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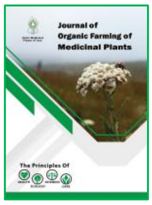

Original Article

Evaluation of N-biofertilizer and irrigation effects on some quantitative characteristics and thymoquinone content of black cumin (*Nigella sativa* L.)

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ABSTRACT

The main objective of this study was to investigate the effects of terminating irrigation at different developmental stages and *Azospirillum* inoculation on quantitative and qualitative yield of black cumin (*Nigella sativa* L.). Treatments consisted of irrigation with three levels (W1 = normal irrigation from emergence to harvest (control), W2 = irrigation terminated at the start of budding and W3 = irrigation terminated at the start of flowering) and *Azospirillum* inoculation at four levels (A1=non-inoculated, A2=seed inoculated and A3= spraying on the plant base at stem elongation stage, A4= seed inoculated + spraying on the plant base at stem elongation stage). The present results have shown that irrigation terminated at the start of budding caused significant reduction in number of follicles per plant, biomass production and seed yield. But, thymoquinone content increased in response to the water stress. *Azospirillum* had positive effects in all measured traits, especially when it used at two times (at seed inoculated + spraying on the plant base at stem elongation stage or A4 treatment).

1. Introduction

One third of the world lands are classified as arid and semi-arid region and the remains are faced with water seasonal or local fluctuations (Beweley and Krochko, 1982). Availability of water rather than land is the main constraint on agricultural production in arid and semi-arid environments (Barnabas et al., 2008). Optimizing irrigation management together with the cultivation of appropriate crops is desirable in these regions. Irrigation scheduling based on developmental stage is the technique of applying water on a timely and accurate basis to the crop (Bannayan et al., 2006), and is the key to conserving water and improving irrigation performance and sustainability of irrigated agriculture (Mpelasoka et al., 2001) without yield and yield components decreases or with an acceptable decrease of it (Bozorgi et al., 2011).
Various studies have shown that water stress has negative effects on yield and yield components of some medicinal plants such as *Nigella sativa* (Bannayan et al., 2008; Karimi Yeganeh and Zeinali, 2010), *Plantago ovate* (Pooryoosef et al., 2010), *Matricaria chamomilla* (Pirzad et al., 2011); *Cuminum cyminum* (Ahmadian et al., 2011); *Mentha piperita* (Khorasaninejad et al., 2011) and *Melissa officinalis* (Ozturk et al., 2004). Also, water stress during budding, flowering and anthesis stages of plants, will cause most reduction in seed number and seed yield (Elikaei Ghanbary et al., 2008).
The potential of medicinal and aromatic plants for growing under limited water conditions make them suitable alternative crops in such agro- ecosystems (Haj Seyed Hadi et al., 2011; Koocheki and Nadjafi, 2003).
Black cumin (*Nigella sativa* L.) is an annual species that have originated from arid and semi-arid zones and is used widely in traditional and industrial pharmacology (Patel et al., 1996).
One of the most important constituents of volatile oil of the *Nigella sativa* seeds are thymoquinone (Badary et al., 2000; Burits and Bucar, 2000). Thymoquinone belongs to class of compounds known as terpenoids (Bannayan et al., 2008). In vivo, it inhibits fore stomach and fibro sarcoma tumor incidence and multiplicity in mice (Badary et al., 1999). Pharmacological investigations explored the effectiveness thymoquinone and carvone against various maladies like oxidative stress, cancer,

immune dysfunction and diabetic complications (Rooney and Ryan, 2005; Ivankovic et al., 2006). Several types of studies in the last 20 years have shown a beneficial effect on crop plants by inoculation of seeds with *Azospirillum* strains (Okon, 1985; Sarig et al., 1988; Das et al., 2007; Verma, 2011; Pereyra et al., 2012). Positive effects of inoculation have been demonstrated on various root parameters, including increase in root length, particularly of the root elongation zone (Kolb and Martin, 1985; Sarig et al., 1988).

Some research has been carried out on responses of black cumin to different irrigation intervals (Babai, 1995; Mozzafari et al., 2000; Bannayan et al., 2008) but irrigation scheduling based on developmental stage has not been studied well. And also, there are a few studies on *Azospirillum* inoculation in black cumin. So, the main objective of this study was to investigate the effects of terminating irrigation at budding and flowering stages and *Azospirillum* inoculation on quantitative and qualitative yield of black cumin.

2. Materials and Methods

2.1Field experiment

The present study was conducted during the growing season of 2018 at the Experimental Station of the Research Institute of Forest and Rangeland, Damavand, Iran (Latitude: 35° 39´ N; Longitude: 52° 05´ E; Elevation: 1800m). The soil of the experimental region was a loamy-clay with pH 7.1, containing total N (0.9 mg/kg), total P (40 mg/kg), total K (550 mg/kg), OC (0.8 %), with an EC of 0.70 (dc/m). Meteorological data on rainfall and mean temperature from January until September and during developmental stages of black cumin are presented at Fig.1 & 2 and Table. 1, respectively.

Table1. Mean temperature and rainfall during various developmental stages of Black cumin.

Date	Developmental stage	Mean	Rainfall
9 April – 23 April	Planting – Seed emergence	23.6	0
23 April – 13 June	Seed Emergence – Budding stage	26.5	5.7
13 June – 25 June	Budding stage – Flowering stage	24.1	2.1
25 June – 7 July	Flowering stage – Seed Maturity	24.8	0.2

The experimental design was split-plot, based on Randomized Complete Block Design (RCBD) with three replications. Treatments consisted of irrigation with three levels (W1 = normal irrigation from emergence to harvest (control), W2 = irrigation terminated at the start of budding and W3 = irrigation terminated at the start of flowering) and *Azospirillum* inoculation at three levels (A1=non-inoculated, A2=seed inoculated and A3= spraying on the plant base at stem elongation stage, A4= seed inoculated + spraying on the plant base at stem elongation stage).

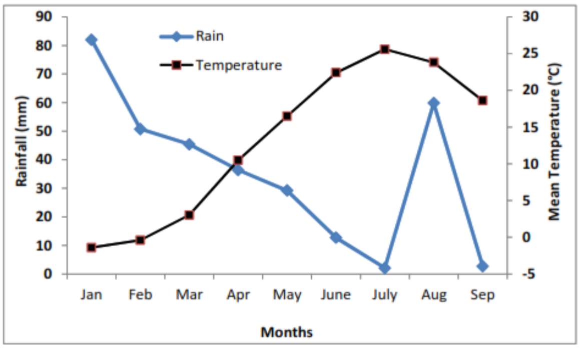

Fig 1. Mean temperature and rainfall during January untill September 2011. Meteorological Data obtained from climatological station of Hamand- Damavand (available on: http: //www. irimet.net).

To record the developmental stages on a weekly basis, the 2 middle rows of each plot was monitored. The developmental stages were determined when one plant in each plot indicated that stage. The developmental stages were: budding, when at least one folded flower bud was observed per plant and flowering, when at least one unfolded flower was observed per plant.

Azospirillum lipoferum was used as the test microorganism. Inoculation was carried out by dipping the black cumin seeds in the cells suspension of 108 CFU/ml for 15 min (Morgenstern and Okon, 1987*)*. Each experimental plot was 3 m long and 2 m wide with the total area of 6 m2. Black cumin seeds were obtained from the Research Center of Medicinal Plants, Isfahan, Iran. Sowing was done manually, 0.5 cm depth and in rows with 25 cm apart on 9 April 2018. Three weeks after sowing, the seedlings were thinned up to 180-plant m−2. Irrigation furrows with uniform slopes were constructed in each experimental plot. A one-time irrigation was applied immediately after sowing for uniform emergence. Soil water content was measured for each development stage weekly across all treatments and was determined on a dry mass basis and converted to a volumetric basis using 1.35 g cm -3 as soil bulk density. In order to determine the soil water content, 24 h after irrigation 5 soil samples were taken by sampling drill, then samples were weighed by electrical scale and placed under 105°C in electrical oven for 48 h (Cassel, 1998; Mahmoodi and Hakimian, 2001):

$$\text{Soil Water Content (Volumetric Basis)} = \frac{\text{Wet Soil (g)} - \text{Dry Soil (g)}}{\text{Dry Soil (g)}} \times \frac{\text{Soil Bulk Density}}{\text{Water Bulk Density}}$$

Based on field capacity (26.5% θ) and wilting point (11% θ) of the study site, 50% of available soil water was considered as the water stress threshold (17.2% θ).

Weeds were controlled manually. All necessary cultural practices and plant protection measures were followed uniformly for all the plots during the entire period of experimentation.

2.2 Measurements

Harvesting was done manually by pulling the dry plant out of the soil and removing the roots. Final seed yield and yield components were measured from 1 and 0.1 m2 of each plot, respectively. Characters consisted of number of follicles per plant, 1000 seed weight, seed yield, harvest index and thymoquinone content.

Harvest index was calculated as follow:

$$HI = \frac{Y}{Ps + Y}$$

Where, HI is harvest index, Y is economical seed yield (kg/ha) and Ps is straw yield (kg/ha)

Weight of seeds and straw were measured in each plot using a digital balance (Sartorius B310S) (±0.01 g) (Azizi et al., 2009; Haj Seyed Hadi et al., 2011).

To determine the amount of essential oil, a sample of 10 g of seeds was mixed with 50 ml of tap water in a flask and the water was distilled for 3 h using a rotavapor instrument and flask put over the heat produced by electro-thermal instrument for 3 hours (for providing direct heat condition). Distillated were put in the centrifuge (at 1000 rpm for 30 min) and supernatants were separated.

UV monitoring of the eluted solutes was carried out at 254 nm for thymoquinone (Ghosheh et al., 1999) by using spectrophotometer UV-visible (model: Cary 100 Boi, VARIAN). Quantities of thymoquinone were determined by measuring the peak area. For standardizing the product, benzoquinone molecule was applied.

2.3 Statistical analysis

All data were subjected to the statistical analysis (one-way ANOVA) using SAS software (SAS Institute Inc, 2002). Means of comparisons were performed by Duncan's Multiple Range Test (DMRT) at 5% probability level. Data were transformed when necessary before analysis to satisfy the assumptions of normality.

However, any values mentioned in this section refer to the original data of present experiment.

3. Results

The results have indicated that measured traits were significantly affected by irrigation and Azospirillum inoculation treatments (Table 2). Interactions was significant only for seed yield.

3.1 Number of follicles per plant

Both, irrigation and *Azospirillum* inoculation treatments had significant effects on number of follicles per plant (Table 2).

The lowest number of follicles per plant was obtained under W3 while the highest number of follicles (6.52) was obtained under W1 or normal water regime.

Bannayan et al (2008) indicated that irrigation had a significant effect on number of follicles per plant and the lowest number of follicles was obtained where the irrigation terminated at budding stage of Black cumin.

Mean comparison has shown significant differences between various levels of *Azospirillum* inoculations. Applying *Azospirillum* at two times (A4 = seed inoculation + spraying on the plant base at stem elongation stage) had the most positive effect on this trait (Table 3).

Bashan and Levanony (1990) have shown that inoculation of plants with *Azospirillum* can result in a significant change in various plant growth parameters. Also, Increased in number of spikes and grains per spike in cereals in response to *Azospirillum* inoculation have been reported (Schank *et al*. 1985; Warembourg *et al*. 1987).

3.2 1000 seed weight

During the present experiment, the one thousand seed weight was not significantly influenced by irrigation treatments (Table 2). Mean seed weight is a relatively stable yield component under water stress treatments and one thousand seed weight rapeseed and faba bean have not been affected by water stress (Xia, 1994; Champolivier and Merrien, 1996).

Table 2. Effects of irrigation treatments and *Azospirillum* inoculation on yield, yield components and plant height of black cumin (*N. sativa* L.)

S.O.V	DF	height	1000 seed weight	Number of follicle / plant	Biological yield	Seed yield
rep	2	0.2686111	0.87861111	1.30707778	112442.274	5058.3333
a	2	1.3119444	4.48178611*	26.41257778**	3602160.419**	359363.5574**
rep×a	4	10.6286111	0.58686944	1.93130694	12851.939	66.6667
b	3	21.5173148*	0.79314815	15.00264074**	336897.115**	57049.7774**
a×b	6	19.5578704*	0.68376759	2.27862963	32652.589	642.1245**
error	18	5.3804630	0.25722778	0.8671306	36468.432	8.3333
CV%		7.98	9.41	12.52	9.66	7.53

ns, * and ** indicate insignificant and the significant differences between traits at P-value<0.05 and P-value<0.01., respectively.

Table 3. Mean comparison of the quantitative characteristics of black cumin at various levels of irrigation treatments and *Azospirillum* inoculation

Treatments	Number follicle/plant	1000 seed weight (g)	Seed yield (kg/ha)	Harvest index	Thymoquinone (mM)*
Irrigation treatments					
W_1	6.52 a	1.98 a	722.85 a	28.20 a	0.012684 b
W_2	4.15 b	1.93 a	377.32 c	25.54 b	0.021964 a
W_3	3.72 b	1.91 a	532.88 b	28.18 a	0.015004 b
Azospirillum lipoferum					
A_1	3.61 c	1.45 b	430.71 d	24.98 b	0.018250 b
A_2	4.82 b	1.67 ab	583.45 b	27.68 a	0.020159 a
A_3	4.27 bc	1.62 ab	551.63 c	28.60 a	0.018747 b

*based on millimolar per 10 g seed weight.

W_1 = normal irrigation from emergence to harvest (control), W_2 = irrigation terminated at the start of budding and W_3 = irrigation terminated at the start of flowering; A_1=non-inoculated, A_2=seed inoculated,

A_3= spraying on the plant base at stem elongation stage and A_4= seed inoculated + spraying on the plant base at stem elongation stage

*Mean values followed by the same letter are not significantly different at $P \le 0.05$.

Also, Irrigation termination at budding, flowering and seed formation stages had not significant effects on one thousands seed weight of Black cumin (Bannayan et al., 2008).

Azospirillum inoculation had significant influence on 1000 seed weight. The highest one thousands seed weight (2.14 g) was obtained from A4 treatment. Several studies have indicated to increased grain weight by inoculation of seeds with *Azospirillum* strains (Pacovsky *et al*. 1985; Schank *et al*. 1985).

3.3 harvest index

Analysis of variance showed that harvest index affected by irrigation treatments and *Azospirillum* inoculation, significantly. Mean comparison, also showed significant differences between various levels of irrigation treatments (Table 2). The highest harvest index (28.20%) was recorded under the W1 treatment. but, there were not significant differences between W1 and W3 treatments. Irrigation termination at start of budding stage caused 9.4% reduction in harvest index compared to the control (W1).

Mean comparison showed significant differences between various levels of *Azospirillum* inoculation.

A1 caused the plant to reach the lowest harvest index (24.98 %). As a general, Azospirillum could increase harvest index significantly in comparison to A1 (control). But, there were not significant changes between A2, A3 and A4 treatment.

According to the interaction between various levels of treatments, W2A2 and W1A4 caused the highest increase in harvest index (Fig. 2). It means that *Azospirillum* could enhance harvest index in water stress conditions.

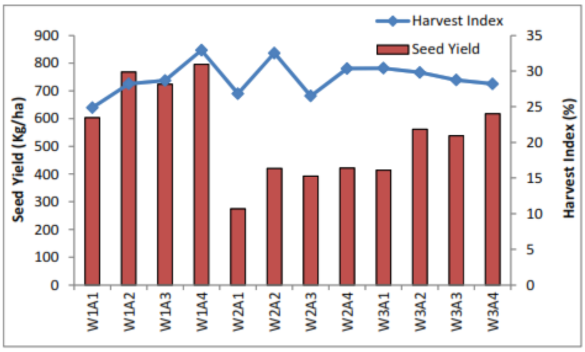

Fig 2. Variation of seed yield and harvest index in response to the various levels of irrigation treatments and Azospirillum inoculation (W_1 = normal irrigation from emergence to harvest (control), W_2 = irrigation terminated at the start of budding and W_3 = irrigation terminated at the start of flowering; A_1=non-inoculated, A_2=seed inoculated, A_3= spraying on the plant base at stem elongation stage and A_4= seed inoculated + spraying on the plant base at stem elongation stage).

3.4 Seed yield

Results showed that irrigation treatments and *Azospirillum* inoculation had significant effects on the seed yield. Mean Comparison showed that seed yield varied between 722.85 and 372.32 kg/ha (Table 3), which was obtained from control (W1) and W2, respectively. Seed yield in response to irrigation cessation at the start of budding (W2) showed the highest decrease compared to controls. The high seed yield of black cumin under W1 treatment might be due to a higher number of follicles per plant and also a higher seed yield (Table 3). Nadjafi and Rezvani (2002) reported that irrigations have significant effects on seed yield.

In combination between various levels of irrigation treatment and Azospirillum inoculation, W1A4 and W1A2 resulted in the highest seed yield (Fig 2).

There were significant differences in seed yield between the plants inoculated with various levels of *Azospirillum lipoferum*. A4 caused the highest seed yield in black cumin (Table 3). In field experiments in Argentina, corn inoculated with *Azospirillum lipoferum* showed double the seeds per ear and increased seed yield (Fulchieri and Frioni 1994).

Inoculation of wheat with various strains of *Azospirillum* caused significant increases over controls in grain yield, ranging from 23 to 63 % (Caballero Mellado et al. 1992).

3.5 Thymoquinone content

Irrigation treatments and *Azospirillum* had significant effects on thymoquinone content. But, it was not affected by interactions between various levels of treatments, significantly.

Maximum thymoquinone content (0.021964 mM/10 g seed) was obtained when irrigation terminated at the start of budding stage. But, there were not significant differences between W1 and W3 treatment for thymoquinone content (Table 1).

Azospirillum could increase thymoquinone content significantly as compared to the control (A1). The highest amount of thymoquinone (0.020159 mM) were obtained at A2. Although, according to the Table 2, there is no significant difference between A2 and A4 treatments.

4. Discussion

Optimizing irrigation management together with the cultivation of appropriate crops is appropriate in arid and semi-arid regions. As it was shown in the results of this study, irrigation treatments had significant effects on most of measured traits. At W2, number of follicle per plant and seed yield were at least. While, the maximum thymoquinone content was obtaind at W2.

The harvest index from W2 was 9.43% and 9.36% lower in comparison with W1 (control) and W3, respectively. Water stress conditions and improper irrigation management, will result in lower assimilation production in the plants (Junqueira and Oliveira, 1997). Also, allocation of assimilates among seeds and vegetative organs will change. Reduction of harvest index under water stress conditions is because of sooner leaves senescence, reduction in seed filling period and lower seed weight (Shakiba et al., 1996). Similar result was obtained by Paknejad et al (2009).

Azospirrilum inoculation could enhance growth and production of black cumin in comparison with control. In A2 treatment (seed inoculated), the highest harvest index was obtained. Although, there were not significant differences between inoculations seeds with *Azospirillum* or spraying at plant base at stem elongation stage.

The most reduction in seed yield obtained from W2 treatment. In other hand, when irrigation terminated at budding stage, its negative effects on seed yield were 29.19% more than W3. The amount of injury caused by the water stress depends on the stage of plant development at which it occurs. Generally at reproductive phase the plant is most sensitive to stresses (Barnabas et al., 2008). The decrease in yield and yield components due to water deficiency has also been reported by other researchers (Dominique et al., 2007; Masoumi et al., 2011; Aiken et al., 2010).

Azospirrilum inoculation could enhance growth and production of black cumin in comparison with control. In A4 treatment (seed inoculated + spraying on the plant base at stem elongation stage), the highest 1000 seed weight, number of follicle per plant, seed yield and thymoquinone content were obtained.

These changes were directly attributed to positive bacterial effects on mineral uptake by the plant. Enhancement in uptake of NO3- , NH4+, P042- , K +, Rb+ and Fe++ by *Azospirillum* (Barton *et al*. 1986; Murty and Ladha 1987) was proposed to cause an increase in foliar dry matter and accumulation of minerals in stems and leaves.

Applying *Azospirillum* only at the base of the plants at stem elongation phase, did not show really any positive effects. For number of follicle per plant and 1000 seed weight, there were not significant differences between A1 and A3. For seed yield, Azospirillum had more positive effects and could increase differences between control (A1) and other levels of bacteria application (A2-A4). *Azospirillum* inoculation could improve water status in stressed plants.

Inoculated plants are less water stressed, having more water in their foliage, higher leaf water potential, and lower canopy temperature than non-inoculated plants. Total extraction of soil moisture by *Azospirillum*-inoculated plants is greater and water extracts from deeper layers in the soil profile (Sarig *et al*. 1988). Therefore, plant yield increase in inoculated plants was attributed primarily to improved utilization of soil moisture. These effects could result in more water uptake, especially in arid and semi-arid regions.

According to the results, the highest thymoquinone content obtained when irrigation terminated at the start of budding stage. In the other hand, water stress increased active compounds of black cumin. Other studies have shown that water stress could enhance active substance and quality of some medicinal plants such as menthol of peppermint (Khorasaninejad et al., 2011), plumbagin of Chitrak (Kharadi et al., 2011), essential oil of balm (Ozturk et al., 2004), essential oil of cumin (Ahmadian et al., 2011) and essential oil of basil (Khalid, 2006).

It must be taken into consideration that a concentration increase of active compounds induced by moderate drought stress in general is associated with a reduction of biomass production. Consequently, it has to be clarified the putative gain in quality by increasing the secondary plant product concentration by applying deliberately drought stress would be compensated by decreasing yields in biomass. Thus, a corresponding decision must strongly be based on the question related to the nature of the desired product.

It is obvious that in the case of medicinal plants which are used as pharmaceuticals, the quality and thus the concentration of active compounds is much more relevant than the total yield, whereas in all cases, where the desired compounds will be extracted, the overall yield has to be very high. A successful and effective application of deliberate drought stress for quality improvement, e.g. by applying special watering regimes is an encouraging new tool for the production of medicinal and pharmaceutical relevant plants (Selmar, 2008).

For thymoquinone content in the seeds, *Azospirillum* had positive effects and could increase differences between control (A1) and other levels of bacteria application (A2-A4). *Azospirillum* inoculation with seeds could improve seed quality.

5. Conclusion

Our study showed that the start of budding was the most sensitive to irrigation termination. Irrigation termination affected flowering, and the number of follicle per plant was also severely affected. Therefore, reduced sink capacity lead to lower seed yield when irrigation was stopped at the beginning of budding stage. On the other hand, the highest thymoquinone and carvone content obtained when irrigation terminated at budding stage. While, increasing the active compounds by W2 treatment compensated by decreasing seed yields. According to the advantages of *Azospirillum* inoculation, which it has mentioned previously; it seems using this *Azospirillum* in agro-ecosystems could increase seed yield and quality of seeds and, of course, help farmers to save water in arid and semi-arid regions.

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