**	111.53**	208.995**	1.803**	0.056^{ns}
a × c	3	0.0571**	0.0052*	0.095**	102.705**	61.94*	57.637**	0.593**	0.307**
b × c	9	0.0010^{ns}	0.00057^{ns}	0.001^{ns}	5.089^{ns}	74.72**	0.751^{ns}	0.020^{ns}	0.011^{ns}
a× b×c	13	0.0008^{ns}	0.00052^{ns}	0.002^{ns}	8.649^{ns}	31.49^{ns}	0.875^{ns}	0.014^{ns}	0.012^{ns}
Main error	3	0.0148	0.0093	0.042	20.338	37.64	5.009	21.120	0.031
Secondary error	86	0.0016	0.0024	0.004	9.394	21.41	4.164	0.011	0.023
CV (%)		5.09	11.76	5.67	7.96	9.93	11.16	8.16	20.2

Note:* and **, significant at 5 and 1% levels respectively and ns is non significant.

Chlorophyll a, Total chlorophyll and Net photosynthetic rate

Results of comparisons of means (Table 2) show that higher amounts of chlorophyll a, total chlorophyll and net photosynthetic rate were obtained at the 15 volumetric percentage under the non-stress condition (irrigation every 7 days) and the least amount of these traits was observed in the control treatment under stress condition (or irrigation every 14 days). Under normal irrigation (irrigation every 7 days or non-stress), the minimum amount of chlorophyll a, total chlorophyll and net photosynthetic rate traits, was achieved by 45 volumetric percentage of methanol spray, and in this condition, 35% (v/v) of methanol and control treatment were placed in the same statistical group and no difference was determined between treatments of 15 and 45 volumetric percentage. But under the condition of stress (or irrigation every14 days), applicaction of methanol at the concentration of 45% (v/v) had the highest evaluation for content of these traits.

According to these results (Table 3) the maximun amounts for chlorophyll a, total chlorophyll and net photosynthetic rate traits were obtained by the use of nano titanium dioxide at the concentration of 0.03% under normal irrigation (non-stress) and the minimum content for these traits was observed by the control treatment. It should be noted that under normal irrigation (non-stress) the least amount of these traits was obtained by the treatment of non-application of nano TiO_2.

Chlorophyll b

According to Table 2, the highest chlorophyll b content, was obtained from 15% (v/v) methanol under normal irrigation and the least was obtained from the control treatment under irrigation every 14 days (or stress condition). Also, it should be noted that under normal irrigation all treatments of control, 35 % and 45 % (v/v) of methanol, were determined as having significant difference and were categorized in the same statistical group. Under conditions of deficit irrigation (every 14 days), all levels of methanol treatment had higher amounts of chlorophyll b in comparison with the control. In this study, it was observed that the highest amount of chlorophyll b, was obtained under the treatment of normal irrigation and 0.03% of titanium dioxide nanoparticle spray and the least was determined in the control treatment under stress condition. Under conditions of drought stress the maximum amount of chlorophyll b was obtained from the treatment of nano TiO_2, at the concentration of 0.05% and the minimum amount of chlorophyll b, was observed in treatments of non-application of nano TiO_2. So that, under irrigation at every 14 days, the concentrations of 0.01% and 0.03% of nano TiO_2 were placed between two treatments of the control and the nano TiO_2 concentration of 0.05%. Under normal irrigation and 0.05 and 0.01% concentrations of nano TiO_2 treatments, there was no significant difference determined between 0.03% and the control treatment (Table 3).

Table 2. Means comparison of deficit irrigation and Methanol intraction effects on traits of Borage (Borago officinalis L.).

Irrigation	Methanol %(v/v)	Chlorophyll a (mg.g^{-1}.fw)	Chlorophyll b (mg.g^{-1}.fw)	Total chlorophyll (mg.g^{-1}.fw)	SPAD value	Anthocyanins (µmol.g^{-1}.fw)	Net photosynthetic rate (µmol CO_2/mg protein min)	Rubisco carboxylase activity (nmol O_2/mgprotein min)
every 7 day	Control	1.050b	0.470b	1.520b	41.40b	37.09d	22.62b	1.689b
every 7 day	15	1.110a	0.512a	1.622a	43.75a	37.89d	24.83a	1.891a
every 7 day	35	1.077b	0.473b	1.550b	42.45ab	37.54d	22.85b	1.749b
every 7 day	45	1.011c	0.448b	1.460c	40.45b	34.75d	19.18c	1.605c
every 14 day	Control	0.442g	0.191e	0.633g	32.86e	53.07c	10.79g	0.529g
every 14 day	15	0.488f	0.232d	0.721f	33.65de	55.38bc	12.26f	0.718f
every 14 day	35	0.545e	0.260dc	0.805e	35.68cd	57.80ab	16.01e	0.999e
every 14 day	45	0.631d	0.285c	0.917d	37.55c	59.24a	17.64c	1.459d

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

Spad value

According to results of comparisons of means (Table 2), foliar application of methanol led to increased Spad values, so that the highest value was related to the use of 15% (v/v) methanol under normal irrigation (every 7 days) and the least was related to the control treatment under stress condition (irrigation once every 14 days). Under the treatment of normal irrigation, the use of 15% (v/v) concentration of methanol was determined as the most effective compared with other treatments of methanol while the Spad evaluation obtained by the treatment of concentration of 45% (v/v) determined no significant difference with that of the control treatment under the same conditions. It is interesting that under stress condition (every 14 days), this concentration (45% v/v) of methanol had the highest Spad value. Also, under normal irrigation the use of nano titanium dioxide at the concentrations of 0.03% and 0.1% and 0.05% were determined as having no significant difference and thus categorized in the same statistical group with the maximum Spad value, but the control treatment was classified in another group. Treatments of 0.03% and 0.05% were in the same statistical group and had the highest Spad values under deficit irrigation (every 14 days) and treatments of 0.01% concentration and the control had no difference but had the lowest Spad values (Table 3).

Anthocyanins

Results of these tests showed that drought stress increased amounts of anthocyanins as an antioxidant, in *Borago officinalis* plants. Results of means comparison (Table 2) demonstrate that the minimun anthocyanins content was determined for the treatment tested under normal irrigation (once every 7 days), so that among all the methanol concentrations that were sprayed on the plant and the control treatment there was no significant difference in terms of anthocyanins. But, under treatments of deficit irrigation (once every 14 days) the highest amount of anthocyanins was achieved by the methanol concentration of 45% (v/v), while the amount of anthocyanin produced in stress conditions by 35% (v/v) and 15% (v/v) of methanol spraying and the control treatments were placed in subsequent groups. However, the overall results showed that all concentrations of methanol that were tested under stress condition increased anthocyanin content in this medicinal plant. The results shown in Table 3 demonstrate that under normal irrigation foliar application of all levels of nano TiO_2 and the control were categorized in one statistical group that had the least amount of antioxidant of anthocyanins compared to the stress condition, but under deficit irrigation, applications of nano TiO_2, had a positive impact on anthocyanins, So that, had the highest amount of anthocyanins and the control treatment in stressful situation, had the minimum content of this trait among all tested concentrations of nano TiO_2. According to the results of the interaction of nano titanium dioxide and methanol (Table 4), application of methanol at concentrations of 45% v/v and 0.05% of titanium dioxide nanoparticles had the highest amount of anthocyanins and no application of methanol and titanium dioxide nanoparticles had the lowest amount of anthocyanins, Also, evaluations for the other treatments fell between levels determined for these above mentioned treatments.

Table 3. Means comparison of irrigation and foliar application of nano Tio2 on traits of Borage (Borago officinalis L.).

Irrigation	Nano TiO_2 (%)	Chlorophyll a (mg.g⁻¹.fw)	Chlorophyll b (mg.g⁻¹.fw)	Total chlorophyll (mg.g⁻¹.fw)	SPAD value	Anthocyanins mol.g⁻) (μ¹.fw	Net photosynthetic rate (μmol CO_2/mg protein min)	Rubisco carboxylase activity (nmol O_2/mgprotein min)	Nitrate reductase (μmol NO_2/mg protein h^{-1})
every 7 day	Control	1.022c	0.455b	1.477c	39.45b	35.92d	21.52b	1.670b	1.016b
every 7 day	0.01	1.081ab	0.477ab	1.558ab	43.00a	37.32d	22.68ab	1.764a	1.116ab
every 7 day	0.03	1.091a	0.500a	1.591a	43.18a	3762d	23.11a	1.785a	1.211a
every 7 day	0.05	1.054b	0.472ab	1.526b	42.41a	36.41d	22.18ab	1.716ab	1.102b ab
every 14 day	Control	0.483f	0.219d	0.702f	32.59d	53.86c	11.42e	0.734e	0.276e
every 14 day	0.01	0.494ef	0.237dc	0.731ef	33.63d	54.95bc	13.75d	0.819d	0.305e
every 14 day	0.03	0.517e	0.254dc	0.771e	36.08c	57.33ab	14.42d	0.837d	0.436d
every 14 day	0.05	0.613d	0.259c	0.872d	37.44bc	59.36a	17.11c	0.734c	0.548c

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

Rubisco carboxylase activity

According to the results of means comparisons, stress induced by irrigation deficit caused a reduction in the amounts of the Rubisco carboxylase activity enzyme but tests showed that plants sprayed with methanol solution did not have an excessive reduction of this enzyme under drought stress.

Based on these results (Table 2) higher levels of this enzyme were achieved by application of methanol spray at the concentration of 15% (v/v), under normal irrigation (once every 7 days); and the the least amount was obtained by the control treatment under deficit irrigation (once every 14 days). Under normal irrigation, 15% (v/v) of methanol, results were superior in comparison with the other treatments, while, 35% (v/v) concentration of methanol and the control treatments had non-significant difference and were categorized in the same statistical group. But at a high concentration of methanol (45% v/v) the effect was less than that of the control or in other words, there was a negative effect on this trait. while, this concentration of methanol had the highest evaluation for enzyme content under conditions of defict irrigation. According to the results on Table 3, the maximum amount of Rubisco carboxylase activity enzyme was achieved by treatments with nano TiO_2 treatment at concentrations at 0.01% and 0.03% under normal irrigation so that, these concentrations were determined as having no difference and the minimum content of this enzyme was observed in the control treatment under irrigation deficit.

Table 4. Means comparison of foliar application of methanol and nano TiO_2 on anthocyanins of borage.

Methanol%(v/v)	Nano TiO_2 (%)	Anthocyanins (µmol.g^{-1}fw)
Control	Control	39.725d
Control	0.01	45.125bc
Control	0.03	47.130bac
Control	0.05	48.365ba
15	Control	48.573ba
15	0.01	45.880bac
15	0.03	49.280ba
15	0.05	42.830dc
35	Control	47.540bac
35	0.01	45.693bac
35	0.03	48.820ba
35	0.05	48.640ba
45	Control	44.725bc
45	0.01	47.850bac
45	0.03	44.688bc
45	0.05	50.740a

Also, it should be noted that, under irrigation deficit the use of the nano titanium dioxide at concentrations of 0.05% 0.03% and 0.01% and the control had the least amount of Rubisco carboxylase activity enzyme, respectively.

Nitrate reductase

Under conditions of normal irrigation, treatment of nano TiO_2 spray at the concentration of 0.03% had the highest amount of this enzyme, while the lowest evaluation for this trait was determined in the control treatment under irrigation deficit. Under conditions of normal irrigation the control treatment had the lowest evaluation for this enzyme and was placed after the nano dioxide treatments with concentrations of 0.01% and 0.05%.

Discussion

The results of photosynthetic pigments (chlorophyll a, b, total), SPAD value, net photosynthetic rate, Rubisco carboxylase activity traits measured in these tests showed that traits decreased under conditions of drought stress (irrigation once 14 days). In fact, results showed that deficit water stress did have an affect on photosynthesis, directly or indirectly, by decreasing CO_2 availability caused by diffusion limitations (Flexas *et al.*, 2007). The amount of photosynthetic pigment was reduced under drought conditions and this can be attributed to chloroplast destruction photosynthetic apparatus, photo oxidation of chlorophyll, interaction of chlorophyll with single oxygen, degradation of chlorophyll substrates, biosynthesis inhibition of new chlorophyll, and an increase in chlorophylase enzyme (El-Tayeb, 2005). In additional, Rubisco activase is susceptible to high temperatures (Craft–Brandner and Salvucci, 2000). This may be associated with drought stress. Severe drought is known to decrease amounts of Rubisco protein in plants (Majumdar *et al.*, 1991). It is important that the mechanism that induces this decrease is linked to Rubisco activity (Salvucci, 1992). However, it has yet to be determined precisely how drought stress affects the expression and activity of Rubisco activase in plants (Law and Crafts-Brandner, 2001). So it is important to note that the amount and properties of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) affect leaf photosynthetic capacity. This enzyme catalyses competing reactions, the carboxylation and the oxygenation of ribulose-1,5-bisphosphate (RuBP), initiating the photosynthetic carbon assimilation and photorespiration, respectively (Bota *et al.*, 2002). Also, a reduction in these traits has been reported in other research, Feng *et al.*, (2007) in wheat and Li *et al.*, (2006) in barley, under irrigation deficit. The results of this experiment have determined that during drought stress, there was a significant increase in anthocyanin content. Furthermore, it should be noted that stimulation of a plant's antioxidant capacity can be determined by a highly efficient protection mechanism against the harmful effects of oxygen radicals (Sherwin and Farrant, 1998). Phenols, mainly antocyanins, are chemical compounds with a high antioxidative effect that plays an important role in plant adaptation to abiotic stress factors (Kruk *et al.*, 2005). Therefore, plant tissues containing anthocyanins, are usually resistant to drought (Chalker-Scott, 2002). Konczak-Islam *et al.*, (2003) reported, a correlation between drought resistance and anthocyanin in sweet potato. Results determined that a decrease in nitrate reductase (NR) activity under deficit irrigation was linked to a decline in the rate of photosynthesis due to stomatal closure (Kaiser and Brendle-Behnisch, 1991). This implies that internal concentration of CO_2 in leaf has a role in NR activity (Fresneau *et al.*, 2007). Accordingly, Foyer *et al.*, (1998) reports that NR transcript levels in maize and tobacco decreased (by about 80% in maize leaves) under drought conditions. They suggested that there was presumably a decrease in protein synthesis due to an inhibition of processes of transcription and formation of nitrate reductase activity of native protein. Results of our experiment showed that application of methanol under conditions of deficit irrigation (irrigation every 14 days) caused an increase in evaluations for plant traits. The results of other research by Zbiec and Karczmarczyk, (1997) confirm the results of this experiment in that they report that an increased percentage of methanol (up to 20 percent) increased the amount of this enzyme, but under an increase of methanol, up to 40% (v/v), amounts of this enzyme decreased. It has been reported that the best effects from methanol application were obtained under dry, desert conditions, but not in temperate climates (Downie *et al.*, 2004).. This increase is probably due to conditions under which methanol application increased CO_2 assimilation. After absorption of methanol by a plant, the conversion of methanol to formaldehyde takes place by activity of the enzyme methanol oxidase that is then converted to form Methanoeic acid then, the format is converted to CO_2 by formation of dehydrogenase, and increased CO_2, inter-cellular (Nonomura and Benson, 1992a). Given that we know that oxygen competes with the dioxide carbon for combination with Rubisco, As we know, under conditions of water stress, intracellular levels of carbon dioxide decrease. So, spray application of methanol serves to increase the intracellular CO_2, and leads to an increase in the rate of photosynthesis. This increase is more tangible under conditions of drought stress, because the plant does not need to increase levels of carbon dioxide under normal irrigation (no water stress), since, in this condition there is sufficient CO_2 for the production of chlorophyll. And, while even the use of a high 45% (v/v) concentration of methanol, may cause toxicity. So that production is much less than the that in the control treatment. Much research has shown that methanol has a significantly positive effect on photosynthesis (Li and Yi, 2004). An increased rate of photosynthesis induced by methanol treatment was also reported in David *et al.*, (2003). The study demonstrated that the rate of oxygen evolution and photosynthetic rate of *Lemna gibba* increased under treatment of methanol spray. Also, these results are consistent with those of Mousavi Bazaz and Tehranifar, (2011) reporting that treatment of methanol spray increased evaluations of Spad in tests of Alstroemeria flowers. The same effects were also found in higher plant species, when plants were treated with a spray application of 10-50% methanol (Li and Yi, 2004). Also, methanol is probably an antioxidant and as such has an affect on plant antioxidant systems by strengthening it. This occurs as the antioxidant system is active in fighting the free radicals produced by stress induced by water deficit with an increase of anthocyanin, as an antioxidant. In accordance to the afore-mentioned the results of these tests are consistent with those reported in Nikolas *et al.*, (2003). Also, the study in Downie *et al.*, (2004) showed that a more than 2-fold increase of anthocyanin content in methanol treated leaf tissue. Results of this experiment show that foliar application of borage, with nano Tio_2, under normal irrigation and especially under deficit irrigation, significantly increased evaluations for the tested plant characteristics. In fact, titanium dioxide (nano) can improve the structure of chlorophyll and better capture sunlight, that facilitates the manufacture of pigments and transformation of light energy to active electron and chemical activity and increase photosynthetic efficiency, stimulate rubisco activase and increase photosynthesis. The results of other research Zheng *et al.*, (2005) confirm the results of these tests. It can be concluded that chlorophyll amounts, in treatments of nanoTiO_2, showed significant increases at 17-times that of the control, also the trait of photosyntetic rate showed a 29% enhancement in evaluations compared to those of the control. An increase in traits of net photosynthetic rate, Rubisco carboxylase activity and chlorophyll with nano TiO_2, was also determined in experiments reported in Gao *et al.*, (2006) and Xuming *et al.*, (2008). In tests reported in Xuming *et al.*, (2008), the protein expression of Rubisco in spinach treated with nano TiO_2, was increased by 40% in comparison with the control. They indicated that Rubisco activity in the nano TiO_2-treated spinach was significantly higher than that in the control, by up to 2.33 times. These scientists stated that nano TiO_2 treatment promotes a molecular carbon reaction such that expression of rbcS and rbcL mRNA is increased as well as activity Rubisco activity that leads to an improvement of Rubisco carboxylation and a higher rate of the photosynthetic carbon reaction, that induces greater efficiency of CO_2 assimilation (Ma *et al.*, 2008). Increasing contents of chlorophyll a, b, total and net photosynthetic rate has been reported in tests in Cui *et al.*, (2013). Also, according to these results, previous research has shown that nano TiO_2, application can increase anthocyanin that affects the antioxidant ability of cell and has a protective role in photosynthetic functioning (Morteza *et al.*, 2013) and these results are in accordance with those reported by Hong *et al.*, (2005b). That research emphasized that, nano TiO_2, promoted antioxidant stress by decreasing accumulations of superoxide radicals, hydrogen peroxide, malonyldialdehyde content and enhanced activities of antioxidant enzymes and thereby increased the rate of oxygen evolution in chloroplasts in tests on spinach under stress. In this study, it was observed that activities of nitrate reductase were significantly increased by nano TiO_2 treatment under deficit irrigation. It can be hypothesized that the improvement of nitrate reductase might be related to the generation of N_2 fixation by nano TiO_2. Therefore, it can be determined that nano TiO_2, induced the reaction of oxidation-reduction and released an energetic electron under light, which might reduce N_2 to NH_3 directly. Therefore, it is thought that the effect of catalyzing N_2 fixation of nano TiO_2 might be more significant than in the control. It is also noteworthy that nitrogen is an important composition of the important plant components of chlorophyll, amino acid, protein such that there is an interrelation between photosynthesis and nitrogen fixation. These results relate to those of Yang *et al.*, (2007). Also, the results of this experiment demonstrate that at the nano TiO_2 concentration of 0.05% and 45% v/v of methanol, evaluations for the tested traits increased by the highest amount under deficit irrigation (irrigation every 14 days). While the highest amount of measured traits, under normal irrigation (every 7 days), was obtained in the treatment of nano TiO_2 at the concentration of 0.03 % and 15% v/v concentration of methanol under normal irrigation, and nano TiO_2 at the concentration of 0.05 % and 45% v/v concentration of methanol was toxic for the plant. However, it is possible that under normal conditions, plants have no need to produce chlorophyll, as there is already an adequate supply of chlorophyll and due to the closure of pores (due to CO_2 production and acidiation of guard cells pore), under stress caused by deficit irrigation, the plant needs more methanol and nano TiO_2 for the process of photosynthesis than it needs under normal and non-stress conditions. Under stress conditions, it is reasonable that, the pores are more constricted so there is less capacity for CO_2 input into the mesophile, while, free radicals of $NADPH_2$ caused by photosystems I and II, for neutralization, required in the calvin cycle. Sufficient amounts of CO_2 and chlorophyll, are necessary for effective performance of the calvin cycle. So, for this reason, plant survival under conditions of drought stress requires higher amounts of methanol, nano TiO_2 and higher levels of chlorophyll. Also in the case of decreases in the measured tarits, high concentrations of methanol and nano TiO_2, can be determined as toxic to plants. The results reported in Albrecht *et al.*, (1995) confirm the results of this experiment in that leaf toxicity was evident at even higher concentrations of methanol (above 60% of the aqueous solution).

Conclusion

Thus according to the final results of this study the following determinations can be made:

Water deficit leads to a reduction of photosynthesis, (chlorophylls- Spad value, net photosynthetic rate, Rubisco carboxylase activity).

Water deficit caused accumulation of total polyphenols (anthocyanins).

C_3 type plants such as borage that produce ribulose 1,5-diphosphate and then 3-phosphoglyceric acid during the proces of photosynthetic carboxylation, respond to methanol by increasing biomass production in the form of carbon dioxide, and this production is caused by the rapid oxidation of methanol that can successfully compete with oxygen for Rubisco.

Plants that grow in a CO_2 enriched atmosphere are less susceptible to drought, this is because their stomata are closed, so transpiration decreases and net photosynthesis is thus elevated.

Methyl alcohol may be an alternate source of carbon for plants.

Exposure to exogenous methanol increases growth in C3 type crops that have experienced drought stress.

Nano Titanium dioxide can improve photosynthetic apparatus and enhance a plant's ability to capture sunlight, that affects the manufacture of pigments and the transformation of light energy to active electron and chemical activity and thus increase photosynthetic efficiency, especially under drough stress.

Methanol and nano Tio_2, by increasing anthocyanins, strengthen a borage plant's immune system under water stress conditions.

Methanol and nano Tio_2 spraying on the plants aerial parts increased traits of plants that suffer a shortage of water compared with the response of plants under normal irrigation conditions.

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