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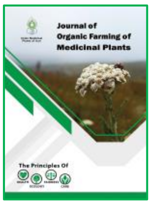

Original Article

Nitroxin improves yield and phenol compound of purple coneflower (*Echinaceae purpurea* L.) root under different irrigation regimes

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ABSTRACT

Purple coneflower (*Echinaceae purpurea* (L.) Munch) root is a rich source of phenolic components used in pharmaceutical industries and the content of phenolic components depends on many factors such as drought stress and nitrogen (N) nutrition. This experiment was conducted in Lordegan, Iran, from 2014 to 2016, to investigate the effect of N and nitroxin on the content of phosphorus, potassium and nitrate, yield, content and yield of phenol compound of purple coneflower root under three irrigation regimes. Irrigation after 25%, 50% and 75% of soil water depletion, as the main factor and 0 kg N ha^{-1} (control), nitroxin (containing *Azotobacter* and *Azospirillium* bacteria), 40 kg N ha^{-1}, a combination of nitroxin and 40 kg N ha^{-1} and 80 kg N ha^{-1} were considered as the sub-factor and arranged as a split plot in the randomized complete block design with three replications. Nitrogen application increased potassium content and root yield and also phosphorus content in all irrigation levels. The highest root nitrate accumulation was obtained by the application of 80 kg N ha^{-1} in all irrigation treatments. Irrigation after 75% of soil water depletion decreased potassium content and root yield. The highest root yield was achieved from nitroxin+40 kg N ha^{-1}. Root phenolic compounds were raised by increasing irrigation intervals, and N consumption decreased them at each irrigation regime. The highest phenolic compound yield was obtained from the application of nitroxin in irrigation after 75% of soil water depletion. Generally, irrigation after 75% of soil water depletion and utilization of nitroxin is suggested for the best quantity and also quality root production in the studied region.

1. Introduction

Purple coneflower as an indigenous North American herb, is one of the three species of *Echinaceae*. *spp.* used to enhance human immune system (Tsai et al., 2012; Wichtl, 2004). Medicinal properties of purple coneflower are due to its secondary metabolites, which are more likely to be phenolic compounds in the aerial parts (stems, leaves, and flowers) and also roots, which show antibacterial and antiviral activity (Montanari et al., 2008).

Environmental factors have a significant influence on fresh and dry weights as well as their secondary metabolites of medicinal plants. Among the various environmental constraints, drought is the most important limiting factor in agricultural production. Many plant activities, including photosynthesis, water uptake and plasma membrane integrity are affected by drought which potentially leads to changes in the yield and chemical and pharmaceutical compositions of the plant organs (Colling et al., 2010; Talbi et al., 2015). Other researchers (Adiku et al., 2001) have shown that drought stress reduced root yield, and proper irrigation increased the root yield of different plants. Gray et al. (2003) and Kleinwächter and Selmar (2014) reported that the drought stress increased the phenolic acid in the purple coneflower.

Improving the nutritional status of plants under drought stress may result in inreased plant resistance and production (Waraich et al., 2011). A better understanding of the role of nutrients in plant resistance to drought is associated with improved fertilizer management in areas that are subjected to drought. Drought stress resistance is related to N supply, so that the physiological and growth status of plants are improved in response to increasing N under reduced water availability in the soil (Drenovsky et al., 2012; Saneoka et al., 2004).

Berti et al. (2002) showed that N intake increased the root yield and total alkylamides in roots of two-years-old purple coneflower as compared with unfertilized plants. On the other hand, application of more chemical fertilizers resulted in an increase in its drainage and finally contamination of biological resources as well as the decrease of the quality of medicinal plants by increasing the nitrate in plant organs. In this regard, the use of bio-fertilizers can be useful in diminishing the use of chemical fertilizers and increasing yield. Nitroxin is a bio-fertilizer brand that contains *Azotobacter* and *Azospirillum* bacteria.

Some researchers indicated that due to the slow release of N by bio-fertilizers, they have higher N uptake efficiency (Yang, et al., 2011; Kizilkaya, 2008). Therefore, to reduce the environmental impacts of chemical N, increase the yield of purple coneflower root and also evaluate the role of N on root secondary metabolites, replacing a part of the chemical N by nitroxin bio-fertilizer may be the important method.

Therefore, this study aimed to investigate the effects of different irrigation regimes and N levels on phenol, potassium, phosphorus, and nitrate accumulation in the roots and root yield of purple coneflower.

2. Material and Method

2.1 Field site description

A two years' field experiment was conducted in Lordegan (31° 31' N, and 50° 48 ' E, 1564 m), Iran, during 2014-2016. The long-term mean annual temperature and precipitation is 15.1°C and 550 mm, respectively. Total monthly rainfall and average monthly air temperature for the two growing seasons are shown in Fig. 1. The soil properties of the location are presented in Table 1.

Table 1. Physicochemical properties of farm soil

Texture	pH	Electrical Conductivity (dS/m)	Organic Matter (%)	Total Nitrogen (%)	Phosphorus (ppm)	Potassium (ppm)	Manganese (ppm)
clay	7.7	0.64	1.14	0.12	51.1	374	6.23
Clay-loam	7.9	0.51	0.78	0.086	45.3	328	7.01

2.2 Field preparation and treatments application

The experiment was conducted as split plot in a randomized complete block design with three replications. Irrigation after 25%, (T25), 50% (T50) and 75% available water depletion (T75) as the main factor was applied from the five-leaf stage. Sub-factor included non N (control), 5 L ha^{-1} nitroxin fertilizer (containing *Azotobacter* (5×10^5 cfu mL^{-1}) and *Azospirillum* (1.4×10^7 cfu/mL), 40 kg N ha^{-1}, the combination of N (40 kg ha^{-1}) and nitroxin (5 L ha^{-1}) and 80 kg N ha^{-1}. Three-leafed (2-month-old) seedlings of coneflower, Roter sonnenhut arterner cultivar, were transplanted to the field. N, as urea, was used at two stages of five-leaf and onset of stem elongation. Nitroxin was provided from the Mehr Asia Biotechnology Co. and applied to the plant by the irrigation water at two stages of five-leaf and onset of stem elongation. Transplanting the coneflower seedlings was performed on May 3, 2014. Each experimental plot consisted of 5 rows with 50 cm spacing. The distance between the plants was 20 cm. The distances between the main plots, sub-plots and blocks were 2, 1, and 2 m, respectively. More detailed farm managements are described in Jalil et al. (2019).

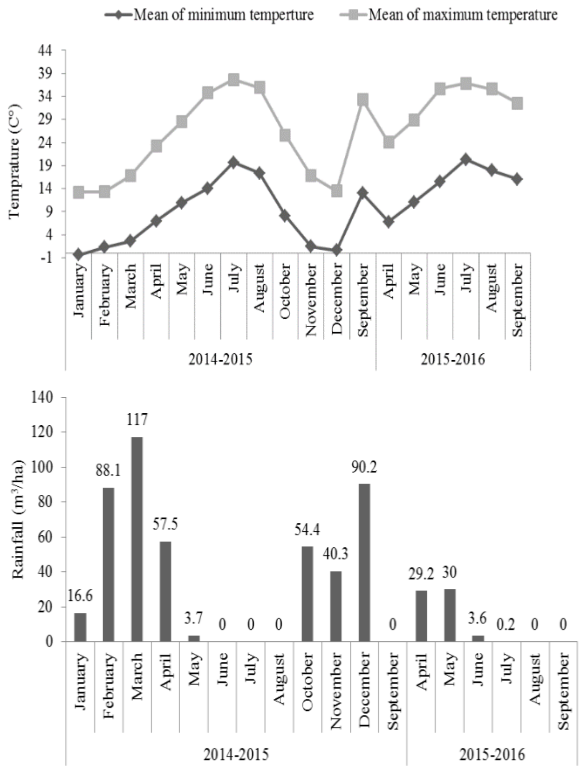

Fig. 1. The mean of minimum and maximum monthly air temperature and total rainfall for the 2014-2015 and 2015-2016 purple coneflower growing seasons in Lordgan, Iran.

2.3 Sampling

At the end of second cropping year, on 24th September 2016, after three shoot harvests at full blooming (Kindscher & Riggs, 2016), roots were sampled from 0-30 cm of the soil. Roots were carefully separated from the soil, washed, dried and ground to the fine powder for phytochemical analysis.

2.4 Root nitrate content

Root sample (0.1 gr) was shacked in 50 mL of 2% acetic acid and filtered. Ten mL of supernatant was added to 0.5 mg mixture agent (37 gr acetic acid, 5 gr manganese sulfate, 2 gr sulfanilamide, 1 gr N (1-naphthyl) ethylendiamine dihydrochloride and 1 gr pure Zn powder) and centrifuged. Absorbance was determined at 550 nm using a spectrophotometer (Lambda EZ 210).

Nitrate concentration was determined by comparing with the potassium nitrate as the standard (Singh, 1988).

2.5 Root phosphorus content

To measure phosphorus content, the ashes were analyzed, forming a complex with molybdate-vanadate and then absorbance at 420 nm was measured (Chapman & Pratt, 1961). Potassium phosphate used to prepare standard phosphorus.

2.6 Root potassium content

Hald (1947) method wasused to measure the potassium content. The amounts of potassium were read by flame diffusion method with a flame photometer, and the readings were adjusted by comparison with the potassium chloride standard.

2.7 Root yield

Purple coneflower roots were harvested in the area of 2 m^2 in autumn (on September 24^{th}) 2016 from 2-year-old plants. The roots were washed with water and then dried at $40^{\circ}C$ in an oven with air convection (Stuart & Wills, 2003), weighted and recorded in kg/ha.

2.8 Determination of total phenolic compound

Total phenolic compound content in the extract were determined using the Folin-Denis method (Ordon˜ez et al., 2006). The extract samples (0.5 mL of different dilutions) were mixed with 2.5 mL of 0.2 N Folin-Ciocalteau reagent for 5 min and 2.0 mL of 75 g L^{-1} sodium carbonate then added. The absorbance of reaction was measured at 760 nm after 2 h of incubation at room temperature. Results were expressed as Gallic acid equivalents.

2.9 Statistical analysis

Variance analysis of data was performed using SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA). The mean of main effects was compared based on the LSD test, and in case of the significant interactions, the slicing and the mean comparison was performed based on the L.S.Means procedure of the SAS program.

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3. Results

The interaction of N and irrigation on phosphorus content, nitrate content and yield of phenolic compounds of root was significant. Also, simple effects of N source and irrigation on root potassium content and root yield were significant (Table 2).

3.1 Root phosphorus content

At all levels of irrigation, N consumption, relative to unfertilized plants, increased the phosphorus content in the root, but in T25, this increase was significant except for 40 kg N ha^{-1} (Fig. 2). In T50 treatment, no significant difference was observed between different N levels. In T75, the highest amount of phosphorus was observed from N and nitroxin combination and consumption of N and nitroxin over control plants increased the amount of phosphorus in the root by 2.05 times. In general, the amount of phosphorus in the root decreased with the change of irrigation level from T25 to T75.

Table 2. Variance analysis of the effect of nitrogen source and irrigation on the studied traits of purple coneflower root.

Sources of variation	df	phosphorus Content	potassium content	nitrate content	yield	phenolic compound Content	phenolic compound yield
Replication	2	$7.21^{ns\,a}$	0.60^{ns}	0.018^{ns}	56269^{ns}	2.60^{ns}	32.19^{ns}
Irrigation (Ir)	2	557.10^{**}	25.05^{**}	1.880**	1551483^{**}	85.85^{**}	136.00^{ns}
Error a	4	6.73	2.08	0.046	58405	2.90	36.28
Nitrogen Source (N)	4	130.08**	10.96^{**}	1.730^{**}	1362842^{**}	32.05^{**}	106.18^{**}
Ir×N	8	9.70^{**}	0.83^{ns}	0.260^{**}	45601^{ns}	7.53^{**}	66.62^{**}
Error b	24	3.50	1.02	0.030	20092	2.17	24.82
C.V. (%)		9.9	12.3	10.2	5.7	12.0	15.3

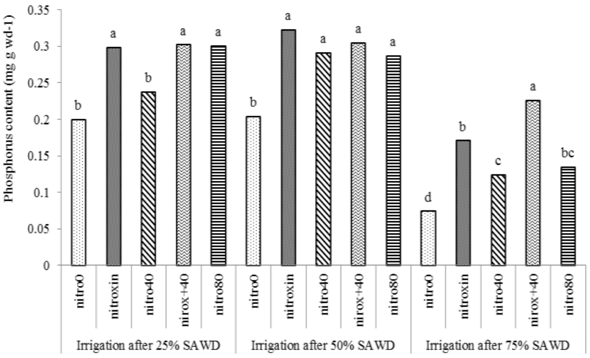

Fig. 2. Means comparison of N levels in each irrigation regime for the root phosphorus content. In each irrigation level, columns followed by the same letters are not significantly different (L.S.Means procedure). SAWD: soil available water depletion percentage. Nitro 0: lack of N and nitroxin; nitroxin (5 L ha^{-1}); nitro 40:40 kg N ha^{-1}; nitroxin+40: nitroxin (5 L ha^{-1}) + 40kg N ha^{-1}; nitro 80: 80 Kg N ha^{-1}.

3.2 Root potassium content

The results indicated that severe drought stress reduces the potassium content of the root. The amount of potassium at T75 level was significantly lower than the T25 and T50 levels (Fig. 3). Also, all N application treatments increased the amount of potassium in the root relative to the control (Fig. 4). Nitroxin and N combination increased the potassium in the root by 24% and 19% relative to the control, respectively.

3.3 Root nitrate content

The results showed that the accumulation of nitrate in the root is closely related to the N consumed at different levels of irrigation. The application of 80 kg N ha^{-1} in all irrigation levels resulted in the highest amount of nitrate in the root (Fig. 5) and increased nitrate content by 129%, 131% and 0.79% in T25, T50 and T75 compared with the control, respectively. In T25 and T50 treatments, the lowest nitrate levels were obtained from control.

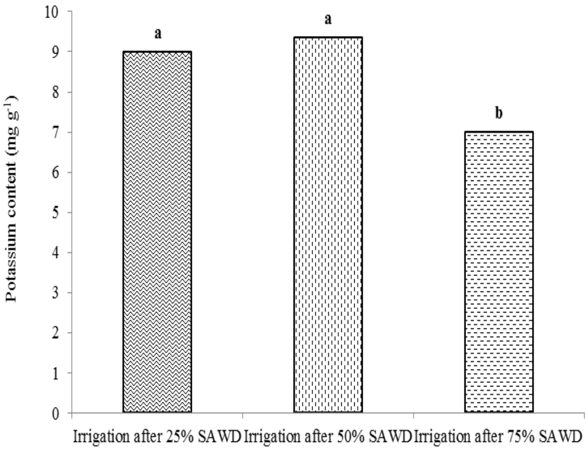

Fig. 3. Mean comparison of irrigation levels for root potassium concentration. SAWD: soil available water depletion percentage. Means followed by the same latter are not significantly different (α=0.05).

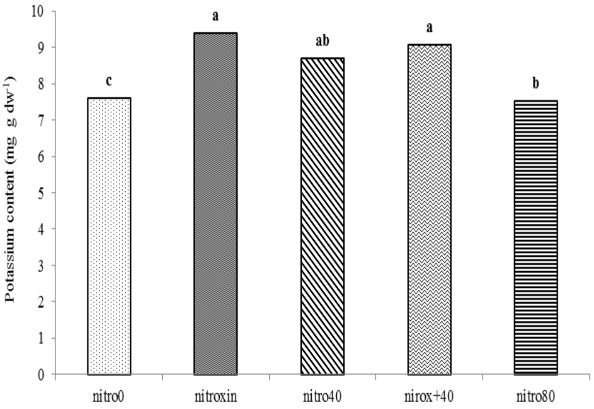

Fig. 4. Mean comparison of N levels for root potassium concentration. Means followed by the same latter are not significantly different (α=0.05). Nitro 0: lack of N and nitroxin; nitroxin (5 L ha^{-1}); nitro 40:40 kg N ha^{-1}; nitroxin+40: nitroxin (5 L ha^{-1}) + 40kg N ha^{-1}; nitro 80: 80 Kg N ha^{-1}.

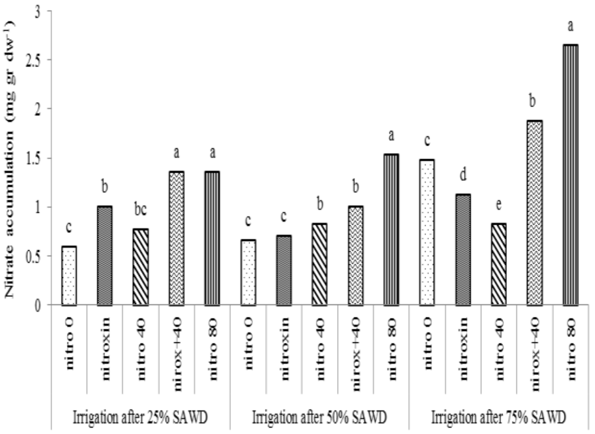

Fig. 5. Means comparison of N levels in each irrigation regime for the root nitrate accumulation. In each irrigation level, columns followed by the same letters are not significantly different (L.S.Means procedure). SAWD: soil available water depletion percentage. Nitro 0: lack of N and nitroxin; nitroxin (5 L ha^{-1}); nitro 40:40 kg N ha^{-1}; nitroxin+40: nitroxin (5 L ha^{-1}) + 40kg N ha^{-1}; nitro 80: 80 Kg N ha^{-1}.

In T75 treatment, the amount of nitrate from nitroxin and 40 kg N/ha decreased in comparison with the control. The consumption of 40 kg of N in T75 treatment caused the lowest accumulation of nitrate in the root.

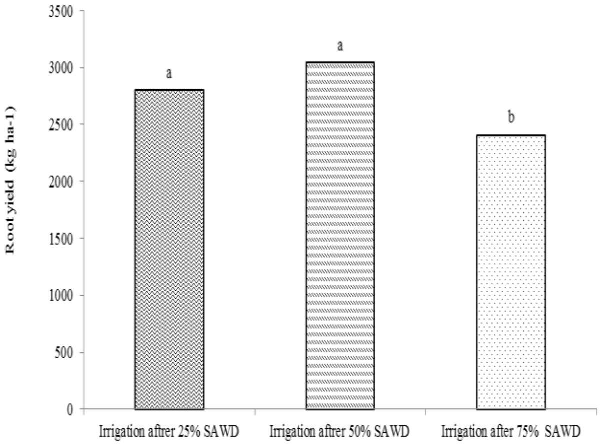

Fig. 6. Mean comparison of irrigation levels for root yield. Means followed by the same latter are not significantly different (α=0.05). SAWD: soil available water depletion percentage.

3.4 Root yield

Irrigation and N had a positive effect on root yield. The highest root yield obtained from T50 and then from T25 (Fig. 6). The results showed that the lowest root yield was derived from T75.

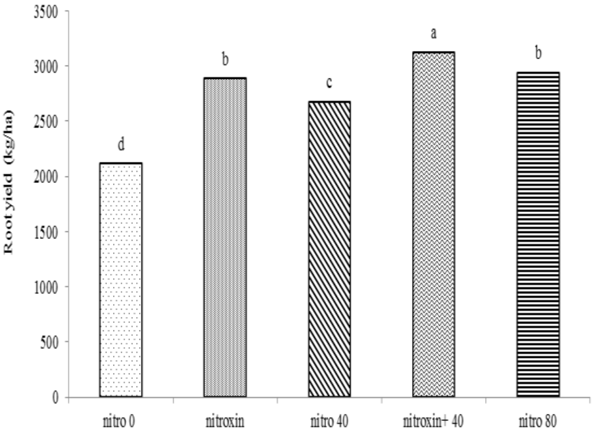

Fig. 7. Mean comparison of N for root yield. Means followed by the same latter are not significantly different (α=0.05). nitro 0: lack of N and nitroxin; nitroxin (5 L ha^{-1}); nitro 40:40 kg N ha^{-1}; nitroxin+40: nitroxin (5 L ha^{-1}) + 40kg N ha^{-1}; nitro 80: 80 Kg N ha^{-1}.

3.5 Root phenol compound content

The application of N reduced the root phenol content (Fig. 8) so that at T25 and T50, minimum root phenol content was gained by 80 kg N/ha. Consumption of 80 kg N/ha relative to non-N reduced phenol content by 25% and 58% at T25 and T50, respectively.

The decrease in phenol content due to N was lower in T75 treatment. But in this treatment, nitroxin and N combinations produced the lowest root phenol content.

By increasing irrigation intervals, root phenol content increased (Fig 8).

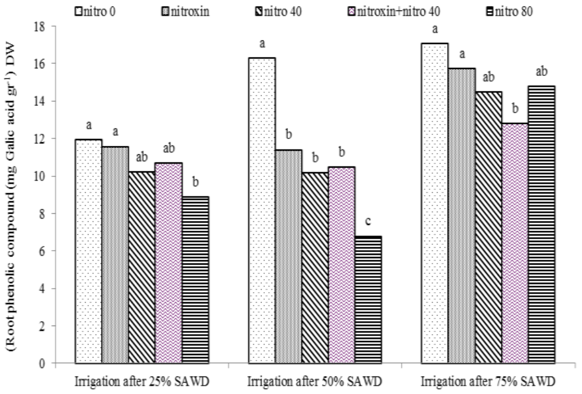

Fig. 8. Means comparison of N levels in each irrigation regime for the root phenolic compounds. In each irrigation level, columns followed by the same letters are not significantly different (L.S.Means procedure). SAWD: soil available water depletion percentage. nitro 0: lack of N and nitroxin; nitroxin (5 L ha^{-1}); nitro 40:40 kg N ha^{-1}; nitroxin+40: nitroxin (5 L ha^{-1}) + 40kg N ha^{-1}; nitro 80: 80 Kg N ha^{-1}.

3.6 Root phenol compound yield

The results showed that the administration of various N sources in the T25 and T75 treatments increased the phenol yield compared with the control (Fig. 9), although this increase was not significant in some N treatments. The application of nitroxin at T75 increased the phenol yield by 40% compared with the non-N treatment. Generally, the highest phenol yield obtained from nitroxin application at T75, although it had no significant difference with some N treatments.

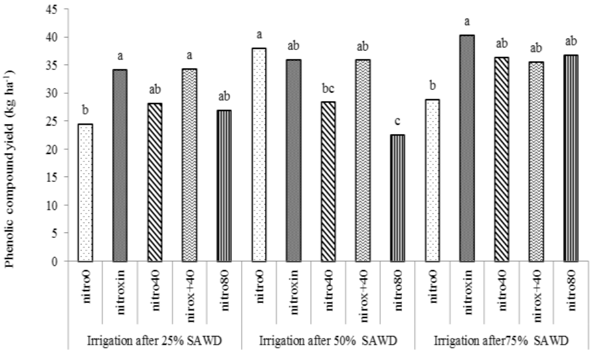

Fig. 9. Means comparison of N levels in each irrigation regime for the root phenolic compounds yield. In each irrigation level, columns followed by the same letters are not significantly different (L.S.Means procedure). SAWD: soil available water depletion percentage. nitro 0: lack of N and nitroxin; nitroxin (5 L ha^{-1}); nitro 40:40 kg N ha^{-1}; nitroxin+40: nitroxin (5 L ha^{-1}) + 40kg N ha^{-1}; nitro 80: 80 Kg N ha^{-1}.

4. Discussion

Results showed that application of N sources enhances the concentration of phosphorus in the root. One of the reasons for increasing phosphorus uptake following N use is to increase plant metabolism and thus increase the need for phosphorus uptake.

On the other hand, nitrogen can be a regulating factor for better absorption of phosphorus in the root (Zeng et al. 2012).

Dobermann and Fairhurst (2000) reported that N application increased the plant's need for other elements, including phosphorus. Therefore, in irrigation treatments N application increased phosphorus content compared with control. Bonomelli (2005) results also show that the use of N in coneflower increased the amount of phosphorus in the plant tissue.

The results showed that the utilization of nitroxin and combination of nitroxin and N resulted in the highest amount of phosphorus in all levels of irrigation. The reason for this increase can be the effect of this bio-fertilizer on the absorption of nutrients through the production of phosphorus solubilizing secretions and the reduction of pH in the rhizosphere, reported by Gilik (2012). The average of root phosphorus in T75 treatment was less than that in T25 and T50 levels. The reason for this result can be the role of moisture in the absorption of phosphorus. In conditions of water shortage, a plant closes its stomata and prevents transpiration and photosynthesis. Therefore, in such a situation, the absorption of most nutrients, such as phosphorus is decreased (Sardans et al., 2012). Of course, absorption of phosphorus is active and is carried out with energy. Under stress conditions, respiration decreases, and ATP supply decreases too (Levitte, 1980).

Fageria et al. (2006) argued that the difference in the absorption and consumption of nutrients, such as potassium, may be due to better root geometry, the ability of plants to absorb sufficient nutrients from lower concentrations, the ability of plants to dissolve nutrients in the root environment, and the equilibrium relationship between sinks and sources. Therefore, increasing potassium levels in T25 and T50 irrigation treatments can be attributed to increased potassium solubility and due to the presence of sufficient moisture and root growth at these irrigation levels.

The T75 treatment reduced potassium uptake; In this treatment (T75), the growth of root and probably the diffusion rate of potassium around the root are reduced, therefore, the absorption of potassium in the root is also reduced. Perhaps the reason is the effect of T75-induced stress on the metabolism, reduced plant photosynthesis, and root growth.

Generally, N consumption raised potassium content in the root. Increasing N enhances photosynthesis and metabolism in the plant and improves the possibility of photosynthate partitioning for root growth. Enhanced root growth leads to an increase in the nutrients uptake, including potassium.

Also, the reason for increasing potassium due to the use of nitroxin and the combination of nitroxin and N is that the bacteria present at these levels of fertilizer may affect potassium dissolution and raise potassium absorption and hence root growth.

The most important reason for increasing nitrate accumulation in plants is increasing available soil N. Therefore, nitrate increase in N application treatments, especially in the highest consumption rate, is expected.

Herencia et al. (2011) reported that organic fertilizers supply N in a simple manner to the plants and also more slowly than mineral fertilizers.

Therefore, it can be concluded that slower fixing and releasing of N in nitroxin treatments compared with other N treatments is the reason for lower nitrate in plant roots in T50 treatment.

Another factor that affects the amount of nitrate is the amount of phosphorus absorbed. Ahmed et al. (2002) reported that nitrate accumulation decreased with increasing phosphorus availability.

Phosphorus can play a role in converting nitrate to amino acids through the activation of some enzymes and also it plays a role in the metabolism, thus it can reduce nitrate accumulation in the tissues (Anjana & Iqbal, 2007). Results of this study showed a significant and negative correlation between nitrate accumulation and phosphorus (*r* = -0.37).

Therefore, considering the role of nitroxin and the combination of nitroxin and N in phosphorus absorption (Table 3) and also the slow release of N, a significant reduction in nitrate in these treatments can be justified.

Table 3. Correlation coefficients between evaluated traits

	1	2	3	4	5	6
1	1					
2	0.56^{*}	1				
3	-0.38^{**}	-0.25^{ns}	1			
4	0.78^{*}	0.50^{**}	-0.004^{ns}	1		
5	-0.69^{**}	-0.35^{**}	0.05^{ns}	-0.65^{**}	1	
6	-0.11^{ns}	0.066^{ns}	0.03^{ns}	0.131^{ns}	0.64^{**}	1

1: Phosphorus content; 2: Potassium content; 3: Nitrate accumulation; 4: Root yield; 5: Phenolic compounds; 6: Yield of phenolic compounds

Drought stress is a factor that affects nitrate accumulation in plants (Anjana & Iqbal, 2007). The reason for the increase in nitrate accumulation at this level of irrigation can be the role of water shortage in reducing photosynthesis. By decreasing the photosynthesis and N metabolism in the plants, the accumulation of nitrate in the cell vacuoles will be more significant. Baričevič and Zupančič (2008) reported that excess nitrate in the soils, coming from intensive N fertilization, resulted in an increase in plants nitrate. Moreover, plants exposure to stress conditions (all factors which affect normal biochemical processes in plant cells) enhances the nitrate accumulation in plant tissues. Therefore, 40 kg N at this irrigation level (T75) caused the least nitrate accumulation. Another role of nitrate is in plant osmotic adjustment; perhaps the reason for a significant increase in nitrate accumulation due to the consumption of 80 kg N ha^{-1} is the role of nitrate in osmotic regulation. In conditions of water shortage and high N, the roots of the plant absorb more nitrates to maintain osmotic pressure.

The root growth in the second level of irrigation was higher than the first level. This increase can be due to a large number of irrigations at the first level of irrigation. When the frequency of irrigation is high, the plant increases its surface roots and is unable to have enough deep root growth; however, with increasing irrigation intervals, root growth is increased to the depths of the soil and as a result, root yield increases, although this increase was not significant.

In addition to the above-mentioned reasons, root yield decrease can be due to the soil dryness and soil resistance to root growth. These results are consistent with the results of Bettaieb et al. (2011) in cumin (*Cuminum cyminum* L.) and Baričevič, and Zupančič (2008) in *Trigonella foenum-graecum* L. cultivars. They reported that drought stress reduced root weight in these plants.

Since increasing the availability of N in the soil improves the development of the root system, it also increases the absorption of water and essential nutrients, especially phosphorus (Dordas, 2009). Our results show that application of N as nitroxin and combination of N and nitroxin, in different levels of irrigation, can improve the positive effects of water through the effect on the absorption of other nutrients such as phosphorus and potassium. A positive and significant correlation was found between root yield and phosphorus (*r*=0.78) and potassium (*r*=0.56) content (Table 3).

The effect of N-fixing bacteria on root growth was observed in this experiment. Other researchers also reported that the use of growth-promoting bacteria increases the growth of plants by increasing the absorption of N and phosphorus as well as reducing fertilizer use and increasing the efficiency of chemical fertilizers (Nain et al., 2012; Arruda et al., 2013). One of the reasons for increasing root yield resulting from the use of nitroxin and the combination of nitroxin and N can be due to the function of bacteria in the production of growth hormones such as auxins. Moreover, their role in increasing the root hairs and so increasing the absorption of nutrients such as phosphorus and potassium, and also N can be critical. By improving the absorption conditions for nutrients, photosynthesis, and thus, the production of carbohydrates and protein substances will increase. Increasing in plant products resulted in the transfer of assimilates and hence further growth of the root.

Gonzalez-Dugo et al. (2010) reported that, under drought stress conditions, an increase in the concentration of N compounds in the rhizosphere caused to increase osmotic pressure and more plant effort to maintain cellular water and reduced transpiration. Many researchers believe that reducing transpiration in these conditions disrupts the normal plant life and leads to the emergence of inhibitory effects, including reduced plant growth and yield. Therefore, it seems that the effect of 80 kg N ha^{-1} in reducing the root yield may be due to the disruption of N uptake and other nutrients due to increased osmotic pressure around the root, decreased transpiration and decreased growth in the plant.

In general, secondary metabolites are the lowest in optimal plant growth conditions and increase with the stresses, especially drought. In this study, by increasing the irrigation intervals, the effect of drought stress on plant growth was increased, and this is the reason for the production of many secondary metabolites, including phenols. These results have a similar trend to Gray et al. (2003) study in which cichoric acid was enhanced by drought stress and to Sabra et al. (2012) research where salinity caused an increase in phenolic compounds.

The different response of 80 kg N/ha in T75 can be due to the reduced N uptake because of the increased osmotic pressure of soil water around the root. Also, the use of abundant N reduces the plant C/N ratio by increasing the amount of N in the plant and hence reducing the production of phenols (Pessarakli, 2001). Berti et al. (2002) reported that the highest root echinacoside and total alkylamides in *E. angustifolia* L. were obtained without the use of any fertilization. The results obtained from this study indicated that although 0 nitro application caused the highest root phenol content, nitroxin and combination of nitroxin and N increased phenol yield by increasing the root yield (Figs. 7, 8 and 9).

5. Conclusion

Producing high quality coneflower roots depends on the quantity and quality of the phenolic compounds of this organ. This experiment revealed that reducing irrigation water as irrigation after 75% of soil water depletion can produce the highest phenolic compound yield. Moreover, in order to use less chemical fertilizers and higher amounts of phenolic compounds, application of bio-fertilizer nitroxin can be related to the high root phenolic compounds.

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