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Physiological Responses of Black Cumin to Chemical and Biological Nitrogen Fertilizers under Different Irrigation Regimes

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ABSTRACT

The objective of this study was to investigate the physiological responses and biological yield of black cumin (*Nigella sativa* L.) to nitroxin biofertilizer and chemical nitrogen fertilizer in the form of urea under different irrigation regimes. A split plot experiment was conducted on the basis of randomized complete block design with three replications. The main factor included four irrigation regimes (i.e., weekly from emergence to harvest and withholding from blooming to maturity, flowering to maturity, and the start of seed formation to maturity) and sub-factor included five levels (i.e., no application of fertilizers, 80 kg N ha^{-1}, 40 kg N ha^{-1}, combination of 40 kg N ha^{-1}+nitroxin biofertilizers, and nitroxin biofertilizer). Application of 80 kg N ha^{-1} under full irrigation and the combined application of 40 kg N ha^{-1} and nitroxin under all withholding irrigations produced the highest glycinebetaine, polyphenol oxidase and catalase enzyme, total chlorophyll, and biological yield. Withholding irrigation from the blooming stage and also the application of 80 kg N ha^{-1} resulted in the highest concentration of malondialdehyde. In combined application of 40 kg N ha^{-1} and nitroxin, polyphenol oxidase, proline, and soluble protein were at the highest levels. Generally, the combined application of 40 kg N ha^{-1} and nitroxin increased the activity of the antioxidant enzymes and the compatible osmolites accumulation under all withholding irrigation treatments and thus decreased the negative effects of drought stress on black cumin, resulting in increased biological yield.

Keywords: Biofertilizer, Chlorophyll, Compatible osmolites, Enzymatic defense system, Withholding irrigation.

INTRODUCTION

Black cumin is one of the herbs that originated from arid and semi-arid regions. This plant is an annual species of the Ranunculaceae family, which is used widely in traditional and industrial pharmacology (D'Antuono *et al*., 2002). Due to the short growth period of black cumin (about 100 to 120 days) and its low input requirement, it is cultivated in arid and semi-arid regions of Iran.

Drought stress is one of the serious problems that reduce plant growth and productivity. This is especially important in the arid and semi-arid regions of the world such as Iran. Drought stress leads to the production of Reactive Oxygen Species (ROS), due to an imbalance between light intake and its consumption. These ROS cause serious damage to plant cell structures. Drought stress can inhibit photosynthesis, limit water absorption, damage the plasma membrane, and decrease potential growth in many plants (Talbi *et al*., 2015). Kabiri *et al*., (2014) reported that oxidative stress is the most important reason for the reduction of photosynthetic pigment and proteins

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content and increment of soluble sugar content in *Nigella sativa* plants under drought stress. Thus, the plants change their metabolic and physiological activities to minimize the negative effects of stress and increase the survival (Nyiraneza *et al*., 2009). Adaptation and response of plants to drought stress is through warning of their cellular metabolism and using different defense mechanisms, such as enzymatic and non-enzymatic antioxidants.

Nitrogen, after water, is the second most important factor affecting the growth and quality of medicinal plants. The improvement of plant nutrition reduces drought stress damage by preserving metabolic activities under low water potential of tissues. Application of N-fertilization increases black cumin growth by promoting processes such as cell division, cell enlargement and metabolic processes (Ali *et al*., 2015).

Currently, there are new trends in agriculture by which the use of chemical fertilizers is reduced by replacing them with bio-fertilizers (Gyaneshwar *et al*., 2002). Some micro-organisms have positive effects on plant growth promotion, including the Plant Growth Promoting Rhizobacteria (PGPR) such as *Azospirillum*, *Azotobacter*, *Pseudomonas fluorescens,* and several gram-positive *Bacillus* spp. (Chen, 2006). Nitroxin, a biofertilizer containing *Azotobacter* and *Azospirillum* bacteria, in addition to fixing atmospheric nitrogen, releases plant hormones such as gibberellins and auxin to stimulate plant growth (Fayez *et al*., 1985). Jalil Sheshbahreh *et al.* (2019) showed that nitroxin provides nitrogen in plants under control and mild stress conditions up to 40 kg N ha^{-1}. Inoculation with Azotobacter can help the growth of black cumin, when the bacteria were established and grown well in the rhizosphere. This increase in plant growth following use of Azotobacter may be due to the synthesis of some plant growth promoting substances (Abdel-Aziez *et al*., 2014). Also, inoculation of black cumin with Azospirillum through the effect on water and mineral absorption can lead to more growth and production of plant under drought stress condition (Hadi *et al*., 2012).

Considering that in many medicinal herbs the severity of undesirable effects of drought varies in different stages of growth, it is important to identify the critical stages of plant growth to drought stress, as well as to use the source of fertilizers that are most effective in low irrigation conditions. Therefore, the present study aimed to investigate the effect of withholding irrigations at different development stages, and impact of chemical and biological nitrogen fertilizer on physiological response and biological yield of black cumin (*Nigella sativa* L.) under field conditions.

MATERIALS AND METHODS

This field study was conducted at the research farm of Yasouj University, Iran (51°33′ E, 30° 38′ N; 1,870 m). The average annual air temperature, humidity, and precipitation in this region are 15.1°C, 43.6%, and 842.6 mm, respectively.

Before planting, physical and chemical characteristics of the soil of the field were measured from the depth of 0 to 30 cm. The soil type was a clay loam. The soil contains 0.03% total N, 10.3 mg kg^{-1} P, 220 mg kg^{-1} available K, and pH of 7.42.

The experimental design was a split plot based on randomized complete block design with three replications. Main plots were four irrigation regimes and subplots were five levels of nitrogen (Table 1). Nitroxin biological fertilizer used in the experiment included *Azotobacter* and *Azospirillum*. Alive cells of each bacterium were 10^8 in each milliliter of nitroxin. The urea fertilizer was used as the source of chemical nitrogen supply.

Seeds were planted on both sides of the stack with row spacing of 25 cm and at the depth of 0.5 cm on early spring in two study years. Drip irrigation was used and water volume was controlled by water meter.

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Table 1. Description of experimental factors

Main plots	
WI_0	Weekly irrigation from emergence to harvest
WI_s	Withholding irrigation from the start of seed formation to maturity
WI_f	Withholding irrigation from flowering to maturity
WI_b	Withholding irrigation from blooming to maturity
Subplots	
N0	No application of fertilizers
N80	80 kg N ha^{-1}
N40	40 kg N ha^{-1}
N40+B	Combination of 40 kg N ha^{-1}+nitroxin biofertilizer
B	Nitroxin biofertilizer

Prior to seed planting, phosphorus fertilizer in the form of triple superphosphate was spread and incorporated into the soil, and nitrogen fertilizer in the form of urea was used as the split in two stages: half as pre-plant and the reminder half one month after emergence (when the plant height reached 15-20 cm). Nitroxin fertilizer, based on the recommendation of the manufacturer (Mehr Asia Biotechnology Company), was added at the rate of 5 L ha^{-1} to the plots at the second and third irrigations.

Proper sampling was made to measure physiological traits at two weeks after the last Withholding Irrigation (WI_s). Afterward, 8-10 plants were randomly selected from each plot and measurements were made on the youngest leaves. One square meter of the center of each plot was hand harvested for biological yield determination at physiological maturity stage.

Malondialdehyde (MDA)

To measure MDA (Heath and Packer, 1968), leaf samples were extracted in Trichloroacetic Acid 0.1% (TCA) and Thiobarbituric Acid 0.5% (TBA). Finally, MDA concentration was measured using spectrophotometer at 532 nm and 600 nm.

Enzyme Assays

In order to assess enzymes activity, frozen leaf samples (0.1 g) were homogenized in 3 mL of extraction buffer, containing 100 mM potassium phosphate (pH= 7.8), 0.1 mM Ethylenediaminetetraacetic Acid (EDTA), and 0.1 M Polyvinyl Pyrrolidone (PVP).

To measure Peroxidase (POX) activity (Ghanati *et al*., 2002), 2 mL potassium phosphate buffer (60 mM, pH= 6.1), 0.5 mL of guaiacol (28 mM), and 0.5 mL of H_2O_2 (5 mM) were added to 100 µL enzyme extract. The absorbance rate variation was measured at 470 nm within 60 seconds and enzyme activity was expressed as unit $min^{-1}\,g^{-1}\,leaf_{fw}$.

Polyphenol Oxidase (PPO) activity was evaluated based on the method of Ghanati *et al.* (2002). The reaction mixture consisted of 100 µL enzyme extract, 500 µL of 5 mM H_2O_2, 500 µL of 0.02 mM methylcatcol, and 1,900 µL of 60 mM potassium phosphate buffer with pH= 6.1. Finally, the absorption changes at 410 nm in spectrophotometry were read at 60 seconds and PPO activity was expressed in unit $min^{-1}\,g^{-1}\,leaf_{fw}$.

To measure Catalase (CAT) (Cakmak and Horst, 1991) about 3 mL of phosphate buffer (50 mM, pH= 7) and 30 mM H_2O_2 were added to 100 µL enzyme extract, followed by reading the absorption rate within 60 seconds at a wavelength of 20 nm by a spectrophotometer and excitation coefficient of 0.0394 $mM^{-1}\,cm^{-1}$. The amount of enzyme activity was reported as mmol $min^{-1}\,g^{-1}\,leaf_{fw}$.

Proline and Total Soluble Sugar (TSS) Contents

To measure proline and TSS content, an alcoholic extract was prepared using ethanol.

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Proline content was measured by adding 3 mL ninhydrin and 5 mL glacial acetic acid to 1 mL alcoholic extract (Paquin and Lechasseur, 1979). Finally, its absorbance was determined at 515 nm in a spectrophotometer and expressed as µmol g^{-1} $leaf_{fw}$. To measure TSS (Irigoyen *et al.*, 1992), 0.1 mL of alcoholic extract was mixed with anthrone reagent (3 mL) and then was measured at an absorbance of 625 nm using a spectrophotometer. The total soluble sugar in the sample was expressed as mg g^{-1} $leaf_{fw}$.

Glycinebetaine Content (GB)

Analysis of glycinebetaine was performed using potassium iodide and 1-2 dichloroethane (Grattan and Grieve, 1998), and then the absorbance was read by a spectrophotometer at 365 nm and expressed as mg g^{-1} dry weight.

Soluble Proteins Content

Soluble protein content was estimated based on the method of Bradford (1976), using the reagent Coomassie Brilliant Blue G-250. The absorbance was read at 595 nm with a spectrophotometer and the amount of soluble protein was calculated using bovine serum albumin as standard and expressed as mg g^{-1} fresh weight.

Total Chlorophyll Contents

Total chlorophyll contents was determined by measuring the absorbance of the acetone extract at 663 and 645 nm and expressed in mg g^{-1} fresh weight (Arnon, 1949).

Statistical Analysis

Uniformity test of variances was performed by Bartlett test and normality test was done before Analysis Of Variance (ANOVA). Statistical analysis was done using SAS software (SAS Institute Inc., 2002). Main effects comparison was carried out using the Least Significant Difference (LSD) at $\alpha < 0.05$. Moreover, the interaction effects of mean comparisons were made by LS Means (Least Significant Means) method in SAS software.

RESULTS

According to Bartlett test results, X^2 was significant for POX, TSS, and GB contents and thus a simple analysis was performed in each year for these traits. The interaction of irrigation regime×nitrogen was significant on POX activity, TSS, and GB contents in both years (Table 2). X^2 was not significant for other traits and, therefore, compound

Table 2. Analysis of variance for the effects of irrigation regime and nitrogen fertilizer on Total Soluble Sugars (TSS), Glycinebetaine (GB) and Peroxidase (POX) of black cumin.[a]

Source of variation	df	TSS		GB		POX	
		2016	2018	2016	2018	2016	2018
Block	2	0.460^{ns}	0.070^{ns}	0.002^{ns}	0.050^{ns}	0.010^{ns}	0.002^{ns}
Irrigation regime	3	269.000^{**}	177.000^{*}	9.420^{**}	8.810^{**}	9.250^{**}	6.930^{**}
Block×Irrigation regime (Error a)	6	0.190	0.090	0.020	0.020	0.007	0.005
Nitrogen	4	7.180^{**}	9.350^{**}	1.110^{**}	1.130^{**}	0.520^{**}	0.520^{**}
Block×Nitrogen	8	0.380^{ns}	0.110^{ns}	0.005^{ns}	0.020^{ns}	0.011^{ns}	0.008^{ns}
Irrigation regime×Nitrogen	12	0.071^{**}	0.320^{**}	0.050^{**}	0.080^{**}	0.018^{**}	0.020^{**}
Error (b)	24	0.240	0.090	0.005	0.020	0.008	0.003
C.V (%)	-	7.610	7.950	6.320	5.100	6.570	5.820

[a]: ns, * and **: Not-significant and significant at 5 and 1% probability levels, respectively.

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analysis was performed for them. The results of the two-year data analysis (Table 4) indicated that irrigation regime×nitrogen interaction was significant on CAT, PPO activities, soluble protein, proline, total chlorophyll contents, and biological yield. Moreover, the interaction of year×irrigation regime was significant for proline and total chlorophyll contents (Table 4). The simple effect of nitrogen application and irrigation regime was significant on MDA (Table 4).

The highest leaf proline content was related to N40+B in all irrigation regimes but it was not significantly different with N80 in WI_0 and WI_s (Table 5). The increase in leaf proline content with N40+B compared to N0 was 49.7, 40.4, 37.7, and 32.5% in WI_0, WI_s, WI_f, and WI_b, respectively. In both years, the highest leaf proline content was obtained in WI_b, which showed 2.5 and 2-times increase compared to WI_0 in the first and second year, respectively (Table 6).

In both years, N0 produced the highest TSS in all irrigation regimes; nevertheless, there was no significant difference with N40 in WI_s and WI_f (Table 3). TSS increased in N0 versus N80 about 43.2, 25.9, 18.1, and 16.7% in the first year and 39.6, 21.2, 28.8, and 15.9% in the second year in WI_0, WI_s, WI_f, and WI_b, respectively.

In both years, the highest GB was obtained in WI_b. Moreover, with a decrease in the duration of irrigation withholding, GB rate decreased as well. N80 increased GB in WI_0; however, it was not significantly different from N40+B. This increase was 12.7 and 13.8% compared with N0 in the first and the second year, respectively (Table 3). In other irrigation regimes, although N40+B resulted in an increase in the GB rate, there was no significant difference with N80 in WI_s and WI_f in the first year and in all withholding irrigation regimes in the second year (Table 3). N40+B compared to N0 increased GB in WI_s, WI_f, and WI_b was 35.3, 27.9, and 25% in the first year and 35.7, 27.5 and 34.3% in the second year, respectively.

The highest CAT activity was related to N40+B in all irrigation regimes (except full irrigation); however, there was no significant difference with N80 in WI_f (Table 5). N80 increased CAT activity compared to N0 (31.63%) in WI_0. The increase of CAT activity with N40+B compared to N0 was equivalent to 29.1, 24.1, and 22.2% in WI_s, WI_f, and WI_b, respectively.

Although N80 resulted in the highest POX activity in WI_0, there was no significant difference with N40+B (Table 3). This increase was approximately 71.4 and 78.4% compared to N0 in the first and the second year, respectively. In other irrigation regimes, N40+B had the greatest POX activity; however, there was no significant difference with N80 in WI_f in the first year and in WI_f and WI_b in the second year (Table 3). The increase in POX activity in N40+B compared to N0 was 69.76, 35.3, and 28.4% in the first year and 69.7, 38.8, and 30.7% in the second year in WI_s, WI_f, and WI_b, respectively.

N40+B treatment was associated with the highest PPO activity in all irrigation regimes but there was no significant difference with N80 in WI_s (Table 5). N40+B compared to N0 resulted in 82.2, 41.5, 38.8, and 38.2% increase in PPO activity in WI_0, WI_s, WI_f, and WI_b, respectively.

The highest MDA was obtained by WI_b, which showed a 35.1% increase compared to WI_0 (Table 7). N80 was associated with the highest MDA and resulted in a slight increase (i.e., 8.3%) compared to N0 (Table 7).

N40+B increased soluble protein content in all irrigation regimes; however, there was no significant difference with N80 in WI_s and WI_f (Table 5). Soluble protein content increased with N40+B compared to N0 44.2, 39.9, 37.2, and 29.2% in WI_0, WI_s, WI_f, and WI_b, respectively.

N80 produced the highest total chlorophyll content in WI_0, which increased 35.7% compared to N0 (Table 5). In other irrigation regimes, N40+B treatment was associated with an increase in total chlorophyll content; nevertheless, there was no significant difference with N80 in WI s and WI_b (Table 5). Total

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Table 3. Mean comparison of the effects of irrigation regime×nitrogen on Total Soluble Sugars (TSS), Glycinebetaine (GB) and Peroxidase (POX) of Black cumin.[a]

Factor[b]		TSS (mg g^{-1})		GB (mg g^{-1})		POX (units min^{-1} g^{-1} $leaf_{fw}$)	
Irrigation regime	Nitrogen fertilizer	2016	2018	2016	2018	2016	2018
WI_o	N0	5.57 a	7.36 a	2.12 d	2.02 b	0.42 b	0.51 c
	N40	4.61 b	6.13 b	2.23 bc	2.14 ab	0.54 b	0.52 c
	B	4.71 b	6.11 b	2.15 cd	2.12 ab	0.54 b	0.72 b
	N80	3.89 b	5.72 bc	2.39 a	2.30 a	0.72 a	0.90 a
	N40+B	4.19 b	5.27 c	2.32 ab	2.22 ab	0.70 ab	0.79 b
WI_s	N0	10.44 a	9.59 a	2.49 c	2.41 b	0.84 c	0.76 c
	N40	9.64 ab	8.61 b	2.76 b	2.60 b	0.86 c	0.78 c
	B	9.43 b	8.48 b	2.73 b	2.58 b	0.88 c	1.06 b
	N80	8.29 c	7.91 c	3.28 a	3.20 a	1.25 b	1.13 b
	N40+B	8.58 c	7.91 c	3.37 a	3.27 a	1.42 a	1.30 a
WI_f	N0	13.16 a	12.87 a	3.08 c	3.05 c	1.54 b	1.31 c
	N40	12.49 ab	12.04 b	3.42 b	3.35 b	1.62 b	1.57 b
	B	12.15 bc	11.84 b	3.32 b	3.24 bc	1.70 b	1.53 b
	N80	11.14 d	9.99 c	3.87 a	3.72 a	1.96 a	1.77 a
	N40+B	11.45 cd	10.21 c	3.94 a	3.89 a	2.09 a	1.82 a
WI_b	N0	15.60 a	15.16 a	3.60 d	3.26 c	2.08 d	1.94 c
	N40	14.91 ab	14.48 b	4.04 c	3.91 b	2.26 c	2.05 b
	B	14.35 bc	14.27 b	3.97 c	3.93 b	2.28 c	2.14 b
	N80	13.65 c	13.08 c	4.36 b	4.16 ab	2.48 b	2.47 a
	N40+B	13.82 c	13.12 c	4.50 a	4.38 a	2.68 a	2.54 a

[a] In each column and irrigation regime level, means with at least one common letter are not statistically significant. [b] Weekly Irrigation from emergence to harvest (WI_0), Withholding Irrigation from blooming to maturity (WI_b), from flowering to maturity (WI_f) and from the start of seed formation to maturity (WI_s) and no application of fertilizers (N0), 80 kg N ha^{-1} (N80), 40 kg N ha^{-1} (N40), combination of 40 kg N ha^{-1}+nitroxin biofertilizer (N40+B) and nitroxin biofertilize (B).

Table 4. Analysis of variance for the effects of withholding irrigation and nitrogen fertilizer on proline, Catalase (CAT), Polyphenol Oxidase (PPO), Malondialdehyde (MDA), soluble protein, total chlorophyll and biological yield of black cumin in two experimental years.

Source of variation	Df	Proline	CAT	PPO	MDA	Soluble protein	Total chlorophyll	Biological yield
Year	1	0.490^{ns}	196.000^{ns}	0.017^{ns}	0.00000002^{ns}	5.380^{ns}	0.210^{ns}	151656.000^{ns}
Block (Year)	4	0.150	4.870	0.002	0.00035000	0.048	0.004	9628.000
Irrigation regime	3	234.000^{**}	9925.000^{**}	13.300^{**}	0.21000000^{**}	38.900^{**}	10.100^{**}	2646747.000^{**}
Year× Irrigation regime	3	6.260^{**}	3.750^{ns}	0.016^{ns}	0.00000021^{ns}	0.110^{ns}	0.019^{*}	1893.000^{ns}
Block×Irrigation regime (Year) (Error a)	12	0.210	2.830	0.014	0.00028000	0.065	0.003	6796.000
Nitrogen	4	26.900^{**}	647.000^{**}	1.290^{**}	0.00760000^{**}	34.600^{**}	0.580^{**}	1936611.000^{**}
Year×Nitrogen	4	0.890^{ns}	9.150^{ns}	0.013^{ns}	0.00000020^{ns}	0.050^{ns}	0.019^{ns}	9110.000^{ns}
Irrigation regime×Nitrogen	12	0.510^{**}	59.600^{**}	0.036^{**}	0.00018000^{ns}	2.320^{**}	0.061^{**}	65922.000^{**}
Year×Irrigation regime×Nitrogen	12	0.920^{ns}	8.180^{ns}	0.014^{ns}	0.00000031^{ns}	0.110^{ns}	0.011^{ns}	1571.000^{ns}
Error (b)	64	0.180	4.070	0.011	0.00026000	0.100	0.003	6129.000
CV (%)	-	7.140	5.040	6.910	7.450	6.670	6.260	9.010

^{ns}, * and **: Not-significant and significant at 5 and 1% probability levels, respectively.

Table 5. Mean comparison of the effects of irrigation regime×nitrogen on Proline, Catalase (CAT), Polyphenol oxidase (PPO), soluble protein, total chlorophyll and biological yield of black cumin.[a]

Irrigation regime[B]	Nitrogen fertilizer[C]	Proline (µmol g^{-1})	CAT (mmol min^{-1} g^{-1} $leaf_{fw}$)	PPO (units min^{-1} g^{-1} $leaf_{fw}$)	Soluble protein (mg g^{-1})	Total chlorophyll (mg g^{-1})	Biological yield (kg ha^{-1})
WI_o	N0	4.20 d	66.70 e	0.62 c	8.27 d	1.90 d	1177.00 d
	N40	5.55 bc	70.60 d	0.71 c	11.30 b	2.35 c	1597.00 c
	B	5.43 c	76.70 c	0.68 c	10.81 c	2.36 c	1637.00 c
	N80	5.98 ab	87.80 a	0.92 b	11.31 b	2.58 a	2190.00 a
	N40+B	6.29 a	83.90 b	1.13 a	11.93 a	2.50 b	2057.00 b
WI_s	N0	6.57 c	55.00 d	1.06 c	8.32 c	1.48 c	940.00 c
	N40	7.40 b	64.10 c	1.21 b	10.31 b	1.70 b	1140.00 b
	B	7.44 b	66.60 b	1.19 b	10.35 b	1.75 b	1167.00 b
	N80	8.96 a	67.50 b	1.39 a	11.57 a	1.85 a	1595.00 a
	N40+B	9.23 a	71.00 a	1.50 a	11.64 a	1.94 a	1679.00 a
WI_f	N0	7.84 d	38.50 c	1.57 d	8.08 d	1.18 c	866.00 c
	N40	9.38 c	43.30 b	1.78 c	9.09 c	1.19 c	1083.00 b
	B	9.47 c	43.00 b	1.71 c	10.07 b	1.26 b	1063.00 b
	N80	9.99 b	47.40 a	1.98 b	10.77 a	1.33 b	1380.00 a
	N40+B	10.80 a	47.80 a	2.18 a	11.09 a	1.50 a	1441.00 a
WI_b	N0	10.31 d	34.20 c	2.04 d	7.52 d	0.92 c	798.00 c
	N40	11.94 c	36.10 c	2.08 d	7.62 cd	0.97 bc	1022.00 b
	B	11.80 c	38.70 b	2.31 c	7.93 c	0.98 b	973.00 b
	N80	13.04 b	39.30 b	2.47 b	9.30 b	1.10 a	1204.00 a
	N40+B	13.67 a	41.80 a	2.82 a	9.72 a	1.10 a	1263.00 a

[a] In each column and irrigation regime level, means with at least one common letter are not statistically significant. [b] Weekly irrigation from emergence to harvest (WI_0), Withholding Irrigation from blooming to maturity (WI_b), from flowering to maturity (WI_f) and from the start of seed formation to maturity (WI_s). [c] No application of fertilizers (N0), 80 kg N ha^{-1} (N80), 40 kg N ha^{-1} (N40), combination of 40 kg N ha^{-1}+nitroxin biofertilizer (N40+B) and nitroxin biofertilize (B).

Table 6. Mean comparison of the effects of year× irrigation regime on Proline and total chlorophyll of black cumin.[a]

Year	Irrigation regime [b]	Proline (µmol g^{-1})	Total Chlorophyll (mg g^{-1})
2016	WI_o	5.01 d	2.27 a
	WI_s	7.79 c	1.73 b
	WI_f	10.03 b	1.26 c
	WI_b	12.48 a	0.96 d
2017	WI_o	5.97 d	2.41 a
	WI_s	8.05 c	1.76 b
	WI_f	8.96 b	1.31 c
	WI_b	11.82 a	1.06 d

[a] In each column and irrigation regime level, means with at least one common letter are not statistically significant. [b] Weekly Irrigation from emergence to harvest (WI_0), Withholding Irrigation from blooming to maturity (WI_b), from flowering to maturity (WI_f) and from the start of seed formation to maturity (WI_s).

Table 7. The effect of nitrogen application (a) and irrigation regime (b) on Malondialdehyde (MDA) of Black cumin.

Factor[A]	MDA (µmol g^{-1})[B]
Nitrogen fertilizer (a)	
N0	0.63 d
N40	0.65 c
B	0.65 c
N80	0.68 a
N40+B	0.66 b
Irrigation regime (b)	
WI_o	0.54 d
WI_s	0.67 c
WI_f	0.69 b
WI_b	0.73 a

[a] Weekly Irrigation from emergence to harvest (WI_0), Withholding Irrigation from blooming to maturity (WI_b), from flowering to maturity (WI_f) and from the start of seed formation to maturity (WI_s) and no application of fertilizers (N0), 80 kg N ha^{-1} (N80), 40 kg N ha^{-1} (N40), combination of 40 kg N ha^{-1}+nitroxin biofertilizer (N40+B) and nitroxin biofertilize (B). [b] In each column means with at least one common letter are not statistically significant.

chlorophyll content in WI_s, WI_f, and WI_b increased 31.1, 27.1, and 19.5% respectively, with N40+B compared to N0. The highest total chlorophyll content in both years was related to WI_0 and the increase in the duration of withholding irrigation resulted in a reduction of this trait, which showed 57.7 and 56% reductions in the first and second year, respectively (Table 6).

As shown in Table 5, N80 produced the highest biological yield compared to other fertilizer levels in WI_0, but with the onset of withholding irrigation at different stages of development, N40+B had a positive effect on increasing the biological yield; nevertheless, there was no significant difference with N80 in all levels of withholding irrigation. The increasing trend of biological yield with N80 in WI_0 and N40+B in WI_s, WI_f, and WI_b was 86.1, 78.6, 66.4, and 58.2% compared to N0, respectively.

DISCUSSION

In general, increasing the duration of withholding irrigation was associated with

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increased leaf proline content (Tables 5 and 6). In this condition, the combined consumption of fertilizers had a positive effect on the incremental trend of this trait (Table 5). The bacteria found in nitroxin bio-fertilizer, in addition to fixing atmospheric N_2 and balancing the absorption of macro and micronutrient elements required by the plant, produced growth stimulant hormones that help leaf proline accumulation by exporting the hormones from root to the leaves (Akhtar and Siddiqui, 2009). The accumulation of proline increases with nitrogen application and water scarcity as one of the physiological responses to stress (Geravandi *et al*., 2011). Proline enhances the activity of various enzymes (Szabados and Savoure, 2010), and serves as storage of organic nitrogen compounds during the reconstruction (Sairam and Tyagi, 2004).

Increasing the duration of withholding irrigation duration increased TSS (Table 3). The basic roles of TSS in plant metabolism are known as a regulator of hydrolytic processes, substrates in biosynthesis processes, energy production, and signaling systems. Other roles of TSS may also be osmotic protection, cell membranes stabilization, and turgor preservation (Mohammadkhani and Heidari, 2008).

In general, an increase in the consumption of nitrogen fertilizer resulted in the declined TSS (Table 3), which can be attributed to the role of nitrogen in increasing chlorophyll content, leaf soluble protein, and amino acids (like proline and glycinebetaine). These processes require the use of certain Krebs cycle metabolites, which needs the consumption of carbohydrates and their derivatives. Plant growth requires sugar consumption in order to reduce the amount of carbohydrates stored (Lastdrager *et al.*, 2014).

An increase in GB content was observed during withholding irrigation (Table 3), which may indicate its role in adaptation. Conditions of water shortage stress may lead to denaturation of proteins and eventually degradation of membrane structures of plants, where GB maintains the activity of various enzymes under a variety of adverse conditions (Ashraf and Foolad, 2007). Schobert (1977) argued that GB, which is linked to hydrophilic proteins, forms a layer of water around the protein that becomes available during stress and prevents protein degradation.

GB increased with the combined application of chemical and biological fertilizers compared to pure fertilizer application (Table 3). Nitrogen sustenance is necessary for biosynthesis of an amino acid derivative such as GB in crop plants (Li, 2007).

Increasing the duration of withholding irrigation reduced CAT activity (Table 5). CAT activity is reduced by inhibition of enzyme synthesis or alteration in the union of subunits enzyme under stress conditions. It may also be related to the degradation caused by induced peroxisomal proteases or due to the photo-inactivation of the enzyme (Abedi and Pakniyat, 2010).

In withholding irrigation condition, the combined use of chemical and biological fertilizers would increase CAT activity (Table 5). The PGPR synthesize enzymes, including CAT, which regulates plant growth and development (Gilck, 2012).

In all withholding irrigation regimes, the combined use of chemical and biological fertilizers in comparison with non-application of fertilizer had an increasing effect on POX activity (Table 3). POX plays a major role in reducing H_2O_2 content and results in decreasing membrane lipids peroxidation and maintaining the integrity of the cell membrane (Hojati *et al*., 2011).

With increasing fertilizer application and withholding irrigation duration, PPO activity increased as well (Table 5). PPO activity was increased under water deficit treatments and nitrogen application (Movludi *et al*., 2014). PPO is present in most of the higher plants and catalyzes the quinine formation from phenols in the presence of O_2. This enzyme plays a role in adventitious root formation, root development organization (Yilmaz *et al*., 2003), cell division, and

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primary differentiation. Increasing the activity of POX and PPO and decreasing the activity of CAT due to water stress has been proven in wheat by Abdalla and El-Khoshiban (2007). Increasing antioxidant enzymes activity may be attributed to the higher capacity of stressed plants for decomposition of toxic H_2O_2 that is accumulated at higher levels due to reduced CO_2 fixation (El-Tayeb, 2006).

Inoculation of PGPR strains improves plant enzyme activity, which reduces the oxidative damage caused by drought and salinity stresses (Wang *et al*., 2012).

In general, it can be stated that the role of CAT enzyme in comparison with POX and PPO enzymes was lower in stress tolerance.

POX (r= 0.95^{**}) and PPO (r= 0.94^{**}) enzymes were positively correlated with leaf proline content (Table 8). Proline can affect the solubility of various cell proteins by interacting with the surface of hydrophobic protein chains, so, it prevents the change of their nature. Enzymes are affected and protected by this proline property due to their protein structure (Kuznetsov and Shevykova, 1999).

Enhancing the activity of antioxidant enzymes helps to reduce MDA and stress damage in cells (Bandeoğlu *et al*., 2004). There was a positive correlation between MDA and POX (r= 0.84^{**}) and PPO (r= 0.86^{**}) enzymes (Table 8), suggesting a decrease in the effects of withholding irrigation through increased activity of these enzymes. Furthermore, MDA was positively correlated with compatible osmolites [i.e., proline (r= 0.85^{**}), GB (r= 0.87^{**}), and TSS (r= 0.74^{**})] (Table 8). Results indicated that MDA and electrolyte leakage of the cell membrane increased by decreasing leaf water, which eventually associated with increased proline, GB, and TSS contents. The accumulation of these compatible osmolites led to reduced cell membrane damage.

Generally, the amount of soluble protein decreased with increasing duration of withholding irrigation (Table 5). There was a significant decrease in protein synthesis in drought-stressed plants, due to a decrease in the number of polysomal complexes in tissues with lower water content (Kabiri *et al*., 2014). Drought stress led to the destruction of proteins and amino acids structure through the production of Activated Oxygen Species (AOS). Furthermore, AOS had a high combinatory tendency with protein and oxidized them, leading to a decrease in the soluble protein of leaf (Parida and Das, 2005).

In all irrigation regimes, the combined application of chemical and biological fertilizers had an increasing effect on the soluble protein content compared to non-application of fertilizer (Table 5). This increase may indicate the role of this biofertilizer in inducing soluble proteins accumulation. Due to the role of nitrogen in the structure of the protein and nucleic acids,

Table 8. Correlation coefficients between Catalase (CAT), Peroxidase (POX), Polyphenol Oxidase (PPO), Proline, Total Soluble Sugar (TSS), Glycinebetaine (GB), Malondialdehyde (MDA), soluble protein, total chlorophyll and biological yield of black cumin in two experimental years.

Trait	1	2	3	4	5	6	7	8	9	10
1- CAT	1									
2- POX	-0.81^{**}	1								
3- PPO	-0.77^{**}	0.95^{**}	1							
4- Proline	-0.78^{**}	0.95^{**}	0.94^{**}	1						
5- TSS	-0.89^{**}	0.80^{**}	0.72^{**}	0.74^{**}	1					
6- GB	-0.80^{**}	0.95^{**}	0.95^{**}	0.93^{**}	0.72^{**}	1				
7- MDA	-0.87^{**}	0.84^{**}	0.86^{**}	0.85^{**}	0.74^{**}	0.87^{**}	1			
8- Soluble protein	0.66^{**}	-0.45^{**}	-0.35^{**}	-0.37^{**}	-0.82^{**}	-0.33^{**}	-0.36^{**}	1		
9- Total Chlorophyll	0.97^{**}	-0.81^{**}	-0.75^{**}	-0.76^{**}	-0.92^{**}	-0.77^{**}	-0.81^{**}	0.74^{**}	1	
10- Biological yield	0.71^{**}	-0.31^{**}	-0.24^{**}	-0.27^{**}	-0.68^{**}	-0.26^{**}	-0.38^{**}	0.80^{**}	0.75^{**}	1

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nitrogen-containing fertilizers increase protein content within plant tissues (Roy and Singh, 2006).

The correlation between soluble protein and proline (r= -0.37**) was negative (Table 8). A significant increase in proline content along with a significant decrease in leaf soluble protein under severe stress conditions can be attributed to protein degradation and reduction of its synthesis (Hsiao, 1973).

Withholding irrigation caused the reduction of total chlorophyll content (Tables 5 and 6). Reducing the chlorophyll content under drought stress may be either due to changes in the lipid/protein ratio of pigment-protein complexes or to increased chlorophyllase activity (Valifard *et al.*, 2012). Drought stress increased the chlorophyll degradation of leaves by breaking the hormonal balance (increasing abscisic acid and decreasing cytokinin) (Shaddad, 2011).

It can be stated that in all withholding irrigation regimes, the combined application of chemical and biological N-fertilizers had an increasing effect on total chlorophyll content compared to the single application of these fertilizers (Table 5). In stress condition, application of nitrogen fertilizer increased the production of cytokinin hormone. In this regard, among growth regulators, cytokinins play a critical role in increasing chlorophyll biosynthesis (Taiz and Zeiger, 2002). Also, bio-fertilizers reduce the damage to chlorophyll and stimulate the synthesis of chlorophyll by encouraging the formation of pyridoxal enzymes, which has an important role in the synthesis of α-amino linolenic acid as a major component in chlorophyll synthesis (Ramadan *et al*., 2003).

Total chlorophyll content had a negative correlation with proline (r= -0.76**) (Table 8). However, accumulation of leaf proline under stress prevented a severe reduction of photosynthetic pigments. Movludi *et al* (2014) reported that chlorophyll indirectly influence the proline accumulation.

Withholding irrigation decreased biological yield (Table 5). The increase in the biological yield of plants under optimal irrigation can be due to the expansion of the leaf area as well as its higher durability, which results in higher biological yield by producing an efficient physiological source for more light absorption (Vafa *et al*., 2014).

In all withholding irrigation regimes, the incremental effect of the combined application of chemical and biological fertilizers was clear on biological yield (Table 5). Through increasing the efficiency and uptake of nitrogen, biofertilizers improve branch growth, and thereby increase biological yield. The use of chemical fertilizer along with providing good nutritional conditions by making nitrogen available also increases the biological yield of corn (Ajami, 2016).

Correlation between biological yield and soluble protein (r= 0.80**) and total chlorophyll (r= 0.75**) was positive (Table 8). Considering that about 30-60% of leaf soluble proteins are from the RUBISCO enzymes, active oxygen forms reduce the content of this enzyme in different plants under drought stress (Surendar *et al*., 2013). The availability of nitrogen can affect the cellular content of RUBISCO with side effects on carbon assimilation (Wilhelm *et al*., 2006). Also, nitrogen is a key element in the chlorophyll that absorbs sunlight that is used in photosynthesis. Therefore, chlorophyll, photosynthesis, soluble protein content, and RUBISCO enzyme tend to increase with nitrogen uptake, thereby increasing the biological yield.

CONCLUSIONS

According to the results, although withholding irrigation reduced physiological traits and biological yield, but also the combined application of chemical and biological fertilizer resulted in increased them in the condition of withholding irrigation. It can be stated that under withholding irrigation condition, the plant

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causes changes in some of its physiological and biochemical characteristics. In that way, this plant applies defensive strategies, such as antioxidant enzymes and the compatible osmolites to overcome the negative effects of withholding irrigation and the removal reactive oxygen species. Besides, the combined application of chemical and biological fertilizers provided an optimal strategy for reducing adverse conditions. Finally, it can be concluded that in drought stress conditions, biofertilizers such as nitroxin, without any contamination to the environment, can provide the plant nutrition required and prepare better condition for plant growth by maintaining the soil microorganism.

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پاسخهای فیزیولوژیک سیاهدانه به کودهای شیمیایی و زیستی نیتروژنه تحت رژیم‌های مختلف آبیاری

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چکیده

این پژوهش با هدف تعیین پاسخ‌های فیزیولوژیک و عملکرد زیستی سیاهدانه (*Nigella sativa* L.) به کودهای شیمیایی و زیستی نیتروژنه تحت رژیم‌های آبیاری مختلف در سال‌های ۱۳۹۵ و ۱۳۹۷ در منطقه یاسوج انجام شد. آزمایش بصورت کرتهای خرد شده در قالب بلوک‌های کامل تصادفی با سه

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تکرار انجام شد. عامل اصلی در چهار سطح شامل رژیم‌های مختلف آبیاری (آبیاری کامل از مرحله سبز شدن تا بلوغ و قطع آبیاری از مراحل غنچه‌دهی، گلدهی و شروع تشکیل دانه تا بلوغ) و عامل فرعی شامل پنج سطح (بدون مصرف کود نیتروژنه، مصرف ۸۰ کیلوگرم نیتروژن در هکتار، ۴۰ کیلوگرم نیتروژن در هکتار، ۴۰ کیلوگرم نیتروژن در هکتار و نیتروکسین و نیتروکسین) بودند. نتایج نشان داد، کاربرد ۸۰ کیلوگرم نیتروژن در هکتار تحت شرایط آبیاری کامل و ترکیب ۴۰ کیلوگرم نیتروژن در هکتار و نیتروکسین تحت تمام رژیم‌های قطع آبیاری بیشترین میزان گلایسین بتایئن، فعالیت پلی‌فنول اکسیداز و کاتالاز، کلروفیل کل و عملکرد زیستی را تولید کرد. قطع آبیاری از مرحله غنچه‌دهی و همچنین کاربرد ۸۰ کیلوگرم نیتروژن در هکتار منجر به حداکثر مالوندی‌آلدهاید شد. ترکیب ۴۰ کیلوگرم نیتروژن در هکتار و نیتروکسین موجب حداکثر فعالیت پلی فنول اکسیداز، پرولین برگ و پروتئین محلول برگ شد. بطور کلی، کاربرد ترکیب ۴۰ کیلوگرم نیتروژن در هکتار و نیتروکسین فعالیت آنزیم‌های آنتی‌اکسیدانت و تجمع اسمولیتهای سازگار را تحت شرایط قطع آبیاری افزایش داد و بنابراین موجب کاهش اثرهای منفی تنش خشکی بر تجمع وزن خشک کل سیاهدانه گردید.

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