THE EFFECTS OF APPLICATION OF BIOLOGICAL FERTILIZERS AND DIFFERENT AMOUNTS OF UREA FERTILIZER SOURCES UNDER LOW WATER STRESS CONDITIONS ON PHYSIOLOGICAL TRAITS OF MEDICINAL PLANT (*CALENDULA OFFICINALIS* L.)

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Abstract. In order to evaluate the effect of application of biological fertilizers and different amounts of urea fertilizer under low water stress conditions on physiological traits of *Calendula officinalis* L. an experiment was conducted during two years of cultivation of 2015-2016 and 2016-2017 at the research farm of Islamic Azad University (IAU), Varamin-Pishva Branch, Iran. The experiment was split-split plot based on randomized complete block design with three replications. The experimental treatments included different levels of dehydration as the main factor in two levels (lack of water stress (control), irrigation interruption during boiling stage), levels of biological fertilizers in four levels as a sub-agent (no use of biological fertilizer, application of *Azotobacter chroococcum*, application of *Azospirillum brasilense*, combination of Azotobacter and Azospirillum), and urea fertilizer sources at four levels as a sub-subtype (non-consumption (control), recommended amount of urea fertilizer with sulphur coating, 75% of the recommended amount of urea fertilizer with sulphur coating was recommended as the most common form of urea fertilizer (157 kg/ha based on soil test). The results showed that irrigation in boiling stage increased activity of the enzyme di-hydroxy Guanuzyn, glutathione peroxidase, superoxide dismutase also, production of the total phenol, total flavonoid and biomarker of di-tyrosine. In this study, drought stress and application of Nitroxin fertilizer resulted in increased malondialdehyde in the first year. In both years, the application of Nitroxin with the recommended amount of urea fertilizer with sulphur coating resulted in an increase in total flavonoid and Glutathione Peroxidase enzymes. The purpose of this study was to investigate the effect of bacteria in the plant, along with the amount and sources of nitrogen fertilizer to reduce nitrogen fertilizer application on plant yield, as well as sustainable agriculture management through integrated nutrition in different plant conditions.
Keywords: *azospirillum, azotobacter, biomarker, total phenol*

Introduction

Calendula officinalis is a plant native to southern Europe and can be found in countryside fields. Calendula is an annual or sometimes biennial plant with erect stems up to 40-70 cm tall and deep taproot. It belongs to the Asteraceae family, its English name is Marygold. It is one of the well-known medicinal plants today, flowers and essential oils are used in pharmaceutical and cosmetic industries. Pharmacological

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studies have confirmed that flowers have a large amount of biological effects and pharmacological activity of the liver and antispasmodic protection (Arora et al., 2013).

Medicinal plants have high economical value due to the presence of effective compounds in them. An agricultural crop is economically valuable medicinal plant when secondary metabolites have reached the optimum level and the purpose of this plant is to exploit the effective ingredients in flowers, especially petals. Nitrogen is one of the elements that is lacking in most arid and semi-arid regions, because the amount of organic matter in these areas is very low as the main source of nitrogen storage or it decomposes rapidly due to high heat (Saneoka, 2004). If available nitrogen is toxic or deficient for the plant, the vital processes of the plant cause a disorder that may occur in various forms such as high growth, reduced transpiration, or even cessation of reproductive growth (Saneoka, 2004). Since most nitrogen fertilizers are wasted shortly after consumption, nitrogen management as well as crop should be done accurately during the growing season. In the meantime, Sulphur Coated Urea fertilizer (S.C.U.) on the one hand is a supplier of nitrogen and on the other hand, it is very important to pay attention to the valuable role of sulphur in plant nutrition and the improvement of agricultural soils, especially in our country, which accounts for over 70% of calcareous agricultural land and high pH (Moallem and Eshghizadeh, 2007). One of the basic pillars of sustainable agriculture is the use of bio-fertilizers in crop ecosystems with a view to eliminating or reducing the use of chemical organs (Shubbra et al., 2004). These bacteria, in addition to the role of elemental absorption, reduce disease, improve soil structure, stimulate more plant growth, increase product quality and quantity, and increase resistance to environmental stress (Nagananda et al., 2010). Drought is the most important limiting factor for plant growth and crop production around the world (Abedi and Pakniyat, 2010).

Dehydration also reduces water absorption by plant root system, reducing transpiration, reducing Stomatal conductance and photosynthesis, as well as breaking the hormonal balance of the plant (Auge et al., 2015). Superoxide dismutase is very important for plant tolerance to oxidative stress, which has been reported by many researchers (McKersie et al., 2000). Therefore, superoxide dismutase seems to be at the forefront of oxidative stress protection and the increase of superoxide dismutase is correlated with increasing the protection of damages from environmental stresses (Pang et al., 2005; Sigaud-Kutner et al., 2002). In a study, drought stress resulted in an increase in antioxidants in an *Calendula officinalis* plant (Sedghi et al., 2012). Oxidative stress during drought stress and increased free radicals by reducing the antioxidant defense results in damage to tissues, lipids, proteins and nucleic acids, and the concentration of biomarkers such as malondialdehyde, di-tyrosine and di-hydroxy Guanuzyn increased (Jose et al., 1999). Data were analyzed using (SAS) statistical software (v.9.12) and the meanings were compared by Duncan's multiple range tests at the 5% level. Draw charts with Excel (2010) software. Considering the above, the purpose of this study was to investigate the effect of application of Bio-fertilizers along with fertilizers of the hunchback on physiological characteristics of an ever-spring medicinal plant under stressed water conditions.

Materials and methods

In order to evaluate the effect of application of biological fertilizers and different amounts of urea fertilizer under low water stress conditions on physiological

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characteristics of *Calendula officinalis* L. an experiment was conducted during two years of cultivation of 2015-2016 and 2016-2017 at the research farm of Islamic Azad University, Varamin-Pishva_Branch in Tehran province (*Table 1*).

Table 1. *Plan*

S1																**S2**																
B1				**B2**				**B3**				**B4**				**B2**				**B3**				**B4**				**B1**				R
N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	2
1	3	2	4	3	2	1	4	4	2	1	3	4	2	3	1	1	3	2	4	4	2	3	1	4	2	1	3	3	4	2	1	
S1																**S2**																
B4				**B2**				**B1**				**B3**				**B2**				**B1**				**B4**				**B3**				R
N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	1
4	2	1	3	1	3	2	4	1	3	2	4	1	3	2	4	4	2	1	3	4	2	3	1	3	4	2	1	4	2	3	1	
S2																**S1**																
B3				**B1**				**B4**				**B2**				**B4**				**B2**				**B1**				**B3**				R
N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	3
4	2	3	1	3	4	2	1	4	2	3	1	1	3	2	4	1	3	2	4	1	3	2	4	1	3	2	4	4	2	1	3	

N1: non-consumption (control), N2: 75% of the recommended amount of urea fertilizer with sulphur coating, N3: recommended amount of urea fertilizer with sulphur coating, N4: (common form) recommended amount of urea fertilizer, S1: (control) lack of water stress, S2: irrigation interruption during boiling stage, B1: (control) no use of biological fertilizer, B2: Azotobacter, B3: Azospirillum, B4: Azot and Azos

Test site located at latitude 35.19 degrees east longitude 51.39 and the altitude is 1050 m above sea level, with dry and desert weather conditions, and steppes with an average annual precipitation of about 175 mm. The experiment was split-split plot based on randomized complete block design with three replications. The experimental treatments included different levels of dehydration as the main factor in two levels (lack of water stress (control), irrigation interruption during boiling stage) levels of biological fertilizers at four levels as a sub-agent (no use of biological fertilizer, application of *Azotobacter chroococcum*, application of *Azospirillum brasilense*, combination of Azotobacter and Azospirillum (Nitroxin) and urea fertilizer sources in four levels as a subtype (non-consumption (control), recommended amount of urea fertilizer with sulphur coating, 75% of recommended amount of urea fertilizer with sulphur coating, was recommended as the most common form (mentioned area) of urea fertilizer (The amount of fertilizer usage based on fertilizer suggestion, 157 kg/ha^{-1}).

Inoculation of seeds took place with bio-fertilizer by (CFU) method (Bapat et al., 2006). Before planting, to determine the physical and chemical characteristics of the soil, from 0-30 cm depth and 30 to 60 cm soil, two locations for sampling were carried out, the full specifications of which are given in *Table 2*. After the leveling of the land, a 6×2 m plot was made, each plot having six rows and a row spacing of 30 cm and a spacing of 10 cm. The cultivation took place in the first week of June. The first drip irrigation was carried out after planting and until the emergence stage, irrigation was carried out with 3-day courses. After emergence of the seeds, the irrigation interval increased to 5 days and continued to 7-8 leaf stage. After the shrubs were fully deployed, at the beginning of budding, the irrigation was stopped for 10 days (Varamin district, Tehran province). The amount of fertilizer application was based on soil test.

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After harvest, the specimens were transferred to the laboratory to measure the traits and there are traits such as total phenol, total flavonoid, di-hydroxy Guanuzyn enzyme, glutathione peroxidase, superoxide dismutase, malondialdehyde biomarker and di-tyrosine were measured. To determine the amount of superoxide dismutase, Sairam et al. (2001) method was used. To prepare the reaction mixture of 13 ml of methionine, 25 μM of Nitroblutterazolium, 6 μM of 0.5 (MEDTA) solution, 1.5 ml of a solution of 1 M buffer phosphate (pH = 7.8), 60 mM Molybucent riboflavin and 50 mM sodium bicarbonate. Then, 2.9 ml of the resulting mixture was poured into a sterilized tube, immediately after adding 2 μM of riboflavin and 0.1 ml of the enzyme extract, for 15 min, a fluorescence lamp of 15 × 2 watts was placed.

Table 2. *Soil field characteristics*

B (ppm)	Mn (ppm)	Cu (ppm)	Zn (ppm)	Fe (ppm)	K (ppm)	P (ppm)	N (%)	OM (%)	OC (%)	TNV (%)	pH	EC ($dS.M^{-1}$)	Silt (%)	Sand (%)	Clay (%)	Type of experiment
1.22	9.74	0.96	0.56	3.44	406.6	12.8	0.06	1.16	0.68	21.4	7.78	1.97	42	36	22	Soil sample

C: Clay, S: Sand, S: Silt, EC: Electrical Conductivity, TNV: Total Neutralising Value, OC: Organic Carbon, OM: Organic Matter, N: Nitrogen, P: Phosphorus, K: Potassium, Fe: Iron, Zn: Zink, Cu: Copper, Mn: Manganese, B: Boron

To determine the amount of activity of the superoxide dismutase enzyme in the mixture, Spectrophotometer was measured at 560 nm at 23 ± 2 °C spectrophotometry (to measure, 5 leaves from each line was cultivar, in the morning they were harvested from the field). The amount of enzyme changes was determined by Paglia (1997) to calculate the Glutathione Peroxidase enzyme (2 leaves of plant from each line). Di-hydroxy Guanzine assay was performed according to the method (fresh plant tissue (leaf) was homogenized by homogenization and then centrifuged at 5000 rpm for 60 minutes with a centrifugal machine) (Bogdanov and Bical, 1999). Measurement of malondialdehyde and di-tyrosine was determined by the method (The extract used to measure 8-oH-dG is based on the thiobarbetabic acid method with MDA) (Steven and Joseph, 1978). The absorption rate of Flavonoids was measured by Krizek et al. (1993) at three wavelengths of 300, 330 and 270 using the (RAY LEIGH UV 1601) spectrophotometer (24 g samples of dried petals were needed). Also, total phenol measurements were dried by extraction of flower samples and at a rate of 2 g of dried matter *Calendula officinalis* (Extraction of polyphenols from flowers collected using the company protocol Naturalin (Professional of Natural Ingredients)). Data were analyzed using (SAS) statistical software (v.9.12) and the meanings were compared by Duncan's multiple range tests at the 5% level. Draw charts with Excel (2010) software.

Results and discussion

Total phenol

In both years of experiment, the effect of drought stress (S), biological fertilizer (B) and urea fertilizer (N) on this trait was significant at 1% level (*Table 3*). Drought stress in both years of experiment led to a significant increase in this trait. So that the Total Phenol in stress conditions increased 8.3% and 8% in the first and second years respectively (*Table 4*). The application of Nitroxin biological fertilizer in both years led to a significant increase in Total Phenol the Total Phenol in terms of Nitroxin

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application increased 19.2% and 14.8% in the first and second year respectively (*Table 5*). Also, application of recommended amount of urea fertilizer with sulphur coating and recommended amount of urea fertilizer in both years of experiment resulted in significant increase of Total Phenol (*Table 6*). High antioxidant Phenolic compounds such as anthocyanin and Flavonoids play a major role in plant resistance to stress and reduce the damage caused by it (Nasibi, 2005; Prasad, 2002).

Table 3. *Analysis of variance of the effect of biological fertilizers and different amounts of urea sources under irrigated conditions of Calendula officinalis L plant during two years of experiment, 2015-2016 and 2016-2017*

Average of squares							
Sources of changes	**Degrees of freedom**	**Total phenol**		**Di-hydroxy Guanuzyn enzyme**		**Total flavonoid**	
		First year	**Second year**	**First year**	**Second year**	**First year**	**Second year**
Repeat	2	14.46^{**}	11.18^{**}	3.26^{**}	1.61^{**}	0.57^{ns}	1.06^{**}
Drought stress (S)	1	124.74^{**}	118.99^{**}	29.06^{**}	30.92^{**}	109.68^{**}	68.12^{**}
Main error	2	0.04	0.35	0.11	0.14	0.67	0.05
Biological fertilizer (B)	3	95.45^{**}	88.66^{**}	7.41^{ns}	2.26^{ns}	122.73^{**}	119^{**}
S×B	3	1.9^{ns}	0.49^{ns}	0.47^{ns}	0.09^{ns}	0.88^{ns}	1.3^{ns}
Sub-error	12	3.36	0.67	3.57	1.6	0.94	1.16
Urea fertilizer sources (N)	3	69.62^{**}	72^{**}	6.23^{ns}	3.95^{ns}	142.44^{**}	128.56^{**}
S×N	3	0.74^{ns}	1.46^{ns}	0.04^{ns}	0.01^{ns}	1.03^{ns}	1.21^{ns}
B×N	9	6.33^{ns}	1.97^{ns}	1.35^{ns}	0.96^{ns}	5.69^{**}	3.7^{*}
S×B×N	9	0.34^{ns}	0.34^{ns}	0.04^{ns}	2.03^{ns}	0.66^{ns}	2.67^{ns}
Sub-error	48	3.25	1.84	2.45	2.08	1.28	1.76
Coefficient of variation (%)		6.8	5	14.4	12.4	6.4	8

ns, *, and ** were non-significant and significant at levels of 5% and 1%, respectively

Table 4. *Comparison of the effect of drought stress on some physiological traits of Calendula officinalis L. in two years of experimentation of 2015-2016 and 2016-2017*

Experimental treatments	**Total phenol (mg/g dry extract)**		**Total flavonoid (mg/g dry extract)**		**Di-hydroxy Guanuzyn enzyme (nano mole per mg protein)**	
	First year	**Second year**	**First year**	**Second year**	**First year**	**Second year**
Tension levels						
Lack of water stress	25.24 b	25.81 b	16.47 b	15.79 b	10.35 b	11.04 b
Irrigation cut in the budding stage	27.52 a	28.04 a	18.6 a	17.47 a	11.45 a	12.17 a

In each column, the same letters indicate that there is no significant difference between the mean of the treatments

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Table 5. *Comparison of the effect of biological fertilizer on some flowers in the two years of experimentation of 2015-2016 and 2016-2017*

Experimental treatments	Total phenol (mg/g fresh extract)		Biomarker Di-tyrosine (nano mole per mg of protein)	
	First year	Second year	First year	Second year
Bio-fertilizer				
Lack of use (control)	24.42 c	25.24 d	97.92 a	106.47 a
Azotobacter	25.54 bc	25.95 c	97.03 a	104.79 a
Azospirillum	26.48 b	26.89 b	84.52 a	90.31 b
Combination of Azotobacter and Azospirillum (Nitroxin)	29.1 a	29.63 a	87.9 a	90.3 b

In each column, the same letters indicate that there is no significant difference between the mean of the treatments

Table 6. *Comparison of the effects of urea fertilizer on some flowering traits in Calendula officinalis L in two years of experiment in 2015-2016 and 2016-2017*

Experimental treatments	Total phenol (mg/g dry extract)		Biomarker malondialdehyde (Nano mol per mg of protein)	
	First year	Second year	First year	Second year
Lack of use (control)	24 c	24.4 c	3.04 c	2.95 d
75% recommended urea fertilizer with sulphur coating	26.35 b	27.19 b	3.98 b	4.15 c
Recommended amount of urea fertilizer with sulphur coating	27.75 a	28.06 a	4.9 a	4.72 a
Recommended amount of common form of urea fertilizer	27.45 a	28.05 a	4.52 a	4.41 b

In each column, the same letters indicate that there is no significant difference between the mean of the treatments

Total flavonoid

In both years of experiment, the effect of drought stress (S), biological fertilizer (B) and urea fertilizer (N) sources on 1% was significant on Total Flavonoid. Also, in the first year of effect (B×N) at 1% level and in the second year at 5% level, this significant trait was significant (*Table 3*). Drought stress in both years of the experiment led to a significant increase in this trait. The total flavonoid in stress conditions increased 11.4% and 9.6%, respectively, in non-stress during the first and second years (*Table 4*).

Comparing the average interactions of biological fertilizers and urea, the results showed that in the first year, the highest total flavonoid in the treatment of Azotobacter and Azospirillum (Nitroxin) and the recommended amount of urea fertilizer (21.91 mg/g dry extract) and combined treatment Azotobacter and Azospirillum (Nitroxin) and application of recommended amount of urea fertilizer with sulphur coating (22.57 mg/g dry extract). In the second year under Nitroxin application, the use of all three sources of urea fertilizer resulted in an increase in total flavonoid (*Table 7*). Flavonoid antioxidants have a protective effect during dry stress. Many Flavonoids are active ingredients in medicinal plants and have medicinal properties. As active physiological compounds, stress-protecting agents and as absorbents play an important role in plant resistance (Tattini et al., 2004).

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Table 7. *Comparison of the intermediate effects of biological fertilizers and different quantities of urea sources on the Calendula officinalis L. plant in two trial years of 2015-2016 and 2016-2017*

Experimental treatments		Total Phenol (mg/g dry extract)		Glutathione Peroxidase enzyme (mg of protein per minute)	
		First year	Second year	First year	Second year
Bio-fertilizer	Urea fertilizer sources				
Lack of use (control)	Lack of use (control)	11.85 i	12 h	21.69 fg	22.24 gh
Lack of use (control)	75% recommended urea fertilizer with sulphur coating	14.02 h	13.81 g	23.36 efg	23.28 gh
Lack of use (control)	Recommended amount of urea fertilizer with sulphur coating	17.77 efg	f16.14	24.22 defg	24.16 efg
Lack of use (control)	Recommended amount of common form of urea fertilizer	15.07 h	16.16 f	23.64 defg	24.06 fg
Azotobacter	Lack of use (control)	12.58 i	gh12.82	22.01 fg	22.58 gh
Azotobacter	75% recommended urea fertilizer with sulphur coating	16.83 fg	13.57 gh	24.1 defg	25.06 defg
Azotobacter	Recommended amount of urea fertilizer with sulphur coating	19.74 ba	17.19 ef	27.54 bcd	27.1 bf
Azotobacter	Recommended amount of common form of urea fertilizer	19.24 bcd	17.65 def	25.54 cdef	27.43 bcde
Azospirillum	Lack of use (control)	16.42 g	13.16 gh	21.81 fg	22.38 gh
Azospirillum	75% recommended urea fertilizer with sulphur coating	18.35 cde	17.43 ef	26.19 bcde	26.64 def
Azospirillum	Recommended amount of urea fertilizer with sulphur coating	20.29 b	18.79 cde	27.61 bcd	28.06 bcd
Azospirillum	Recommended amount of common form of urea fertilizer	18.16 def	19.26 bcd	26.46 bcde	26.9 cdef
The combination of Azotobacter and Azospirillum	Lack of use (control)	16.69 g	16.12 f	21.01 g	20.56 h
The combination of Azotobacter and Azospirillum	75% recommended urea fertilizer with sulphur coating	19.08 b-e	20.02 abc	29.6 b	30.08 bc
The combination of Azotobacter and Azospirillum	Recommended amount of urea fertilizer with sulphur coating	22.57 a	21.25 a	33.47 a	34.28 a
The combination of Azotobacter and Azospirillum	Recommended amount of common form of urea fertilizer	21.91 a	20.73 ab	28.99 bc	30.28 b

In each column, the same letters indicate that there is no significant difference between the mean of the treatments

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Di-hydroxy guanuzyn enzyme

In both years of experiment, the effect of drought stress (S) on the level of 1% was significant on the amount of Di-hydroxy Guanuzyn enzyme (*Table 3*). Drought stress in both years of the experiment led to a significant increase in this trait. The di-hydroxy Guanzine enzyme in stress conditions increased 10.6% and 10.2% in non-stress during the first and second years respectively (*Table 4*). Increasing the amount of di-hydroxy Guanuzyn increased production of superoxide radicals as a result of the occurrence of oxidative stress in the plant. Increasing of di-hydroxy Guanosine under drought stress indicates damage to plant synthetic structures and destruction of nucleic acids (Wenho and Russel, 2000). The results of Davoudi Fard et al. (2012) indicate a significant increase in di-hydroxy Guanuzyn due to drought stress.

Glutathione peroxidase enzyme

In both years of experiment, the effect of drought stress (S), biological fertilizer (B) and urea fertilizer (N) sources on Glutathione Peroxidase enzyme activity was significant at 1% level. Also in the first year of effect (B×N) at 5% level and in the second year at 1% level this trait was significant (*Table 8*). Drought stress in both years of the experiment led to a significant increase in this trait so that the activity of Glutathione Peroxidase enzyme in stress conditions increased 56.6% and 56.2%, respectively, compared to non-stress conditions in the first and second year (*Table 9*). Results showed that in both years, the effect of Glutathione Peroxidase in the treatment of Azotobacter and Azospirillum (Nitroxin) and application of recommended amount of urea fertilizer with sulphur content was highest (*Table 7*).

One of the mechanisms can increase the tolerance of the plant to stress, by activation of antioxidant systems. A large number of physiological disorders in plants are due to the increased production of active oxygen species due to environmental stresses such as drought (Sgherri and Navari-Lzzo, 1995; Zhang and Kirkham, 1994; Miller et al., 2010). Since one of the common effects of drought stress, like other environmental stresses, is primarily oxidative damage (Chen, 2000), plants to cope with oxidative stress and overcome these active species, antioxidant defense system with enzymatic and non-enzymatic mechanisms such as superoxide dismutase, catalaze..., along with a number of non-enzyme antioxidants such as Ascorbic acid, Glutathione, Alpha-tocopherol, and Cartenoids constitute the bulk of this defense system (Nasibi, 2005; Prasad, 2002). The results of Sedghi et al. (2012) indicate that antioxidant enzymes increase the activity of drought stress in an evergreen plant.

Enzyme activity of superoxide dismutase

The results of analysis of variance showed that in both years the effect of drought stress (S) on 1% level was significant on the activity of the enzyme superoxide dismutase (*Table 8*). Drought stress in both years of the experiment led to a significant increase in this trait. The level of enzyme activity of superoxide dismutase in stress conditions increased to 85.7% and 100.6% in the first and second year respectively (*Table 9*). Kawakami et al. (2010) reported that superoxide dismutase enzyme activity increased significantly under drought stress. In this regard, drought stress has been reported to increase the activity of superoxide dismutase in wheat (Tian and Lei, 2007) and rice (Sharma and Dubey, 2005). The results of Sedghi et al. (2012) indicate increased activity of the enzyme superoxide dismutase due to drought stress.

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Table 8. *Analysis of variance of the effect of biological fertilizers and different amounts of urea sources under dehydrating conditions of Calendula officinalis L plant during two years of experiment, 2015-2016 and 2016-2017*

Sources of changes	Degrees of freedom	Average of squares							
		Biomarker malondialdehyde		Glutathione peroxidase enzyme		Superoxide dismutase enzyme		Biomarker di-tyrosine	
		First year	Second year	First year	Second year	First year	Second year	First year	Second year
Repeat	2	0.04^{ns}	0.2^{ns}	5.04^{**}	15.02^{**}	3.3^{**}	3.33^{**}	182.3^{**}	92.05^{**}
Drought stress (S)	1	34.62^{**}	50.13^{**}	3043.13^{**}	3109.35^{**}	3029.85^{**}	3573.87^{**}	2146.76^{**}	2227.42^{**}
Main error	2	0.04	0.39	0.19	1.43	0.11	0.11	10.06	8.89
Biological fertilizer (B)	3	118.05^{**}	7.27^{**}	106.22^{**}	117^{**}	8.51^{ns}	5.14^{ns}	1062.97^{**}	1889.49^{**}
S×B	3	1.15^{*}	0.31^{ns}	1.18^{ns}	2.19^{ns}	0.62^{ns}	0.37^{ns}	69.25^{ns}	19.86^{ns}
Sub-error	12	0.26	0.24	18.94	14.91	4.47	4.22	216.86	99.13
Urea fertilizer sources (N)	3	15.49^{**}	14.37^{**}	182.72^{ns}	189.35^{**}	14.07^{ns}	9.64^{ns}	105.15^{ns}	229.81^{ns}
S×N	3	0.58^{ns}	0.6^{**}	10.9^{ns}	9.4^{ns}	0.31^{ns}	0.96^{ns}	0.97^{ns}	1.11^{ns}
B×N	9	0.74^{ns}	0.38^{**}	20^{ns}	28.71^{**}	2.82^{ns}	2.47^{ns}	185.06^{ns}	164.25^{ns}
S×B×N	9	0.27^{ns}	0.1^{ns}	1.48^{ns}	2.53^{ns}	0.9^{ns}	0.42^{ns}	3.79^{ns}	2.5^{ns}
Sub-error	48	0.47	0.15	8.79	6.35	5.7	5.52	173.84	152.96
Coefficient of variation (%)		16.6	9.4	11.6	9.7	12.8	12.9	14.4	12.6

ns, *, and ** were non-significant and significant at levels of 5% and 1%

Table 9. *Comparison of the effect of drought stress on some physiological traits of Calendula officinalis L in two years of experimentation 2015- 2016 and 2016-2017*

Experimental treatments	Glutathione peroxidase enzyme		Superoxide dismutase enzyme		Biomarker di-tyrosine (nano mole per mg of protein)	
	First year	Second year	First year	Second year	First year	Second year
Stress levels						
Lack of water stress	19.82 b	20.25 b	13.11 b	12.14 b	87.11 b	93.15 b
Irrigation cut in the budding stage	31.08 a	31.63 a	24.35 a	24.35 a	96.57 a	102.79 a

In each column, the same letters indicate that there is no significant difference between the mean of the treatments

Biomarker malondialdehyde

In both years of experiment, the effect of drought stress (S), biological fertilizer (B) and urea (N) fertilizer sources on 1% level was significant on malondialdehyde

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biomarker. Also in the first year of interaction (B×N) at 5% level and in the second year of effect (B×N) and (S×N) at 1% level, this significant trait was significant (*Table 8*). Application of recommended amount of urea fertilizer with sulphur coating in both years of experiment resulted in significant increase of this trait. So, the amount of biomarker of carotene Dialledium in application of recommended amount of urea fertilizer with sulphur coating increased 61.2% and 60%, respectively, compared to control treatment in the first and second year. In the first year, there was no significant difference between the application of the recommended amount of urea fertilizer with sulphur coating and the application of the recommended amount of urea fertilizer in this regard (*Table 6*).

Comparison of the mean interactions of drought stress and biological fertilizer showed that in the first year of experiment, the highest trait in terms of stress and the combination of Azotobacter and Azospirillium (11.99 Nmol/mg protein) and stress and application of Azospirillum (59.11 nmol/mg^{-1} protein) was obtained (*Fig. 1*). Comparing the mean interactions between drought stress and urea fertilizer sources, the highest biomarker of calcium diphtheria was obtained in the second year under stress conditions and application of recommended amount of urea fertilizer with sulphur coating (5.43 nmol/mg protein) and the lowest in terms non-stress and non-use of urea fertilizers were obtained (*Fig. 2*). Results showed that in the second year, the combination of Azotobacter and Azospirillum (Nitroxin) with three sources of urea fertilizer resulted in a significant increase in this trait (*Fig. 3*). Many plants, when placed in a dry environment, are injured seriously and increase their malondialdehyde content (Grmaetxe, 1998; Saneoka, 2004).

There are reports of reduced malondialdehyde due to the maintenance of membrane lipids from damage induced by free oxygen radicals (Ozdamir et al., 2004). The results of Davoudi Fard et al. (2012), (Pan et al., 2006), (Zhang et al., 2006), (Habibi et al., 2009) and (Li Wen et al., 2006) indicate a significant increase in malondialdehyde due to drought stress.

Biomarker di-tyrosine

In both years of experiment, the effect of drought stress (S) and biological fertilizer (B) on the amount of di-tyrosine biomarker was significant at 1% level (*Table 8*). Drought stress in both years of the experiment led to a significant increase in this trait. So, the amount of biomarker di-tyrosine in stress conditions increased 10.9 and 15.7% in non-stress during the first and second years respectively (*Table 9*).

The application of Nitroxin biological fertilizer in both years led to a significant reduction in the amount of di-tyrosine biomarker. The highest amount of this trait was obtained every two years in non-use of biological fertilizers. In the second year, there was no significant difference between the absence of Bio-fertilizer application and Azotobacter application (*Table 5*). The results of Davoudi Fard et al. (2012) indicate a significant increase in di-prozine due to drought stress. In this study, the use of Bio-fertilizer resulted in the reduction of this trait.

This is consistent with the findings of Davoudi Fard et al. (2012). This conclusion is likely to indicate that bacteria reduce the effects of drought stress through another defense mechanism, which has been prevented from rising levels of Di-tyrosine and antioxidant enzymes. Because antioxidant enzymes activity is reported to be the only defense mechanism against free radical oxidants produced under stress conditions increasing proline can also reduce the free radicals produced under drought stress

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conditions (Aktas et al., 2007). As a result, due to the reduction of the production of free radicals, the amount of protein degradation and, consequently, the production of biomarker of di-tyrosine is reduced (Jose et al., 1999).

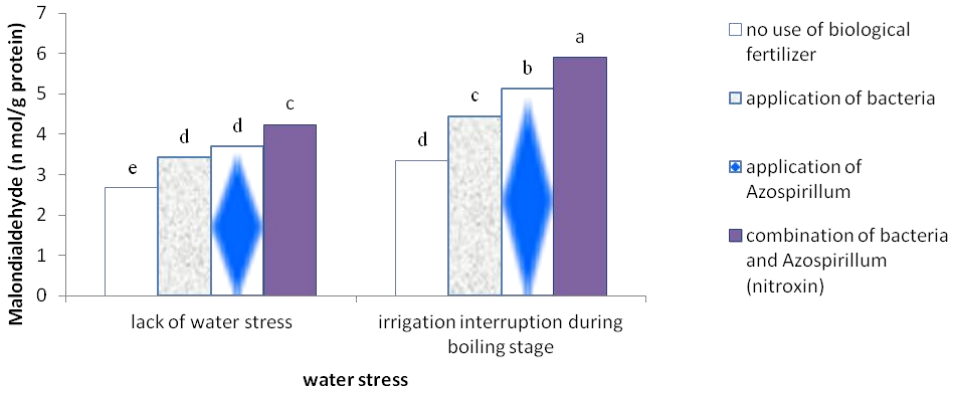

Figure 1. *Effect of drought stress and biological fertilizer on malondialdehyde biomarker in the first year*

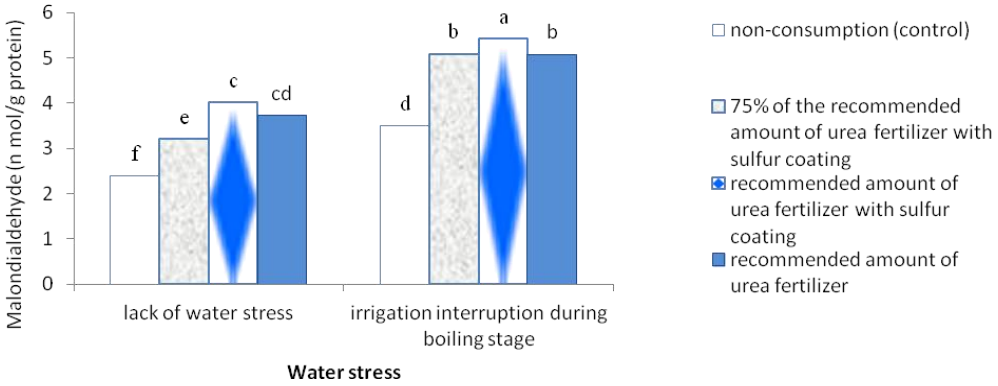

Figure 2. *Effect of drought stress and biological fertilizer on malondialdehyde biomarker in the second year*

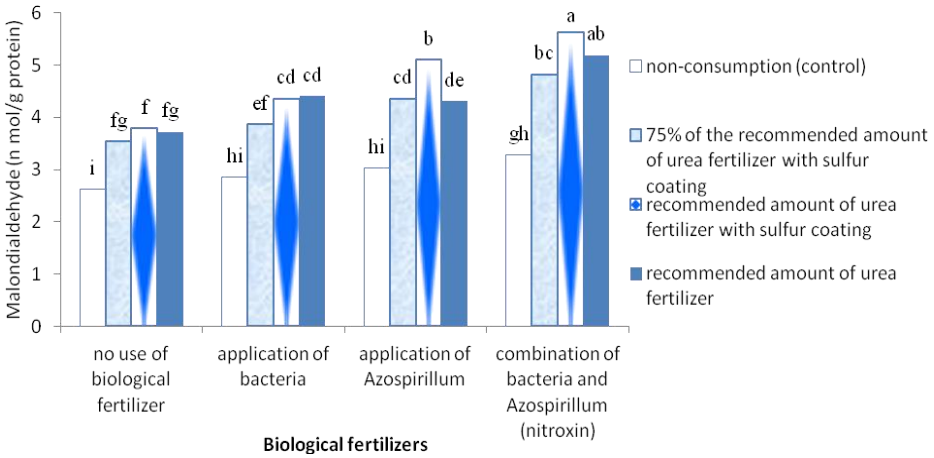

Figure 3. *Effect of biological fertilizer and urea fertilizer source on malondialdehyde biomarker in the second year*

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Overall conclusion

The results showed that irrigation cut in the boiling stage led to increases in the enzyme di-hydroxy Guanuzyn, glutathione peroxidase, superoxide dismutase, total phenol, total flavonoid and biomarker di-tyrosine. In general, the use of Nitroxin led to an increase in all of these traits except di-tyrosine.

The application of the recommended amount of urea fertilizer with sulphur coating resulted in an increase in total phenol and biomarker malonaldehyde. There was no significant difference between in malonaldehyde, application of recommended amount of urea fertilizer with sulphur coating and the usual form of recommended urea fertilizer. Whilst, results showed that in both years, the effect of Glutathione Peroxidase in the treatment of Azotobacter and Azospirillum (Nitroxin) and application of recommended amount of urea fertilizer with sulphur content was highest. Also, application of recommended amount of urea fertilizer with sulphur coating and recommended amount of urea fertilizer in both years of experiment resulted in significant increase of Total Phenol. In this study, drought stress and application of Nitroxin fertilizer resulted in increased malondialdehyde in the first year. In the second year, malondialdehyde was affected by the interaction of drought stress and urea fertilizer resources. Drought stress and application of recommended amount of urea fertilizer with sulphur coating resulted in increased malondialdehyde. In both years, the application of Nitroxin with the recommended amount of urea fertilizer with sulphur coating resulted in an increase in total flavonoid and glutathione peroxidase enzymes. Comparing the average interactions of biological fertilizers and urea, the results showed that in the first year, the highest total flavonoid in the treatment of Azotobacter and Azospirillum (Nitroxin) and the recommended amount of urea fertilizer and combined treatment Azotobacter and Azospirillum (Nitroxin) and application of recommended amount of urea fertilizer with sulphur coating. In the second year under Nitroxin application, the use of all three sources of urea fertilizer resulted in an increase in Total Flavonoid. There was no significant difference between the recommended amount of urea fertilizer with sulphur coating and urea fertilizer with sulphur coating for Total Flavonoids. Future debate should promote the promotion of information and culture in the field of medicinal plants, as well as the expansion of international cooperation in medicinal plant science and technology, the protection of monolithic plant species, planning for the development of cultivation and domestication of species Important medicinal plants with economical value, the transfer and absorption of the advanced sciences and technologies of other countries in the field of medicinal plants.

REFERENCES

[1] Abedi, T., Pakniyat, H. (2010): Antioxidant enzyme changes in response to drought stress in ten cultivars of oilseed rape (*Brassica napus* L.). – Czech J. Genet. Plant Breed 46(1): 27-34.

[2] Aktas, L. Y., Turkyilmaz, H., Akca, S. P. (2007): Role of abscisic acid and proline treatment on induction of antioxidant enzyme activities and drought tolerance responses of Laurus nobilis L. – Grainlings 19(2): 14-27.

[3] Arora, D. A., Rani, A. S. (2013): A review on phytochemistry and ethnopharmacological aspects of genus Calendula. – *Pharmacognosy Reviews*. 7: 179-187.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1604_48134827

[4] Auge, R. M. H. Toler, A. M., Saxton. A. (2015): Arbuscular mycorrhizal symbiosis alter stomatal conductance of host plants more under drought than under amply watered conditions: a meta-analysis. – Mycorrhiza 25(1): 13-24.
[5] Bapat, P., Nandy, SK., Wangikar, P., Venkatesh, K. (2006): Quantification of metabolically active biomass using Methylene Blue dye Reduction test (MBRT): Measurement of CFU in about 200 seconds! – J. Microbiol. Methods 65: 107-116.
[6] Bogdanov, M., Bical, M. B. (1999): Acarbon column based LCEG approach to routine 8-OH-dG measurements in biological matrices. – Free Radical Biology & Medicine 27: 647-666.
[7] Chen, W. P., Li, P. H., Chen, T. H. (2000): Glycinebetaine increases chilling tolerance and reduces chilling induced lipid peroxidation in Zea mays L. – Plant Cell Environ 23: 609-618.
[8] Davoudi Fard, M., Habibi, D., Paknejad, F., Fazeli, F. (2012): Investigating the effect of growth stimulators and spraying of amino acids and silicic acid on biochemical biomarkers activity under drought stress conditions in wheat. – Agronomy and Plant Breeding. 8(4): 99-83.
[9] Grmaetxe, I., Escurede, P. R., Arrese-Igor, C. (1998): Oxidative damage in pea plants exposed to water deficit or paraquat. – Plant Physiology 116: 173-181.
[10] Habibi, D., Rahmani, A. H., Shirani, J., Daneshian, A., Valadabadi, P., Moaveni, M., Boojar, M. A. (2009): Effect of superabsorbent polymers on yield and antioxidant enzymes activity of mustard (*Sinapis alba* L.) under drought stress. – The 3rd International Conference on Integrated Approaches to Improve Crop Production under Drought-prone Environments. Shanghai, China.
[11] Jose, M., Gomez, C. (1999): Antioxidant enzyme and human disease. – Chemical Biochemistry 32(8): 596-603.
[12] Kawakami, E. M., Oosterhuis, D. M., Snider, J. L. (2010): Physiological effects of 1-methylcyclopropene on well-watered and water-stressed cotton plants. – Plant Growth Regulation 29: 280-288.
[13] Krizek, D. T., Kramer, A., Upadaya, R., Mirecki, M. (1993): UV B response of cucumber seedling grown under metal halide and high pressure sodium/deluxe lamps. – Physiol. Plant. 88: 350-358.
[14] Li, W. R., Zhang, S. L., Shan, R. (2006): Effect of water stress on chlorophyll fluorescence parameters and activity of antioxidant enzyme in Alfalfa (*Medicago sativa* L.) seedlings. – The First International Conference on the Theory and Practices in Biological Water Saving (ICTPB), Beijing, China.
[15] Martin, R. J., Deo, B. (2000): Effect of plant population on *calendula (Calendula officinalis* L.) flower production. – New Zealand J. Crop Hort. Science 28: 37-44.
[16] McKersie, B. D., Murnaghan, J., Jones, K. S., Bowley. S. R. (2000): Iron superoxidase dismutase expression in transgenic Alfalfa increases winter survival without a detectable increase in photosynthetic oxidative stress tolerance. – Plant Physiol. 122: 1427-1437.
[17] Miller, G., Suzuki, N., Yilmaz, S., Mittler, R. (2010): Reactive oxygen species homeostasis and signaling during drought and salinity stresses. – Plant, Cell & Environment 33: 453-467.
[18] Moallem, A., Eshghizadeh, H. R. (2007): The use of biological fertilizers: benefits and limitations. – Proceedings of the 2nd National Conference on AgroEcology in Iran, Gorgan, Iran, National Conference of Iranian Ecology 2(3): 47-51.
[19] Moghadasan, S. H., Safipour, A. A., Saeid Nematpour, F. (2015): The role of mycorrhiza in drought tolerance is always spring (*Calendula officinalis* L.) – Journal of Crop Ecophysiology 9(4): 521-532.
[20] Moslemi, Z. (2010): Effect of super absorbent polymer and growth stimulating bacteria on yield and yield components, forage yield and some biochemical and physiological traits of forage corn. – Master's Thesis, Faculty of Agriculture. Islamic Azad University of Karaj Branch.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1604_48134827

[21] Nagananda, G. S., Das, A., Bhattacharya, S., Kalpana, T. (2010): In vitro studies on the effects of biofertilizer (Azetobacter & Rizobium) on seed germination and development of Trigonella foemum graecum 1. using a novel glass marble containing liquid medium. – Int. J. Botany 6: 394-403.
[22] Nasibi, F., M-Kalantari, K. (2005): The effects of UV-a, UV-b and UV-c on protein and ascorbate content, lipid peroxidation and biosynthesis of screening compounds in *Brassica napus*. – Iranian Journal of Science & Technology 29: 40-48.
[23] Ozdamir. F., Bor, M., Demiral, T., Turkan, I. (2004): Effects of 24-epibrassinolide on seed germination, seedling growth, lipid peroxidation, prolin content and antioxidative system of rice (*Oriza sativa* L.) Under Salinity Stress. – Plant Growth Regulation 42: 203-211.
[24] Paglia, D. (1997): Studies on the quantitative trait Dase. – J. Lab. Med. 70: 158-165.
[25] Pan, Y. L., Wu, Z., Yu, L. (2006): Effect of salt and drought stress on antioxidant enzymes activities and SOD isoenzymes of liquorice (*Glycyrrhiza uralensis* Fisch). – Plant Growth Regul 49: 157-165.
[26] Pang, C. H., Zhang, S. J., Gong, Z. Z., Wang, B. S. (2005): NaCl treatment markedly enhances H_2O_2-scavenging system in leaves of halophyte Suaeda salsa. – Physiol Plant 125: 490-499.
[27] Prasad, B. (2002): Emerging global market scenario: opportunity and challenges for drug industries of Indian systems of medicine. – Proceedings of Whom Training Cum Workshop on Quality, Safety and Efficacy of Drugs of Indian System of Medicine 10: 113-119.
[28] Sairam, R. K., Srivastava, G. C. (2001): Water stress tolerance of wheat *Triticum aestivum* L.: Variation in hydrogen peroxide accumulation and antioxidant activiy in tolerant and susceptible genotype. – Journal of Agronomy and Crop Science 186: 63-70.
[29] Saneoka, H., Moghaieb, R. E., Premachandra, G. S., Fujita, K. (2004): Nitrogen nutrition and water stress effects on cell membrane stability and leaf water relations in Agrosist palutris Huds. – Enveronmental and Experimental Botany 52: 131-138.
[30] Sedghi, M., Seyed Sharifi, R. A., Amanpour-Balaneji, B. (2012): Phytohormonal regulation of antioxidant systems in petals of drought stressed pot marigold (*Calendula officinalis* L.). – J. Agr. Science. Tech. 14: 869-878.
[31] Sgherri, C. L., Navari-Izzo, F. (1995): Sunflower seedlings subjected to increasing water deficit stress: oxidative stress and defence mechanisms. – Physiol Planta 93: 25-30.
[32] Sharma, P., Dubey, R. S. (2005): Drought induces oxidative stress and enhances the activities of antioxidant enzymes in growing rice seedlings. – Plant Growth Regu. 46: 209-221.
[33] Shubhra, K., Dayal, J., Goswami, C. L., Munjal, R. (2004): Effects of water-deficit on oil of Calendula aerial parts. – Biologia Plantarum 48(3): 445-448.
[34] Sigaud-Kutner, T. C. S., Pinto, E., Okamoto, O. K., Latorre, L. R., Colepicolo, P. (2002): Changes in superoxide dismutase activity and photosynthetic pigment content during growth of marine phytoplankters in batch-cultures. – Physiol Plant 114: 566 571. DOI: 10.1034/ j.1399-3054.2002.1140409.x.
[35] Steven, A. K., Joseph, M. H. (1978): Lipid peroxidase in sample as measured by liquid choromatography separation. – Elin. Chem. 32: 217-220.
[36] Tattini, M., Galardi, C., Pinelli, P., Massai, R., Remorini, D., Agati, G. (2004): Differential accumulation of Flavonoids and hydroxycinnamates in leaves of *Ligustrum vulgare* under excess light and drought stress. – New Phytologist 163: 547-561.
[37] Tian, X. R., Lei, Y. B. (2007): Physiological responses of wheat seedlings to drough and UV‑B radiation. Effect of exogenous sodium nitroprusside application. – Russ J Plant Physiol. 54: 676-682
[38] Wenho, Q., Russel, J. (2000): 8-OH-dg in calf thymus DNA indubead with CrCI3 PL H_2O_2. – Int. Cong. 6: 45-53.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1604_48134827

[39] Zhang, J. X., Kirkham, M. B. (1994): Water relations of water-stressed, splitroot C_4 (*Sorghum bicolor*; Poaceae) and C_3 (*Helianthus annuus*; Asteraceae) plants. – Am. J. Bot. 82: 1220-1229.

[40] Zhang, M., Duan, X., Tian, Z., He, J., Li, B., Wang, Z. (2006): Uniconazole-induced tolerance of soybean to water deficit stress in relation to changes in photosynthesis, hormones and antioxidant system. – Journal of Plant Physiology 164: 709-717.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1604_48134827