Journal of Elementology ISSN 1644-2296

Selem E. Tuncturk R., Nohutcu L., Tuncturk M. 2022.
Effects of rhizobacteria and algal species on physiological and biochemical parameters in Calendula officinalis L. *under different irrigation regimes.*
J. Elem., 27(1): 87-97. DOI: 10.5601/jelem.2022.27.1.2173

RECEIVED: 30 July 2021
ACCEPTED: 17 February 2022

ORIGINAL PAPER

EFFECTS OF RHIZOBACTERIA AND ALGAL SPECIES ON PHYSIOLOGICAL AND BIOCHEMICAL PARAMETERS IN *CALENDULA OFFICINALIS* L. UNDER DIFFERENT IRRIGATION REGIMES

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Abstract

Drought is one of the main limiting factors affecting the growth and productivity of plants. Drought stress affects plant growth in different ways through a series of anatomical, morphological, physiological and biochemical changes. Therefore, it is important to develop alternative methods to protect plants against drought stress. Turkey has one the most unique cultivated areas of different medicinal and aromatic plants including *Calendula officinalis*. Despite its considerable importance and use as a medicinal plant, its commercial production is very limited. In this study, the effects of different rhizobacteria and algal species treatments (B_0 – control, B_1 – *Azospirillum lipoferum*, B_2 – *Bacillus megaterium*, B_3 – *Chlorella saccharophila*) on several physiological and biochemical parameters in *C. officinalis* were studied under irrigation regimes – normal irrigation (K_1), 50% irrigation (K_2) and 25% irrigation (K_3). The results showed that the application of different rhizobacterial and algal species had positive effects by reducing the limited irrigation stress. Also, the range of different physiological and biochemical parameters values, including leaf relative water content (RWC), ion leakage in leaf tissues (ILLT), membrane endurance index (MEI), chlorophyll content, nitrogen balance index, MDA, total antioxidant activity, total phenolic content and total amount of flavonoids were determined as 61.8-77.2%, 29.2-42.8%, 57.2-68.9%, 26.8-38.2 μg cm^{-2}, 110.6-158.0 mg g^{-1}, 0.276-573.0 nmol g^{-1}, 19.92-55.92 Mmol TE g^{-1}, 27.37-68.75 mg GAE g^{-1} and 9.49-17.88 mg QE 100 g^{-1}, respectively. The results revealed that *C. officinalis* could be commercially cultivated as a drought-resistant plant species in arid and semi-arid regions of Turkey.

Keywords: marigold, *Calendula officinalis* L., drought stress, rhizobacteria.

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INTRODUCTION

Calendula officinalis L. known as "Aynısafa" or medicinal daffodil in Turkey belongs to the Asteraceae family. It is widely used worldwide owing to its important phytochemicals (carbohydrates, amino acids, lipids, fatty acids, carotenoids, terpenoids, flavonoids, quinones, coumarins etc.) as well as different medicinal properties, such as wound healing, immune-enhancing, spasmogenic and spasmolytic, hepatoprotective, genotoxic and antigenotoxic, anti-amylase, anti-inflammatory, anti-edematous, anti-bacterial, anti-fungal, antioxidant, antidiabetic, anti-HIV, anti-cancer and nephron protective characteristics (Jan et al. 2017).

Water deficiency is one of the most important environmental stress factors, which is considered a serious threat to plant growth and yield, especially in arid and semi-arid regions, and it affects various physiological processes, such as transpiration, photosynthesis and cell elongation (Chaves et al. 2002, Abideen et al. 2019, Shakib et al. 2019, Zulfiqar et al. 2020). The ability of plants to benefit from irrigation water at the maximum level depends on the soil structure and the amount of organic matter in the soil. Alternative methods, such as using plant growth promoting rhizobacteria (PGPRs), have been developed to reduce the negative effects of water stress on morphological, physiological and biochemical parameters in plants. Studies have shown that PGPRs contribute to plant growth through hormone production (cytokinin, auxin and gibberellin), nitrogen fixation, phosphate solubility, nutrient uptake, and water-use efficiency (Grichko, Glick 2001, Samancioglu et al. 2016, Widnyana 2018, Uysal Sahin, Donmez 2020). There are many studies showing positive effects of different types of PGPR species on many medicinal and aromatic plants, such as *Rosmarinus officinalis* L., *Ociumum basilicum* L., *Mentha piperita* L., *Melissa officinalis* L., *Echinaceae purpurea* L., according to different growth parameters (Heidari et al. 2011, Kazeminasab et al. 2016, Bat et al. 2019, Bidgoli et al. 2019, Chiappero et al. 2019).

In this study, the effects of different rhizobacteria and alga species treatments on several physiological and biochemical parameters of *C. officinalis* were studied under irrigation regimes in order to find a promising treatment to reduce the negative effects of drought stress.

MATERIALS AND METHODS

This study was carried out in May 2020, in a growth chamber at the Department of Field Crops, Faculty of Agriculture, Van Yüzüncü Yıl University (Van Yyu). Seeds of *C. officinalis*, obtained from the garden of medicinal and aromatic plants of Van YYU, were planted in 500 cc pots in a mixture of peat + perlite + soil (1: 1: 2) and kept in a growth chamber (65% RH; 8/16 h dark/light intervals; 25°C). When they had 5-6 leaves, the cultivated plants were subjected to water stress based on determination of pot field capacity according to Ünlükara et al. (2010) in 3 different regimes: normal irrigation (K_1), 50% irrigation (K_2) and 25% irrigation (K_3).

Two rhizobacterial species including *Azospirillum lipoferum* which is a biological nitrogen fixer (1x10^6 CFU ml^{-1}) and *Bacillus megaterium* as a phosphate dissolving bacterium (1x10^5 CFU ml^{-1}) as well as a freshwater alga *Chlorella saccharophila* (2x10^4 CFU ml^{-1}) were applied. The plant seeds were inoculated with bacterial and algal solutions for 2 h and then planted in pots. One week after planting, 50 ml of bacterial and algal solutions were applied again to the pots. This study was performed as a factorial experiment based on a completely randomized design with 4 replicates. No bacterial and algal species were applied in the control treatments.

Several physiological and biochemical parameters were measured after a month. The chlorophyll content and nitrogen balance index were measured according to Cerovic et al. (2015) using a Dualex scientific + (FORCE-A, France) instrument. Relative water content (RWC) was determined according to the method proposed by Arora et al. (2002). Ion leakage (ILLT) and membrane endurance index (MEI) were also determined according to the methods developed by Premchandra et al. (1990) and Sairam (1994).

The level of lipid peroxidation was measured based on a malondialdehyde (MDA) content assay. For this purpose, leaf samples (0.5 g) were homogenized in 10 mL of 0.1% trichloro acetic acid (TCA) followed by centrifugation at 15,000 g for 5 minutes. Then, 1.0 mL of the supernatant was mixed with 4.0 mL of 0.5% thiobarbituric acid (TBA) in 20% TCA. The mixture was heated at 95°C for 30 min and then quickly cooled in ice. After centrifugation at 10,000 g for 10 min, the absorbance of the supernatant was recorded at 532 nm and 600 nm. The MDA content was calculated according to Güneri, Bagci (2010). Total phenolic compounds content was measured according to Obanda, Owuor (1997) method. The antioxidant activity was also based on the antioxidant power (FRAP) [iron (III) antioxidant power reduction] method (Benzie, Strain 1996) followed by readings of the absorbance at 593 nm, and the antioxidant activity values were recorded as Trolox equivalent (TE) mg^{-1}. The total flavonoids content was determined with some modifications according to the method developed by Quettier et al. (2000). For this purpose, 1 ml of 2% $AlCl_3$ was added to 1 ml of plant extract and kept for 70 min at room temperature in the darkness. The total amount of flavonoids was measured at 415 nm and recorded as mg 100 g^{-1}.

All the recorded data were subjected to analysis of variance using the Costat (6.3 version) software. The average data were also compared by Duncan Multiple Range Test at $P<0.05$ (Düzgünes et al. 1987).

RESULTS AND DISCUSSION

The results showed significant effects of bacterial and algal treatments on several physiological and biochemical growth parameters of *C. officinalis* under water stress (Tables 1 and 2). It is well known that water stress

Table 1

The effects of irrigation regimes and application of bacterial and/or algal species on some physiological parameters of *C. officinalis*

Irrigation regimes	Bacterial and/or algal applications	RWC (%)	ILLT (%)	MEI (%)	Chlorophyll (µg cm^{-2})	NBI (mg g^{-1})
K_1	B_0	68.3	31.1	68.9	35.2	139.5
	B_1	77.2	31.4	68.5	27.9	139.8
	B_2	73.8	42.8	57.2	26.8	137.8
	B_3	74.1	31.8	68.1	38.2	158.0
K_1 average		73.4*a*	34.3	65.7	35.6*a*	143.7*a*
K_2	B_0	65.9	31.0	63.4	30.8	110.6
	B_1	67.8	31.4	63.4	28.5	120.3
	B_2	73.2	42.8	62.5	33.7	139.4
	B_3	70.3	31.8	64.6	33.5	143.2
K_2 average		69.3*ab*	35.7	63.5	30.8*b*	128.4*b*
K_3	B_0	61.8	39.5	60.5	28.8	122.3
	B_1	66.3	32.7	67.3	35.5	152.4
	B_2	68.8	29.2	70.8	31.2	125.7
	B_3	62.9	31.4	68.5	28.6	148.2
K_3 average		65.0*b*	33.2	66.8	28.2*c*	137.1*ab*
Bacterial and algal application	B_0	65.4*b*	35.7	64.3	29.9*b*	124.1*b*
	B_1	70.4*ab*	32.5	66.4	32.5*a*	137.5*ab*
	B_2	72.0*a*	36.5	63.5	32.0*a*	134.3*ab*
	B_3	69.1*ab*	32.9	67.1	31.8*ab*	149.8*a*
CV(%)		8.90	15.89	8.29	6.04	9.92
K		*	ns	ns	**	*
B		ns	ns	ns	*	**
K x B		ns	ns	ns	ns	ns

Irrigation regimes: K_1 – control (normal irrigation), K_2 – 50% irrigation, K_3 – 25% irrigation; Bacterial and algal applications: B_0 – control (no bacteria or algae), B_1 – *Azospirillum lipoferum,* B_2 – *Bacillus megaterium,* B_3 – *Chlorella saccharophila,* B – Bacterial and algal applications, K – irrigation Regimes, ns – not significant, K x B – interaction of bacterial and algal applications and irrigation regimes, CV – coefficient of variation, * significant at $P<0.05$, ** significant at $P<0.01$, the values with the same letter are not significantly different based on Duncan Multiple Range Test at $P<0.05$.

Table 2

The effects of irrigation regimes and application of bacterial and/or algal species on some biochemical parameters of *C. officinalis*.

İrrigation regimes	Bacterial and/or algal applications	MDA (mol g^{-1})	Total antioxidant activity (Mmol TE g^{-1})	Total phenolic content (mg GAE g^{-1})	Total flavonoid (mg QE 100 g^{-1})
K_1	B_0	0.485*abc*	32.55*cd*	43.25*cd*	17.88*a*
	B_1	0.425*bc*	22.92*efg*	34.25*fg*	9.49*f*
	B_2	0.300*e*	41.80*b*	68.75*a*	13.02*cde*
	B_3	0.315*de*	23.92*efg*	45.75*c*	13.95*bcde*
K_1 average		0.381*c*	30.24*b*	48.18*a*	14.08*b*
K_2	B_0	0.573*a*	21.92*fg*	38.62*def*	12.91*de*
	B_1	0.276*e*	19.92*g*	36.37*ef*	13.45*cde*
	B_2	0.378*cd*	24.8*ef*	27.37*h*	12.11*ef*
	B_3	0.493*ab*	25.42*ef*	30.62*g*	15.48*ab*
K_2 average		0.430*b*	22.10*c*	33.24*b*	13.57*b*
$K_{3,}$	B_0	0.567*a*	30.92*d*	43.50*c*	13.96*bcd*
	B_1	0.475*bc*	34.55*c*	47.62*c*	17.45*a*
	B_2	0.416*c*	25.67*de*	39.12*de*	14.95*abc*
	B_3	0.454*bc*	55.92*a*	58.87*b*	16.51*a*
K_3 average		0.478*a*	37.43*a*	47.28*a*	15.71*a*
Bacterial and algal application	B_0	0.542*a*	28.38*b*	42.04*b*	15.47*a*
	B_1	0.392*bc*	25.79*b*	39.41*b*	13.46*a*
	B_2	0.365*c*	31.64*a*	45.08*a*	13.35*b*
	B_3	0.421*b*	33.86*a*	45.08*a*	15.53*a*
CV (%)		9.52	10.81	6.47	11.26
K		**	**	**	**
B		**	**	**	**
K x B		**	**	**	**

Irrigation regimes: K_1 – control (normal irrigation), K_2 – 50% irrigation, K_3 – 25% irrigation; Bacterial and algal applications: B_0 – control (no bacteria and algae), B_1 – *Azospirillum lipoferum,* B_2 – *Bacillus megaterium,* B_3 – *Chlorella saccharophila,* B – bacterial and algal applications, K – irrigation regimes, K x B – interaction of bacterial and algal applications and irrigation regimes, CV – coefficient of variation. * Significant at $P<0.05$, ** Significant at $P<0.01$,the values with the same letter are not significantly different based on the Duncan multiple Range Test at $P<0.05$.

significantly reduces the relative water content and leaf water potential in plants. Also, the ion leakage would be increased by water stress due to both cellular deterioration and oxidative damage (Janiak et al. 2015, Assaha et al. 2016).

In this study, it was observed that bacterial and algal applications as well as the interaction of irrigation regimes with bacterial or algal treatments did not affect significantly the relative water content, while the effect of irrigation regimes alone was significant at $P<0.05$. In bacteria and algal applications, the maximum value of the relative water content measured in the B_2 application was 72.0% and the lowest value (65.4%) was observed in the control. On the other hand, the highest RWC value was determined in normal irrigation conditions (73.4%), while the lowest value was observed in 25% irrigation (65.0%) in different irrigation regimes. The studied factors had no significant effects on ion leakage (ILLT) parameter. The ion leakage values in irrigation regime treatments as well as bacterial and algal treatments were measured between 33.2%-35.7% and 32.5%-36.5%, respectively. The effects of bacteria and algae applications, irrigation regimes and their interactions on the membrane durability index (MEI) were not statistically significant. The membrane endurance index values in bacterial and algal treatments and irrigation regimes were between 63.5%-67.1% and 63.5%-66.8%, respectively. No damage occurred in cellular functions due to drought stress in the tissues; on the other hand, it was observed that bacterial and algal applications had a positive effect on the host plant compared to the control groups.. While Kara (2019) reported the maximum RWC rate as 90.37% in the control group in echinacea (*Echinaceae purpurea* L.) plant exposed to salt stress, the RWC rate was measured as 76.56% (6cc L^{-1}) when seaweed was used in the same study. Several studies showed that ion leakage and cellular membrane damage values were elevated by increasing of stress conditions (Kara 2019, Bahereh 2020, Oral et al. 2020).

The effect of different irrigation regimes as well as bacterial and algal application on the total chlorophyll content was significant at 1% and 5% levels, respectively, but their interactions were not significant. The highest chlorophyll content value (32.5 μg cm^{-2}) was observed in B_1 treatment. On the other hand, the minimum amount of chlorophyll (29.9 μg cm^{-2}) was shown in the control. Considering the irrigation regimes, the highest chlorophyll content (35.6 μg cm^{-2}) was detected in the control group, while the minimum content (28.2 μg cm^{-2}) was observed in K_3 treatment. Our results are similar to previous studies, which revealed that the chlorophyll content in different plant species (*Foeniculum vulgare, Salvia sinaloensis, Carthamus tinctorius*) decreased due to an elevated drought stress and negatively affected the plant growth parameters under water stress conditions (Caser et al. 2018, Gholami-Zali, Ehsanzadeh 2018, Yeloojeh et al. 2020).

The application of bacterial and algal treatments and irrigation regimes had significant effects on the NBI parameter at $P<0.01$ and $P<0.05$ levels, respectively, while their interactions were not significant. The highest nitrogen balance index value (149.8 mg g^{-1}) was obtained in B3 applications, while the minimum value (124.1 mg g^{-1}) was observed in the control group. Considering the irrigation regimes, the highest and the lowest nitrogen balance index values (143.7 mg g^{-1} and 128.4 mg g^{-1}) were obtained under normal irrigation conditions and 50% irrigation, respectively.

In this study, the interaction of irrigation regimes and bacterial or algal applications had significant effects (P<0.01) on the MDA content, total antioxidant, total phenolic and total flavonoids content. Under stress conditions, the highest value of the MDA content (0.478 nmol g^{-1}) was determined in K_3 treatment, while the lowest value (0.381 nmol g^{-1}) was obtained in the control. As for the interaction of the applications, the highest value (0.573 nmol g^{-1}) was obtained in the K2 x B0 treatment and therefore it was evaluated in the same group as the K3 x B0 treatment.. Samancioglu et al. (2016) found that the MDA content which is the final product of unsaturated fatty acid peroxidation in plants increased due to water stress and the application of PGPRs decreased its value. Similarly, Bat et al. (2020) found that seaweed application to echinacea plant reduced the negative effects caused by drought stress and decreased the MDA level. Similarly, Yaban, Kabay (2019) indicated in their study the resistance of different pepper genotypes to drought stress induced by bacterial applications in that the MDA content increased under water stress. Zarrinabadi et al. (2019) found that the amounts of MDA in the *Calendula* plant were 26.3 nmol g^{-1}, 48.6 nmol g^{-1} and 59.0 nmol g^{-1} in the control group, moderate drought and high-severity drought stress conditions, respectively. Our study yielded results consistent with the previous studies mentioned above. The studies have shown that different species of *Calendula* as well as different varieties of the same species display significant differences in the content of flavonoids, phenolic and antioxidant activities. The antioxidant properties stated by Ercetin et al. (2012) are in correlation with the total flavonoid and phenolic content, which is also correlated with our results (Table 2). Considering the irrigation regimes, the highest total antioxidant and flavonoid amounts (37.43 Mmol TE g^{-1} and 15.71 mg QE 100 g^{-1}) were obtained in K_3 treatment. Also, the minimum values (22.10 Mmol TE g^{-1} and 13.57 mg QE 100 g^{-1}) were observed in K_2 treatment. No significant difference was found between the control and K_2 treatment in terms of the total flavonoids content, so they were included in the same group. The highest value of total phenolic substances (48.18 mg GAE g^{-1}) was observed in the control group, which was fell in the same statistical group with K_3 treatment. Erçetin et al. (2012) found that the amount of antioxidants in the leaf extracts were 0.281±0.01 μg mL^{-1} in *C. arvensis* and 0.286±0.01 μg mL^{-1} in *C. officinalis*. Preethi et al. (2006) also stated that the best antioxidant activity was obtained from aqueous extraction of *C. officinalis* with 750 μg mL^{-1}. Among the bacterial and algal applications, the highest total antioxidant, phenolic and flavonoid content (33.86 Mmol TE g^{-1}, 45.08 mg GAE g^{-1} and 6215.53.7 mg 100 g^{-1}, respectively) was observed in B_3 treatment. However, no significant difference was found between B_2 and B_3 treatments in the total amount of antioxidants and phenolic substances, and between B3 and control treatments in terms of the total flavonoids content. As for the interactions between the treatments, the highest antioxidant value (55.92 Mmol TE g^{-1}) was shown in K_3 x B_3 treatment. Also, the highest value of total phenolic substances (68.75 mg GAE g^{-1}) was indicated in K_1 x B_2 treatment. Considering the total flavonoid substance accumulation, the highest value (17.88 mg 100 g^{-1}) was determined in K_1 x B_0 treatment, and it was included in the same group with K_3 x B_1 treatment. Cetkovic et al. (2004) found that the total phenol composition of *C. officinalis* provided by herbalists was in the range of 57.47-14.49 mg g^{-1}. Yetis (2019) also reported that the total phenol compounds in the *C. officinalis* plants grown in Samsun ecological conditions varied between 42.34-12.80 mg g^{-1}. Erçetin et al. (2012) showed that the methanol extract of *C. arvensis* had a total phenol content of 118.18±10.29 mg g^{-1}.

It is known that *C. officinalis* is rich in flavonoids and carotenoids that are effective in the formation of yellow and orange colour pigments (Khalid, Da Silva 2010). It has also been reported that flavonoids are effective in phytopathogen defence, protection against UV, neutralizing free radicals, seed germination and antioxidant activities (Fini et al. 2011, Agati et al. 2012). Flavonoids have antioxidant properties that play an important role in food preservation and human health by combating the damage caused by oxidizing agents (Meda et al. 2005). Yetis (2019) found that the total amount of flavonoids in *Calendula* plant was 5.31 mg g^{-1} - 19.91 mg g^{-1}. Cetkovic et al. (2004) reported the amounts as 5.26 mg g^{-1} - 18.62 mg g^{-1}, Fonseca et al. (2010) showed the amount as 18.8 mg g^{-1}. Ercetin et al. (2012) also found that the flavonoid contents in the methanol extract of *C. arvensis* were 74.14±3.09 mg g^{-1}.

CONCLUSION

The cultivated land where medicinal and aromatic plants are grown has increased in total acreage worldwide, but several biotic and abiotic stresses, such as climate changes, drought, natural disasters, sustainable land management, market preference and demand limit their production.

It is important to increase the efficiency of water use in agricultural production, especially in arid and semi-arid areas. One alternative is through breeding resistant varieties. However, breeding strategies are mainly used for plant species with high commercial and food supply values. Therefore, a limited number of breeding studies have been carried out on medicinal and aromatic plants. Other alternative methods have been brough to the focus to increase the adaptation, yield and quality of these plants. It is well known that biochemical, physiological and morphological parameters are adversely affected by water deficiency. it has been shown that some biological agents such as PGPRs could be useful to minimize these negative effects.

In this study, bacterial and algal treatments were applied under different irrigation regimes, and it was concluded that they had positive effects on the stress physiology of the host plant. The results indicated that water stress significantly affected several physiological and biochemical parameters such as RWC (65.0%-73.4%), chlorophyll (28.2-35.6 μg cm^{-2}), NBI (128.4-143.7 mg g^{-1}), MDA (0.381-0.478 nmol g^{-1}), total antioxidant activity (890.7-1510.7 mg Trolox g^{-1}), total phenolic content (133.0-192.4 mg 100 g^{-1}) and total flavonoid amount (53.95-62.90 mg g^{-1}). By the application of bacterial and algal treatments, the above parameters were found to have changed as follows: chlorophyll (29.9-32.5 μg cm^{-2}), NBI (124.1-149.8 mg g^{-1}), MDA (0.365-0.542 nmol g^{-1}), total antioxidant activity (19.92-55.92 Mmol TE g^{-1}), total phenolic content (27.37-68.75 mg GAE g^{-1}) and total flavonoid content (17.88 mg QE 100 g^{-1}). It was also observed that there was no damage to the physiological parameters of the *C. officinalis* plants under water stress conditions, which indicates that the plant species is resistant to drought stress. It was also determined that the biochemical parameters were increased due to the increasing water stress, an effect which could be mollified by bacterial or algal applications. Based on the results, it can be concluded that the plant species could be cultivated with bacterial or algal treatments in arid and semi-arid regions where irrigation could be problematic.

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