Physiological and yield responses of purple coneflower (*Echinacea purpurea* (L.) Moench) to nitrogen sources at different levels of irrigation

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Abstract *Echinacea purpurea* (L.) is one of the most important medicinal plants in the world showing different biochemical reactions as affected by drought stress and nitrogen fertilizer. The purpose of this study was to determine the effect of nitrogen on soluble protein, enzyme activities, carotenoids metabolism, greenness and biological yield of the *Echinacea purpurea* under different levels of irrigation. The experiment was conducted in a research field in Iran during 2013–2015. Irrigation treatments included irrigation after 25, 50 and 75% soil water depletion, and nitrogen sources were no nitrogen (N), nitroxin, 40 kg N ha⁻¹, 40 kg N ha⁻¹ + nitroxin and 80 kg N ha⁻¹. Nitroxin is a biofertilizer including *Azotobacter* and *Azospirillum*. The activity of catalase (11.9–21.3 mmol g⁻¹fw min⁻¹), peroxidase (40.1–48.9 μmol g⁻¹fw min⁻¹), polyphenoloxidase (0.79–3.63 mmol g⁻¹fw min⁻¹) increased in both years under drought stress conditions. The lowest greenness (50.1–45.9) was achieved from no-application of nitrogen in the irrigation treatment after 75% water depletion. The results demonstrated the beneficial effects of nitrogen sources on physiological reactions, especially peroxidase, polyphenol oxidase and carotenoids metabolism. It is concluded that irrigation

of *E. purpurea* based on 50% water depletion and 80 kg nitrogen as well as the combination of nitroxin and 40 kg nitrogen treatments should be an appropriate choice for 2 years.

Keywords Antioxidant enzymes · Biological yield · Drought stress · Soluble proteins · Carotenoids

Introduction

Purple coneflower (*Echinacea purpurea* L.) is a perennial and herbaceous medicinal plant belonging to the family Asteraceae. Although all parts of *Echinacea purpurea* can be used for medicinal purpose, leaves, flowers, and roots are widely used in the preparation of pharmaceutical tablets and syrups. These products are used to stimulate immune system and for treating respiratory disorders and viral infections (Tsai et al. 2012). *Echinacea purpurea* has been cultivated in some parts of Iran (e.g., Isfahan and Shiraz) and used in some herbal drugs (Zolgharnein et al. 2010).

Drought is a major factor limiting crop production, particularly in arid and semiarid regions (Zhang et al. 2007). Drought stress can reduce regular growth and plants productivity in several ways. The production of reactive oxygen species (ROS) under stress conditions is one of these ways. In drought stress condition, plants close the stomata to control water loss by reducing the transpiration. Subsequently, the concentration of CO₂ is reduced in mesophyll tissue. Consequently, photosynthesis dark reactions are disturbed, and products of the light reaction, including ATP and NADPH, are not used. Due to lack of oxidation of NADPH molecules, the usage of NADP to receive electron would decrease. Therefore, oxygen molecule in the path of electron transfer serves as a receiver of

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electron replacement, leading to the formation of superoxide radical ($O^{\cdot-2}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$) (Sairam and Srivastava 2001; Turkan et al. 2005).

The protection against oxidative stress is through enzymatic and non-enzymatic antioxidant mechanisms with high efficiency that can destroy or neutralize free radicals and prevent the oxidative damage to the plant (Furtana and Tipirdamaz 2010). Enzymatic defense system, includes superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), polyphenol oxidase (PPO) and other enzymes (Appel and Hirt 2004). The results of Kotapati et al. (2014) in finger millet and Freire de Sousa et al. (2016) in maize showed that with increasing drought stress, peroxidase and catalase enzyme activity enhanced. Also, effects of drought stress on catalase, peroxidase and polyphenol oxidase in maize (Zhang et al. 2007) and in creeping bent grass plant (Chang et al. 2016) have been reported. CAT and POX are enzymes that catalyze the conversion of H_2O_2 to water and O_2 under oxidative stress condition (Wang et al. 2012). The results of other researchers indicate that plants with higher levels of catalase and peroxidase are more resistant to drought. The *o*-hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to quinones in the presence of oxygen are carried out by copper-containing enzymes called polyphenol oxidases (PPOs). According to Zolgharnein et al. (2010), the most active compounds of *E. purpurea* are polyphenols-caffeic acid derivatives. Changing the activity of polyphenol oxidases will affect the content of phenolic compounds in the plant cells (Saiedian et al. 2007).

Carotenoids play an important role in the light-harvesting complex and the photo-protection of photosystems (Inze 2008). Several studies have shown that carotenoids are highly important in protecting the photosynthesis apparatus against photodamage, by inter-conversions among the xanthophyll molecules (Ort 2001; Ramel et al. 2012).

Nitrogen is important in the development and physiological processes of plants (Purves et al. 2004), playing a role in the structure of proteins, enzymes, coenzymes and cytochromes (Hasegawa et al. 2008). The use of an appropriate amount of nitrogen is required to regulate plant growth, and increase yield and resistance to environmental stresses (Zhu et al. 2014; Wang et al. 2012; Boeckx et al. 2015). Nitrogen as well plays a crucial role in the metabolism of antioxidant enzymes under drought stress (Zhang and Liu 2001). Furthermore, Zhang et al. (2007) reported that application of nitrogen increased the activity of peroxidase and catalase compared with control in maize under drought stress.

The chlorophyll meter SPAD (Soil–Plant Analysis Development) is a portable device that was first used for

estimation of nitrogen in rice (*Oryza sativa*) leaves. Due to the high correlation between the amount of nitrogen and greenness, chlorophyll meter was used to measure the greenness in leaves and estimate the relative chlorophyll concentration of leaves (Reis et al. 2009). Reis et al. (2009) showed the high correlation between the nitrogen and SPAD value in the coffee plant. However, there are few studies on the physiological and biochemical mechanisms of medicinal plants, especially purple coneflower, under drought and nitrogen deficiencies. This study aimed to investigate changes in leaf soluble proteins, CAT, POX and PPO activity, carotenoids metabolism, greenness level and their relationship with the biological yield of purple coneflower in response to nitrogen sources under different levels of irrigation.

Materials and methods

Field site description

This field experiment was conducted in 2013–2014 and 2014–2015 cropping years in Lordegan region, Iran. The geographical location of the station was 31°31'N and 50°48'E with altitude 1564 m. The mean annual temperature and precipitation in the region are 15.1 °C and 550 mm, respectively. Table 1 shows the chemical and physical properties of the soil from the depth of 0–30 cm.

Design of the experiment

The experiment was a split plot in randomized complete blocks with three replications. Irrigation treatments were scheduled based on the maximum allowable depletion (MAD) percentage of the soil available water (SAW) between – 0.03 and – 1.5 MPa (Kramer and Boyer 1995). Irrigation treatments based on MAD of SAW were allocated as main-plot. Irrigation treatments applied were irrigation after 25% MAD of SAW which was considered as control and irrigation after 50% MAD of SAW and 75% MAD of SAW which corresponded to moderate and severe drought stress, respectively. Irrigation treatments started at the stem elongation stage. The rows and plants in each row space were adjusted on 50 and 20 cm, respectively. Adjacent plots and replications were 2 m apart. Each experimental plot size was 2.5 × 3.5 m. To achieve irrigation treatments, a soil sample from the depth of root zone, 24 h after daily irrigation, was obtained. The amount of available water was calculated in the soil by weighting method (Sanchez et al. 1998).

Five levels of nitrogen fertilizer, including lack of nitrogen (N) (control), nitroxin (5 L ha⁻¹), 40 kg N ha⁻¹, 40 kg N ha⁻¹ + nitroxin (5 L ha⁻¹) and 80 kg N ha⁻¹

Table 1 Physicochemical properties of farm soil

Character	Texture	pH	EC (ds m ⁻¹)	Organic matter (%)	Total nitrogen (%)	Phosphorus (mg kg ⁻¹)	Potassium (mg kg ⁻¹)	Manganese (mg kg ⁻¹)	Iron (mg kg ⁻¹)	Zinc (mg kg ⁻¹)
Value	Clay-loam	7.7	0.84	1.14	0.19	51.1	274	6.23	4.11	0.52

were allocated as sub-plot. Urea fertilizer was used as a source of mineral nitrogen so that $\frac{1}{2}$ was applied at the 5th leaf stage and $\frac{1}{2}$ at stem elongation. Nitroxin is a bio-fertilizer including *Azotobacter* and *Azospirillum*. Leaf samples were taken after complete application of nitrogen and two cycles of irrigation treatments at the flowering stage for biochemical measurements.

Determination of soluble protein

For the determination of antioxidant enzyme activities and the contents of soluble proteins, 200 mg frozen leaves (fixed with liquid nitrogen and stored at -40°C) were extracted in 2 ml buffer phosphate (0.1 M and pH = 6.8). Then, extract was centrifuged at $13,000\times g$ for 15 min at 4°C and supernatant was collected. Soluble protein contents were determined according to the method of Bradford (1976), using the reagent Coomassie Brilliant Blue G-250, followed by absorbance readings at 595 nm using bovine serum albumin as standard.

Determination of enzyme activities

Catalase activity was measured according to Cakmak and Horst (1991). The reaction mixture contained 0.5 ml H_2O_2 (10 mM), 2.5 ml sodium phosphate buffer (pH = 6.8) and 100 μl extracted solution. CAT activity was determined by the decrease in absorbance at 240 nm due to the application of H_2O_2 .

The activity of peroxidase enzymes was determined according to the protocol proposed by Ghanati et al. (2002), the reaction mixture contained 2 ml of potassium phosphate buffer (60 mM with pH = 6.1), 0.5 ml of guaiacol (28 mM), 0.5 ml of H_2O_2 (5 mM) and 100 μl of extracted solution (such as extract of protein measurement). The increase in absorbance at 470 nm (by spectrophotometer (UV-2550, Shimadzu, Japan) was recorded.

Polyphenol oxidase activity was assayed according to Ghanati et al. (2002). The reaction mixture contained 100 μl of extracted solution, 500 μl methyl catechol (0.02 mM), 1900 μl of potassium phosphate buffer (60 mM with pH = 6.1) and 500 μl of H_2O_2 (5 mM), and then the absorbance at 410 nm was recorded.

Carotenoids content

Carotenoids were determined using the method of Lichtenthder (1987). A total of 0.5 g of frozen leaves was extracted, using 80% acetone, and the absorbance of the supernatants was measured using spectrophotometer (UV-2550, Shimadzu, Japan) at 470 nm.

Leaf greenness

Chlorophyll meter device (SPAD-502Plus—KONICA MINOLTA) was used to measure leaf greenness by averaging five plants per treatment and three measurements per plant on the youngest fully expanded leaf at stem elongation.

Biological yield

Plant harvest was done at flowering stage, one time in the first year and three times in the second year. After drying at room temperature ($20\text{--}30^{\circ}\text{C}$), plants weighed in each plot.

Statistical analysis

Data analysis was performed using the GLM procedure in SAS 9.1 (SAS Institute 2009). Means were compared using LSD at P value = 5%. SAS correlations program were used to analyze the relationships between the protein, catalase, peroxidase, polyphenol oxidase, SPAD value and biological yield.

Results

Leaf soluble protein

Severe drought stress caused reductions in the content of leaf soluble protein (Fig. 1a). In this treatment, the average soluble protein content was 15% and 27% lower than the control (irrigation after 25% MAD of SAW) in the first and second year respectively. There were no significant differences in soluble protein between the irrigation after 25% and 50% MAD of SAW conditions (Fig. 1a).

Fig. 1 Mean comparison of different levels of irrigation (a) and nitrogen (b) in each year for leaf protein content. MAD of SAW: Maximum allowable depletion percentage of the soil available water. nitro0: lack of nitrogen; nitroxin: nitroxin (5 L ha^{-1}); nitro40:

40 kg N ha^{-1} ; nitroxin + 40: $40 \text{ kg N ha}^{-1} + \text{nitroxin } (5 \text{ L ha}^{-1})$; nitro80: 80 kg N ha^{-1} . In each year, columns followed by the same letters are not significantly different (L.S.Means Procedure)

It was observed that application of nitrogen increased soluble protein in leaf compared to the lack of nitrogen in both years (Fig. 1b). Therefore, using combined nitrogen and nitroxin, enhanced soluble protein in leaf by 2.3 times compared to the control in the first year (Fig. 1b). Generally, leaf soluble protein content increased in the second year.

Enzyme activity

Figure 2 depicts the activity of antioxidative enzymes in the leaf of *E. purpurea*. Catalase activity had a different response to nitrogen application in 2 years. In the first year, application of 80 kg N ha^{-1} , nitroxin and nitrogen combination and nitroxin increased CAT activity approximately 4.6 times as much as that in control under irrigation after 25% MAD of SAW (Fig. 2a). Also, CAT activity was increased by nitroxin (57% higher than control) and nitrogen combination and nitroxin (89% higher than control) treatment under irrigation after 50% MAD of SAW. Drought stress led to an increase in catalase activity. Application of 80 kg N ha^{-1} significantly increased the catalase activity compared to other treatments in irrigation

after 75% MAD of SAW. Under irrigation after 25% MAD of SAW condition, there were significantly higher activities of catalase in 40 and 80 kg N ha^{-1} treated in the second year. For CAT activity, under irrigation after 50% and 75% MAD of SAW conditions, nitroxin application led to a significant increase in the catalase activity. However, in irrigation after 75% MAD of SAW treatment, the highest activity of catalase was obtained from the application of nitroxin, but other nitrogen source levels cause a significant decline in the catalase activity compared to the control (Fig. 2a). In general nitroxin application led to a significant increase in the CAT activity in the second year, which can significantly improve antioxidant defense.

Different nitrogen sources during the 2 years of the experiment increased the POX activity compared to the control (no nitrogen fertilizer) (Fig. 2b). In the first year, although different sources of nitrogen increased peroxidase activity than control, there was no significant difference between the different sources of nitrogen under the irrigation after 25% and 50% MAD of SAW conditions. In irrigation after 75% MAD of SAW condition, application of nitroxin and nitrogen combination caused increase in the activity of peroxidase compared to other nitrogen sources

Fig. 2 Mean comparison of the nitrogen levels in each irrigation levels for Catalase (a), peroxidase (b) and polyphenoloxidase (c) activity in 2 years experiment. MAD of SAW: Maximum allowable depletion percentage of the soil available water. nitro0:

lack of nitrogen; nitroxin: nitroxin (5 L ha⁻¹); nitro40: 40 kg N ha⁻¹; nitroxin + 40: 40 kg N ha⁻¹ + nitroxin (5 L ha⁻¹); nitro80: 80 kg N ha⁻¹. In each year, columns followed by the same letters are not significantly different (L.S.Means Procedure)

in both years (Fig. 2b). In the second year, POX activity increased 101% and 44% by nitroxin under irrigation after 25% and 50% MAD of SAW conditions (Fig. 2b). This increase was not significant from 40 and 80 kg N ha⁻¹ in this irrigation treatment. For POX activity under irrigation

after 75% MAD of SAW condition, the combination of nitroxin and nitrogen application led to a significant increase in the POX activity.

Although nitrogen application increased the activity of polyphenol oxidase, this increase was not significant with

control in irrigation after 25% and 50% MAD of SAW conditions at first year. However, in the irrigation after 75% MAD of SAW condition, application of nitrogen sources increased enzyme activity; application of nitroxin and 80 kg N ha⁻¹ increased PPO activity by 50% compared to the control (Fig. 2c). In general, nitroxin has a positive effect on PPO activity in the first year.

PPO activity in the second year was higher than in the first year. In the second year, PPO activity in both non-stressed and stressed conditions increased by adding nitrogen nutrition. In irrigation after 25% MAD of SAW conditions, nitrogen and nitroxin combination increased the activity of PPO activity compared to control by 1.5 times. In irrigation after 50% MAD of SAW, the highest activity was obtained by combining nitroxin and nitrogen and 80 kg N ha⁻¹, which was not different significantly. In addition, the use of different sources of nitrogen significantly increased PPO activity compared to control in irrigation after 75% MAD of SAW condition. However, there was no significant difference between nitroxin and nitrogen combination and control (Fig. 2c).

Leaf carotenoids

Nitrogen sources caused to increase leaf carotenoids compared to control in all irrigation levels (Fig. 3). There was no significant difference between different sources of nitrogen in irrigation after 25% MAD of SAW. The maximum of carotenoids was obtained using 80 kg N ha⁻¹ in irrigation after 50% MAD of SAW; however, there was no significant difference with 40 kg N ha⁻¹ and nitroxin. The combined 40 kg N ha⁻¹ and nitroxin increased carotenoids by 101% compared to the control in irrigation after 75% MAD of SAW condition. However, it did not differ from 40 kg N ha⁻¹.

Leaf greenness

Leaf greenness was affected by irrigation in the first year. It was observed that drought stress reduced the leaf greenness. The leaf greenness in irrigation after 25% MAD of SAW treatment was 4% and 20% higher than moderate and severe stresses in the first year (Fig. 4a). In the second year, there was no significant difference between irrigation after 25% and 50% MAD of SAW. In irrigation after 75% MAD of SAW, the greenness was 12% lower than irrigation after 25% MAD of SAW (Fig. 4a). Nitrogen source caused a considerable increase in leaf greenness. Thus, application of 80 kg of nitrogen and the nitroxin and nitrogen combination increased leaf greenness approximately 27% compared to the control (Fig. 4b).

Biological yield

It was observed that application of nitrogen sources significantly increased biological yield in all of the irrigation treatments in both years (Fig. 5). Hence, the application of 80 kg N ha⁻¹ increased biological yield (100%) compared to the control under irrigation after 25% MAD of SAW conditions in the first year. In this year, maximum of biological yield was achieved from application of nitrogen and nitroxin combination in irrigation after 50% MAD of SAW treatment, which was 108% higher than the control. However, the application of 80 kg N ha⁻¹ nitrogen did not have a significant difference with the nitrogen and nitroxin combination. Under irrigation after 75% MAD of SAW conditions, application of different nitrogen sources enhanced biological yield compared to the control, but there was no significant difference between different nitrogen sources. In the second year, the effect of nitrogen treatments was similar to that of the first year under irrigation after 25% MAD of SAW conditions. Combination

Fig. 3 Mean comparison of the nitrogen levels in each irrigation levels for carotenoids content. MAD of SAW: Maximum allowable depletion percentage of the soil available water. nitro0: lack of nitrogen; nitroxin: nitroxin (5 L ha⁻¹); nitro40: 40 kg N ha⁻¹;

nitroxin + 40: 40 kg N ha⁻¹ + nitroxin (5 L ha⁻¹); nitro80: 80 kg N ha⁻¹. In each year, columns followed by the same letters are not significantly different (L.S.Means Procedure)

Fig. 4 Mean comparison of different levels of irrigation (a) in each year and different levels of nitrogen (b) for leaf greenness. MAD of SAW: Maximum allowable depletion percentage of the soil available water. nitro0: lack of nitrogen; nitroxin: nitroxin (5 L ha⁻¹); nitro40:

40 kg N ha⁻¹; nitroxin + 40: 40 kg N ha⁻¹ + nitroxin (5 L ha⁻¹); nitro80: 80 kg N ha⁻¹. In each year, columns followed by the same letters are not significantly different (L.S.Means Procedure)

Fig. 5 Mean comparison of the nitrogen levels in each irrigation levels for biological yield in 2 years experiment. MAD of SAW: Maximum allowable depletion percentage of the soil available water. nitro0: lack of nitrogen; nitroxin: nitroxin (5 L ha⁻¹); nitro40:

40 kg N ha⁻¹; nitroxin + 40: 40 kg N ha⁻¹ + nitroxin (5 L ha⁻¹); nitro80: 80 kg N ha⁻¹. In each year, columns followed by the same letters are not significantly different (L.S.Means Procedure)

of nitrogen and nitroxin and 80 kg N ha⁻¹ treatments had the highest yield under irrigation after 50% MAD of SAW

conditions, while the biological yield in nitroxin treatment was 8.5% higher than 40 kg ha⁻¹ in this irrigation level.

However, in irrigation after 75% MAD of SAW conditions, application of nitroxin and nitrogen combination, and 40 kg N ha⁻¹ had the highest biological yield. Generally, the second year had higher biological yield than the first year.

Discussions

Our results show that the use of nitrogen sources would increase leaf protein content. Since nitrogen is one of the main components of amino acids, increased application and absorption of nitrogen lead to increment in protein synthesis. A reason for significant reduction of protein in 80 kg N ha⁻¹ treatment can be attributed to the greater volume of biomass produced in irrigation after 25% and 50% MAD of SAW conditions (Fig. 5). Additionally, the application of 80 kg N ha⁻¹ in irrigation after 75% MAD of SAW condition disrupts nitrogen uptake due to lack of moisture. Mohammadkhani and Heidari (2008) reported that reducing the amount of soluble proteins in corn grown under the influence of drought stress was associated with a sharp drop in photosynthesis process and subsequent reduction of protein production and, finally, reduction of protein synthesis. The reasons for the high protein in nitroxin application are better and continuous absorption of nitrogen by the plant and the resulting increase in nitrogen in the shoot.

The results show that drought stress reduced the leaf soluble proteins. Feller et al. (2008) reported that drought stress induced the expression of the genes coding for intracellular proteases, resulting in the decomposition of proteins, reutilization of nitrogen and subsequent synthesis of soluble materials. Therefore, reduction of protein content under drought stress seems to be related to the reduction of synthesis and the higher activity of protein-degrading enzymes. The results of Jalilian et al. (2012) showed that a combination of nitrogen and biofertilizer increased the amount of soluble protein in the leaves of sunflower. Regarding our results, although the content of the soluble proteins was decreased by drought stress, enzymes activity (CAT, POX, and PPO) was up-regulated under drought stress (Figs. 1a, 2 a, b, c). Therefore, this protein reduction can be related to protein degradation and reduction in synthesis under drought stress conditions. Besides, the positive and significant correlation between the protein and CAT, POX, and PPO enzymes (Table 2) indicate that increasing the amount of leaf protein increases the activity of these enzymes.

Our results show that drought stress can be oxidative stress for the *Echinacea purpurea*. Investigating the diagrams of enzymes activity shows the significant enhanced leaf antioxidant enzymes activity in stress conditions

compared to the control in both years. Moreover, high N treatments did not increase the activities of CAT in *Echinacea* under irrigation after 50% and 75% MAD of SAW conditions soil moisture regimes in the second year, indicating the negative effect of nitrogen on enhancing drought resistance by increasing the antioxidant capacity in severe drought conditions. Catalase is one of the antioxidant enzymes playing a key role in eliminating H₂O₂ from the cells. According to the results, we observed the higher constitutive activity of CAT from nitroxin treatment in the second year. As a consequence, it may be due to the direct and indirect role of nitroxin in the absorption of other nutrients and moisture absorption under stress conditions. Our results demonstrated that the CAT activities were increased in the second year. This increase might be related to the plant age.

Our study indicates that under all nitrogen treatment, POX activity were higher than control (no nitrogen), whereas under irrigation after 75% MAD of SAW, application of nitrogen and nitroxin combination have the best effect on POX activity. This result can be due to the role of nitroxin in increasing the growth of hairy roots and the better and continuous absorption of water, nitrogen and other elements (Gilik et al. 2001) since by increasing the hairy roots, it is possible to absorb more nitrogen and other elements. All in all, the increase in POX might be considered as a key point for the catalysis of H₂O₂, especially in the case of reduced CAT compared to control in both years.

The present study indicates a significant increase in PPO activity in leaf under drought stress by nitrogen in irrigation after 75% MAD of SAW treatment in the first year, whereas PPO enzyme activity was significantly affected by nitrogen in all irrigation treatments in the second year.

Nitrogen had a considerable influence on the activity of enzymes involved in photosynthesis such as RUBISCO (Xiong et al. 2015). In the absence of sufficient nitrogen, RUBISCO activity reduced. Under these conditions, the amount of electron in the oxidative reduction pathway was enhanced through the photo-inhibition reaction within the chloroplast, so, ultimately raising the production of ROS. In these conditions, it is expected that the enzymatic and non-enzymatic combinations of antioxidants will increase in the leaves.

Wang et al. (2012) reported that an increase in the application of nitrogen led to enhancing the production of antioxidant enzymes such as CAT, POX, and SOD in *Populus yunnanensis*. Boeckx et al. (2015) reported that one of the reasons for increasing activity of PPO was the effect of winter passing phase on this enzyme. Therefore, increasing the activity of PPO in the second year may be due to the effect of seasonal changes and winter on *Echinacea purpurea*.

Table 2 Correlation coefficients between evaluated traits

	1	2	3	4	5	6
1. Leaf soluble protein	1					
2. SPAD value	0.02 ^{ns}	1				
3. Leaf carotenoids	− 0.42**	0.41**	1			
4. Catalase activity	0.372**	− 0.42**	− 0.21*	1		
5. Peroxidase activity	0.482**	− 0.05 ^{ns}	0.15 ^{ns}	0.594**	1	
6. Polyphenol oxidase activity	0.732**	− 0.29**	− 0.38**	0.674**	0.536**	1
7. Biological yield	0.882**	0.94**	− 0.38**	0.365**	0.61**	0.8*

^{ns} non-significant

*, **Significant at 5 and 1% probability level of error

The correlation analysis (Table 2) shows that PPO is positively correlated with protein ($r = 0.73$) and the biological yield ($r = 0.8$). Some pieces of evidence suggest that the PPO enzyme has an effective role in the photosynthesis processes due to its relationship with photosystems and the role played by chlorophyll protecting against O_2 (Boeckx et al. 2015). Therefore, according to the results, PPO plays a crucial role in protecting photosystems and increasing biological yield in *Echinacea purpurea*. Lee et al. (2009) reported that the activity of PPO enhances the production of dry matter in red clover plants. In general, increasing catalase, peroxidase and polyphenol oxidase activity can be an effective strategy to adapt to drought stress conditions. Indeed, these enzymes can help to overcome the damage to the tissues metabolism by reducing toxic levels of H_2O_2 produced during cell metabolism and protection against oxidative stress. The results showed that application of nitroxin and nitrogen affected PPO activity in the second year. It seems that nitroxin treatment can increase drought tolerance through osmotic adjustment (increasing soluble proteins) and also can increase antioxidant capacity (through increasing CAT, POX, and PPO activity) in *Echinacea purpurea* under irrigation after 50% and 75% MAD of SAW conditions. These results confirm that nitroxin application, in addition to reducing the use of chemical fertilizers and their destructive effects, can increase the plant's ability to cope drought stress. In current study, positive correlations between activities of CAT, POX and PPO (Table 2) indicated that the increase of CAT activity was accompanied by increases of CAT and POD activities as a result of scavenging H_2O_2 .

One of the most important functions of carotenoids is protecting the photosynthetic system against the ROS under drought stress conditions (Bartwal et al. 2013). According to our results, the maximum increase in the carotenoids was observed in the application of nitroxin and nitrogen combination treatment under irrigation after 75% MAD of SAW conditions. Carotenoids are hydrocarbon structures and plants need nitrogen and photosynthates to

produce these structures. Therefore, with increasing nitrogen, the production of carotenoids also increases. As an antioxidant, carotenoids are considered to be the first line of defense of chlorophyll against superoxide radical (O^{2-}) damage by scavenging them. The positive correlation ($r = 0.41$) between carotenoids and greenness (Table 2) confirms that carotenoids can play an important role in protecting chlorophyll, especially under drought stress. These results are consistent with the findings of Abdalla and El-Khoshiban (2007). They reported that carotenoids increased in *Triticum aestivum* cultivars under drought stress.

The chlorophyll meter results show that greenness was decreased under limited irrigation. Reduction of nitrogen uptake and consequently chlorophyll production lead to decline greenness under limited irrigation (irrigation after 75% MAD of SAW). On the other hand, our results show that drought stress can be oxidative stress for the *Echinacea purpurea*. Drought stress induced excess active oxygen species in cells, which may cause damage to chlorophyll and other pigments content. Application of nitrogen improves the production of chlorophyll since nitrogen is a key constituent of chlorophyll. Also, the results show that nitrogen and nitroxin increased the activity of enzymes and carotenoids content (Figs. 2 a, b, c, 3), thereby preventing chlorophyll oxidation under drought stress. Also, the results showed that greenness in the first year was more than the second year. It is probably related to leaf thickness or leaf area. Due to the presence of new secondary stems and consequently higher plant shoot volume in the second year, nitrogen and greenness spread over the larger leaf area in the second year.

The negative correlation among activities of CAT, PPO and SPAD value suggests the increasing in CAT and PPO activities is a result of high demand for scavenging H_2O_2 . So, the presence of high H_2O_2 increases the oxidation of pigments. Reduction of pigments content is a typical symptom of oxidative stress. The high and positive correlation ($r = 0.94$) between biological yield and greenness confirms that SPAD values can be a suitable tool for plant

production (biomass) estimation since it can measure the amount of nitrogen and chlorophyll in the plant. Many studies have shown that a linear relationship exists between chlorophyll content, photosynthesis, nitrogen and SPAD values (Reis et al. 2009).

Many researchers maintain that reduced transpiration interrupts the normal plant life under drought stress conditions, leading to the emergence of inhibitory effects, including reduction of plant growth and biological yields. Therefore, it seems that reduction in biological yield with application of 80 kg N ha⁻¹ under irrigation after 75% MAD of SAW condition, especially in the second year can be due to increased osmotic pressure around the roots, impairment in nitrogen and other elements absorption and finally decreased transpiration and consequently decreased growth in the plant. Furthermore, the experiment results indicate that nitroxin activity decreases in drought stress conditions; however, nitroxin significantly increases biological yield compared to the control in drought stress conditions. The application of nitroxin and nitrogen combination significantly increased biological yield. This increase can be attributed to the role of bacteria in enhancing the absorption of nutrients by improving root growth and increasing the antioxidant capacity (increasing the activity of enzymes and carotenoid content) of the plant as a result of better plant growth.

Supplying of the bacteria nitrogen requirement by nitrogen and nitroxin combination treatments provided a basis for the better activity of nitroxin's bacteria. Decreased efficiency of resources by water and nitrogen limitations ultimately reduces the biological yield. There are several reasons for decreasing growth under water stress conditions, including reducing the amount of the soluble protein and enhancing ROS in stress conditions. High and positive correlation ($r = 0.88$) of the leaf proteins content with the biological yield is due to the role of protein in the leaves (Table 2). The RUBISCO consists of approximately 30–60% of the total soluble proteins in the leaves of a plant (Feller et al. 2008). This enzyme has an important role in the photosynthesis process. Since RUBISCO is an important and the most abundant protein in the leaves, any reduction in soluble proteins content is indicative of a decrease in the concentration of RUBISCO, which can lead to a reduction in the photosynthesis rate. Experimental observations confirm that deficiency of water and nitrogen due to their impacts on the amount of leaf proteins and also the activity of free radicals can affect plant growth. Nitrogen, as one of the main constituents of proteins and enzymes can be able to improve the effects of water deficiency stress by increasing the activity of antioxidant enzymes and leaf protein and carotenoids content. Our results were generally in line with Bonomelli et al. (2005) in *Echinacea purpurea*. Nabizadeh et al.

(2012) in millet also reported that nitroxin use improves biological yield.

Generally, it was found that nitrogen application reduced the destructive effects of drought stress on *Echinacea purpurea*. However, lower levels of chemical fertilizer are more effective in increasing antioxidant capacity and better growth under irrigation after 75% MAD of SAW condition. Nitroxin and combination of nitroxin and nitrogen proved to be favorable for antioxidant defense strategy and higher protein content in *E. purpurea*. In this experiment, the highest biological yield was obtained from irrigation after 50% MAD of SAW and using 80 kg N ha⁻¹ as well as the combination of nitroxin and 40 kg N ha⁻¹ in 2 years. However, due to the risk of environmental pollution by chemical fertilizers and urgency of decreasing chemical nitrogen fertilizer for sustainability in agriculture, the combination of nitroxin and 40 kg N ha⁻¹, and nitroxin can be suitable alternative nitrogen nutrition instead of 80 kg N ha⁻¹ and 40 kg N ha⁻¹ respectively.

References

- Abdalla M, El-Khoshiban N (2007) The influence of water stress on growth, relative water content, photosynthetic pigments, some metabolic and hormonal contents of two *Triticum aestivum* cultivars. *J Appl Sci Res* 3:2062–2074
- Appel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress and signal transduction. *J Plant Biol* 55:373–399
- Bartwal A, Mall R, Lohani P, Guru SK, Arora S (2013) Role of secondary metabolites and brassinosteroids in plant defense against environmental stresses. *Plant Growth Regul* 32:216–232
- Boeckx T, Winters AL, Judith Webb K, Kingston-Smith AH (2015) Polyphenol oxidase in leaves: is there any significance to the chloroplast localization? *J Exp Bot* 66(12):3571–3579
- Bonomelli C, Cisterna D, Reciné C (2005) Effect of nitrogen fertilization on *Echinacea purpurea* mineral composition. *Cien Invest Agrar* 32(2):85–91
- Bradford M (1976) A rapid and sensitive method for the quantitation of protein utilizing the principle of protein-dye binding. *Annu Rev Biochem* 72:248–254
- Cakmak I, Horst W (1991) Effect of aluminum on lipid peroxidation, superoxide dismutase, catalase and peroxidase activities in root tip soybean. *J Plant Physiol* 83:463–468
- Chang Z, Liu Y, Dong H, Teng K, Han L, Zhang X (2016) Effects of cytokinin and nitrogen on drought tolerance of creeping bentgrass. *J PLoS ONE* 11(4):e0154005
- Feller U, Anders I, Mae T (2008) Rubiscolytics: fate of Rubisco after its enzymatic function in a cell is terminated. *J Exp Bot* 59:1615–1624
- Freire de Sousa DP, Braga BB, Gondim FA, Gomes-Filho E, Martins K, Batista de Brito PO (2016) Increased drought tolerance in maize plants induced by H₂O₂ is closely related to an enhanced enzymatic antioxidant system and higher soluble protein and organic solutes contents. *Theor Exp Plant Physiol* 28:297–306
- Furtana GB, Tipirdamaz R (2010) Physiological and antioxidant response of three cultivars of cucumber (*Cucumis sativus* L.) to salinity. *Turk J Biol* 34:287–296

- Ghanati F, Morita A, Yokota H (2002) Induction of suberin and increase of lignin content by excess Boron in Tobacco cell. *J Soil Sci Plant Nutr* 48:357–364
- Gilik BR, Penrose D, Wenbo M (2001) Bacterial promotion of plant growth. *Biotechnol Adv* 19(2):135–138
- Hassegawa RH, Fonseca H, Fancelli AL, Silva VN, Schammas EA, Reis TA, Corrêa B (2008) Influence of macro- and micronutrient fertilization on fungal contamination and fumonisin production in corn grains. *Food Control* 19:36–43
- Inze D (2008) Oxidative stress in plant. *Environ Exp Bot* 48(5):351–358
- Jalilian J, Mohammad Modarres-Sanavy SA, Saberali SF, Sadat-Asilan K (2012) Effects of the combination of beneficial microbes and nitrogen on sunflower seed yields and seed quality traits under different irrigation regimes. *Field Crop Res* 127:26–34
- Kotapati KV, Kumar Palaka B, Kandukuri A, Pamuru RR, Ampasala DR (2014) Response of anti-oxidative enzymes and lipoxygenases to drought stress in Finger Millet leaves (*Eleusine coracana* (L.) Gaertn). *IJPAES* 4(3):644–653
- Kramer PJ, Boyer JS (1995) Water relation of plants and soils. Academic Press, New York
- Lee MRF, Tweed JKS, Minchin FR, Winters AL (2009) Red clover polyphenol oxidase: activation, activity and efficacy under grazing. *J Anim Feed Sci Technol* 149:250–264
- Lichtenthder HK (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Methods Enzymol* 147:350–382
- Mohammadkhani N, Heidari R (2008) Drought-induced accumulation of soluble sugars and proline in two maize varieties. *WASJ* 3:448–453
- Nabizadeh E, Habibi H, Hosainpour M (2012) The effect of fertilizers and biological nitrogen and planting density on yield quality and quantity *Pimpinella anisum* L. *Eur J Exp Biol* 2(4):1326–1336
- Ort D (2001) When there is too much light. *Plant Physiol* 125:29–32
- Purves WK, Savada S, Orians GH, Heller HC (2004) Life: the science of biology. Sinauer Associates's, Sunderland, p 1121
- Ramel F, Birtic S, Ginies C, Soubigou-Taconnat L, Triantaphylidès C, Havaux M (2012) Carotenoid oxidation products are stress signals that mediate gene responses to singlet oxygen in plants. *Proc Natl Acad Sci U S A* 109(14):5535–5540
- Reis AR, Favarin JL, Malavolta E, Júnior JL, Moraes MF (2009) Photosynthesis, chlorophylls, and SPAD readings in coffee leaves in relation to nitrogen supply. *Commun Soil Sci Plant Anal* 40:1512–1528
- Saiedian S, Keyhani E, Keyhani J (2007) Polyphenol oxidase activity in dormant saffron (*Crocus sativus* L.) corn. *Acta Physiol Plant* 29:463–471
- Sairam RK, Srivastava GC (2001) Water stress tolerance of wheat *Triticum aestivum* L.: variation in hydrogen peroxide accumulation and antioxidant activity in tolerant and susceptible genotype. *Agron Crop Sci* 186:63–70
- Sanchez FJ, Manzanares M, Tenorio JL, Ayerbe L (1998) Turgor maintenance, osmotic adjustment and soluble sugar and proline accumulation in 49 pea cultivars in response to water stress. *Field Crops Res* 59:225–235
- Tsai YL, Chiou SY, Chan KC, Sung JM, Lin SD (2012) Caffeic acid derivatives, total phenols, antioxidant and anti-mutagenic activities of *Echinacea purpurea* flower extracts. *Food Sci Technol* 46:169–176
- Turkan I, Bor M, Ozdemir F, Koca H (2005) Differential response of lipid peroxidation and antioxidants in the leaves of drought-tolerant *P. Acutifolius* Gray and drought-sensitive *P. vulgaris* L. subjected to polyethylene glycol mediated water stress. *Plant Sci* 168:223–231
- Wang K, Zhang X, Ervin E (2012) Antioxidative responses in roots and shoots of creeping bentgrass under high temperature: effects of nitrogen and cytokinin. *Plant Physiol* 169(5):492–500
- Xiong D, Liu X, Liu L, Douthe C, Li Y, Peng S, Huang J (2015) Rapid responses of mesophyll conductance to changes of CO₂ concentration, temperature and irradiance are affected by N supplements in rice. *Plant Cell Environ* 38:2541–2550
- Zhang SG, Liu GL (2001) Plant nutrition and drought resistance of crops. *Chin Bull Bot* 1:64–69
- Zhang LX, Li SX, Zhang H, Liang ZS (2007) Nitrogen rates and water stress effects on production, lipid peroxidation and antioxidant enzyme activities in two maize (*Zea mays* L.) genotypes. *J Agron Crop Sci* 193:387–397
- Zhu Y, Fan X, Wu J, Wang T (2014) Effect of different levels of nitrogen deficiency on switch grass seedling growth. *Crop J* 2(4):223–234
- Zolgharnein J, Niazi A, Afiuni-Zadeh S, Zamani K (2010) Determination of cichoric acid as a biomarker in *Echinaceae purpurea* cultivated in Iran using high performance liquid chromatography. *Chin Med J* 1:23–27