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Research Article

Biochemical response of rainfed chicory (*Cichorium intybus* L.) to vermicompost-enriched biofertilizers and supplemental irrigation

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Abstract

Chicory, blue-flowered perennial plant, Native to Europe, is cultivated extensively in the Netherlands, Belgium, France, and Germany and to some extent in North America. Its leaves are eaten as a vegetable or in a salad, and the roots may be boiled and eaten. The plant is grown as a fodder or herbage crop for cattle. Supplemental irrigation, an impressive strategy, diminishes the detrimental effects of drought on the productivity of rainfed plants. A factorial experiment was performed at Urmia University during 2016-2017. The experiment was included three factors; irrigation [without irrigation (rainfed) and supplemental irrigation], growing stage (vegetative and flowering) and fertilizers (control, mycorrhizal fungi (M, *Funneliformis mosseae*), *Thiobacillus thiooxidans* bacteria (T), M+T, vermicompost (V; 10 Mg ha^{-1}), M+V, T+V and M+T+V). By supplemental irrigation, the amount of hydrogen peroxide, the activities of ascorbate peroxidase (APX), superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidase (G-POD) were significantly reduced. Dual inoculation of plants resulted in a significant decrease of electrolyte leakage and malondialdehyde (MDA). Also, in co-inoculation conditions, the amount of total phenolics content (TPC), soluble proteins, catalase, ascorbate peroxidase and guaiacol peroxidase activity were significantly increased, which this increment was more noticeable with vermicompost application. Biological yield was increased by 74.7%, in dually-inoculated and irrigated plants. The rate of root colonization in mycorrhized plants was significantly increased, which probably had a major role in the above results. Accordingly, combined use of mycorrhiza, *Thiobacillus* and vermicompost, as well as supplemental irrigation can improve the yield of chicory forage in rainfed condition.

Keywords: Antioxidant, Enzymatic defense, Forage, *Funneliformis mosseae*, *Thiobacillus thiooxidans*, Vermicompost

Introduction

Drought, as the harshest environmental stress, limits agricultural crop yield and quality worldwide (Reddy *et al.*, 2004) through the considerable morphological, biochemical and physiological changes (Pandey and Shukla, 2015). Nevertheless, plants use different strategies to cope with drought, including the accumulation of osmolyte compounds (Wu *et al.*, 2016), increasing the activity of antioxidant enzymes (Kamarudin *et al.*, 2018), and increasing phenolic compounds (Okello *et al.*, 2017).

Supplemental irrigation, irrigation in the most sensitive stage of growth, is an impressive strategy to diminish the detrimental effects of drought on the productivity of rainfed plants. It may be defined as "the addition of limited amounts of water to soil" during times when precipitations are not sufficient for normal plant growth (Oweis and Hachum, 2012; Saadat *et al.*, 2019). This increasing productivity can be for enzymatic and non-enzymatic antioxidants, leaf chlorophyll, and leaf relative water content improvements of mycorrhized plants under water deficit conditions (Saadat *et al.*, 2021).

The plant can establish beneficial associations with rhizospheric microorganisms that can alleviate stress symptoms (Ruiz-Lozano *et al.*, 2016). Arbuscular mycorrhizal fungi (AMF), making symbiotic interactions with most terrestrial plants (Zhang *et al.*, 2018), may help plants resist drought through different mechanisms (Saadat *et al.*, 2019; Saadat *et al.*, 2021; Tao *et al.*, 2014). These mechanisms include the accumulation of soluble proteins (Rahimzadeh and Pirzad, 2017; Tuo *et al.*, 2017), reduction of malondialdehyde (Singh, 2015) and H_2O_2 content through the enhancement of antioxidant levels (Huang *et al.*, 2017) and chlorophyll contents (Saadat *et al.*, 2021).

Thiobacillus bacteria are one of the sulfur-oxidizing microorganisms that make it available in soil (Masoodi and Hakimi, 2017), as one of the important plant

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nutrients. These microorganisms solubilize other soil minerals such as Mg, K and P through the production of sulphuric acid (Mohamed *et al.*, 2014). Sulfur is used in the formation of amino acids, proteins and chlorophyll and helps to develop and activate certain enzymes in plants (Shadravan *et al.*, 2018).

Application of vermicompost, a nutritive organic fertilizer with high levels of absorbable minerals, aeration, water holding capacity, and microbial activity (Shirkhani and Nasrolahzadeh, 2016), to soil affects crop growth and yield (Salehi *et al.*, 2016). Additionally, vermicompost contains humic substances that can stimulate protein synthesis in plant organs. These versatile compounds also induce the activity of defensive enzymes such ad catalase, ascorbate peroxidase and superoxide dismutase in plants under drought stress (Garcia *et al.*, 2012).

Chicory (*Cichorium intybus* L., Asteraceae family), a small Mediterranean aromatic plant, chiefly grown throughout Europe, in Southwestern Asia, and in limited areas of Australia, South Africa, and North America (Mehmood *et al.*, 2012; Barcaccia *et al.*, 2016). It is variously used as a cooked food, a vegetable or salad green, an industrial crop for the extraction of polysaccharide, a medicinal plant, a possible biomonitor of heavy metal pollution, and a forage crop (Perovic *et al.*, 2021). Forage chicory produces a large quantity of high quality feed (Abbas *et al.*, 2015), is palatable for ruminants, is high in nonstructural carbohydrates, and low in fiber and can remain green longer than other forage species (Piluzza *et al.*, 2014).

There is little knowledge about the reactions of chicory to bio-inoculation with some beneficial microorganisms, organic manure, as well as an additional irrigation under rainfed conditions. Accordingly, this study was conducted to evaluate the effects of *Thiobacillus thiooxidans* and mycorrhiza, vermicompost, and supplemental irrigation on the physiological and biochemical responses of rainfed chicory.

Materials and methods

Field condition and experimental design: A 2-year experiment was performed in the research farm of Urmia University, West Azerbaijan, Iran (37′ 39′ 24.28″ N latitude, 44′ 58′ 12.24″ E longitude) during 2016-2017. Some physicochemical properties of the soil (0–30 cm) and vermicompost were shown in Table 1. Climatological parameters were shown in Figure 1.

The experiment, factorial based on a randomized complete block design with three replications, included three factors; irrigation [without irrigation (rainfed), growing stage (vegetative and flowering) and supplemental irrigation] and fertilizers were included: control, mycorrhiza fungi (M, *Funneliformis mosseae*), *Thiobacillus thiooxidans* bacteria (T), M+T, vermicompost (V), M+V, T+V and M+T+V. Fertilizers were prepared for us in cooperation with Green Biotech Company. In order to prevent water seepage, 2 meter spacing was considered between adjacent plots and blocks. Chicory seeds were sown at 20 cm intra-row spacing in rows and 40 cm inter-row on the 10 March 2016 and 6 April 2017.

In the respective treatments, vermicompost was used at the rate of 10 Mg ha^{-1}. In plots containing mycorrhiza, the AMF inoculum (20 g m^{-2}) was placed in planting rows. Before planting, the seeds of chicory were inoculated with *T. thiooxidans* bacteria (10^7 cfu g^{-1}) using *sucrose solution and then dried in shade*. In plots containing *T. thiooxidans*, powdered sulfur (300 kg ha^{-1}) was first placed in planting rows, and after covering using a thin layer of soil, the pre-inoculated seeds of chicory were sown at a depth of 1-2 cm. Non-inoculated seeds were considered as controls. Supplemental irrigation was performed only once before flowering stage (105 and 95 days after sowing in 2016 and 2017, respectively) in both years. The amount of water used in supplemental irrigation was calculated using the equation $V_n=(F_c-\theta)\times(A\times h)$ where V_n the amount of water required for each plot (m^3), F_c field capacity of the soil, θ soil moisture content at irrigation time, A plot area and h penetration depth of the root (Benami and Ofen, 1984). In both years, plants were harvested at two growth stages (vegetative and full flowering). More details of the harvest schedule are presented in Table 2.

Biological yield: Seven plants from each treatment were randomly selected at harvest (Table 2) and removed and the shoot was separated from the root system. The shoot was dried in a forced-air oven at 75 °C for 48 h and the biological yield of samples was measured by a digital scale.

Root colonization: Fresh roots of five plants were washed to remove soil, placed in 10% KOH (w/v) for 10 min at 90 °C, washed again with water, immersed in 4% lactic acid for acidification, and then stained in 0.05% Trypan blue in lactoglycerol (Phillips and Hayman, 1970). Using the method of gridline intersection, the amount of root colonization was determined (Giovannetti and Mosse, 1980). The root colonization percentage was determined as the ratio of the number of roots colonized with AM to the total number of root.

Soluble proteins: The soluble proteins content was determined by the method of Bradford (1976) with minor modifications. 250 mg of leaf samples was homogenized with 5 ml phosphate buffer (pH 7.6). The mixture was centrifuged at 6000 g for 20 min at 4 °C. 20 µl of the supernatant was added to 80 µl distilled water, and then 2.9 ml of Bradford reagent was mixed with the solution and vortexed for 2 min. After incubation for 10 min at room temperature, the absorbance was read at 595 nm. By using BSA (Bovine Serum Albumin) as standard, the content of soluble proteins (mg g^{-1} FW) was calculated.

Anthocyanin: Anthocyanin content was measured using the method described by Gitelson *et al.* (2001). The absorbance of the supernatant was read at 550 nm,

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Table 1. Some properties of the soil and vermicompost

	N	P	K	Ca	Organic carbon	pH	Texture
	%						
Soil (0-30 cm)	0.14	0.002	0.068	4	1.76	7.84	Silty-Clay-Loam
Vermicompost	2.96	1.72	1.98	12.88	22.1	7.5	-

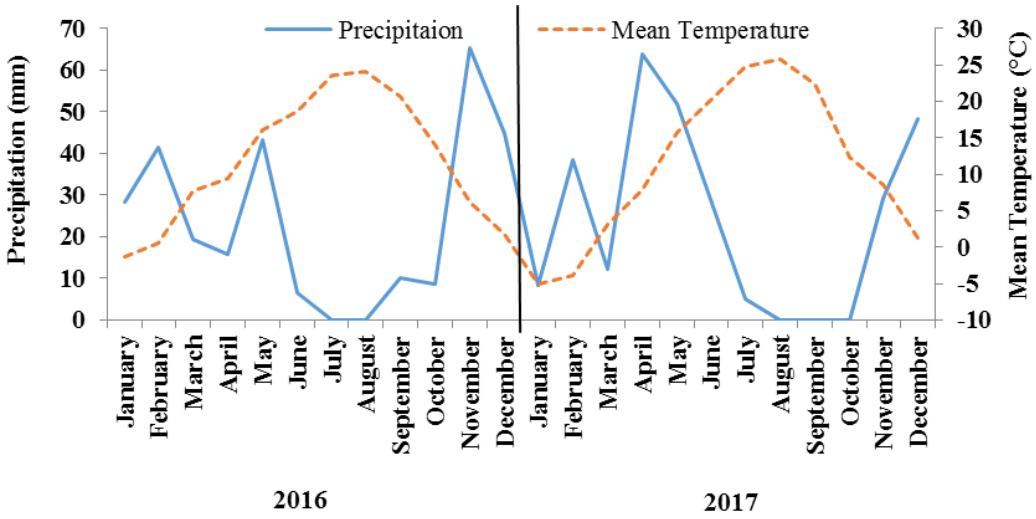

Figure 1 Average monthly precipitations and air mean temperature of the experimental site during 2016-2017.

Table 2. The harvest schedule of plants (days after sowing) at two growth stages during 2016-2016.

	Rainfed		Supplemental Irrigation	
	Vegetative	Full Flowering	Vegetative	Full Flowering
2016	80	115	80	135
2017	70	100	70	125

and the amount of anthocyanin (µmol/g FW) was calculated by the formula: A = εbc, where A is the absorbance of the sample, ε is the extinction coefficient (33000 cm mol^{-1}), b is cuvette width and c is the extract concentration.

Total phenolics: Total phenolics content (TPC) was quantified using the method described by Dogru and Bayram (2016) with some modifications. The mixture of Folin-Ciocalteu's reagent and the supernatant was incubated at room temperature in a dark place for 30 min. Absorption was read at 765 nm, and total phenolic content (mg GAE/g FW) was determined using Gallic acid as standard.

GAE= Gallic Acid Equivalent

Antioxidant enzyme extraction and assays: Using a pestle in an ice-cold mortar, 500 mg frozen leaf sample was powdered in 5 ml 50 mM potassium phosphate buffer (pH 7.0) including 1 mM ethylene-diamine tetraacetic acid (EDTA), 2 mM ascorbic acid and 1% polyvinylpyrrolidone (PVP). After centrifuge at 12000 g for 20 min at 4 °C, the supernatant was collected and used for enzymatic assays (Yang *et al.*, 2015).

Ascorbate peroxidase: Ascorbate peroxidase (APX) activity was assayed according to Nakano and Asada (1981). One unit of the enzyme is defined as the amount of the enzyme required for oxidation of 1 µmol ascorbate per milligram of protein.

Catalase: Catalase (CAT) activity was assayed by using the method of Maehly and Chance (1959). Decrease in hydrogen peroxide (H_2O_2) was monitored at 240 nm for 1 min.

Guaiacole peroxidase: Guaiacole peroxidase (G-POD) activity was assayed by the method of Cakmak and Marschner (1992). The activity was determined by monitoring the increase in absorbance caused by guaiacole oxidation at 470 nm for 1 min. G-POD activity was expressed as enzyme unit (U) per milligram of protein.

Superoxide dismutase: Superoxide dismutase (SOD) activity was assayed according to the method as described by Giannopolitis and Ries (1977). One unit of SOD is defined as the quantity of enzyme required for inhibition of photochemical reduction of nitroblue tetrazolium (NBT) by 50%. SOD activity was expressed as enzyme unit (U) per milligram of protein.

Hydrogen peroxide (H_2O_2): The amount of H_2O_2 was estimated according to the method described by Loreto and Velikova (2001) with slight modifications. Leaf sample (200 mg) was homogenized in an ice-cold porcelain mortar with 5 ml of 1% (w/v) TCA (trichloroacetic acid). After centrifuging at 12000 g for 15 min, 0.5 ml of the supernatant was mixed with 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1M potassium iodide (KI). After reading the absorbance at 390 nm, the content of H_2O_2 was

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determined using a standard calibration curve made by different amounts of H_2O_2.

Malondialdehyde (MDA): Malondialdehyde content was estimated using thiobarbituric acid (TBA) reaction according to the method used by Hodges *et al*. (1999). The absorbance of the prepared sample was read at 450, 532 and 600 nm (A_{450}, A_{532} and A_{600}, respectively), and the content of MDA was calculated by the formula:

MDA content (µmol/g FW) = $6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}$

Electrolyte leakage: To measure electrolyte leakage (EL), leaf samples were prepared from expanded leaves and cut into 1-cm long segments. The samples were washed three times with distilled water, placed in tubes with 30 ml of deionized water, and incubated for 24 h at 25 °C. Then, the initial electrical conductivity (L_1) was measured with an electrical conductivity meter. The samples were placed in an autoclave for 30 min at 120 °C, and after equilibrating at 25 °C, the last conductivity (L_2) was determined (Lutts *et al.*, 1996). The electrical leakage was calculated as EL (%) = $(L_1/L_2) \times 100$.

Statistical analysis: Before analysis of variance, the normality of data distribution was confirmed by using the Kolmograf Smirnov test. The combined analysis of 2-year data was done using the statistical software SAS (ver. 9.1). The Duncan's multiple range test was used at the 0.05 probability level for detection of significant differences among means.

Results

The results of the combined analysis of 2-year data were shown in Table 3.

Root colonization: Root colonization of rainfed plants was increased through supplemental irrigation (Fig. 2A). At the flowering stage, root colonization was higher than vegetative stage in all the treatments. Application of vermicompost along with bio-inoculants resulted in an increase in root colonization under both vegetative and flowering stages. Singly inoculated plants by *T. thiooxidans* had a higher level of root colonization compared to control, whereas higher levels of root colonization were obtained in the presence of mycorrhiza, regardless of using vermicompost (Fig. 3A). These above results were obtained for two years (Table 4).

Soluble proteins: The quantity of soluble proteins was decreased by supplemental irrigation at the flowering stage (Fig. 2B). All inoculated plants had higher levels of soluble proteins over control, regardless, using vermicompost. Soluble protein content of the co-inoculated plants, regardless of using vermicompost, was the highest among all other inoculation treatments, as well as control. Singly inoculated plants with mycorrhiza had a higher concentration of soluble proteins compared to the plants singly inoculated with *T. thiooxidans* (Table 5). In two years, the use of vermicompost alone resulted in an increase in soluble protein content in comparison with control. At both two years, soluble proteins were the same for rainfed and supplemental irrigation for the vegetative stage, but they were reduced for the flowering stage by irrigation (Table 6).

Anthocyanins: A significant reduction was observed in anthocyanin content at the flowering stage by performing supplemental irrigation (Fig. 2C). Application of vermicompost alone resulted in an increase in anthocyanin content compared with untreated control. The lowest concentration of anthocyanin was observed in control plants, and the highest amount of anthocyanin was recorded for combined treatment of T_8 (mycorrhiza + *Thiobacillus* +vermicompost), followed by T_6 (mycorrhiza + vermicompost). Irrespective of the vermicompost application, the anthocyanin content of the singly inoculated plants with mycorrhiza was significantly higher than that of those plants singly inoculated with *T. thiooxidans* (Table 5).

Total phenolics content: The lowest amount of total phenolic content (TPC) was observed in supplementally irrigated plants at the flowering stage, but it was non-decreasing at the vegetative stage. (Fig. 2D). The application of vermicompost alone produced more total phenolic content compared to the control. Regardless of using vermicompost, the quantity of total phenolics was increased with the application of mycorrhiza and *Thiobacillus*, alone or in combination with each other, in comparison to the control. The maximum total phenolics content was recorded in mycorrhiza+vermicompost, followed by mycorrhiza+ *Thiobacillus*+vermicompost (Table 5), which was the same for both 2016 and 2017 years (Table 6).

Ascorbate peroxidase: The activity of ascorbate peroxidase (APX) at the flowering stage of rainfed plants was decreased by supplemental irrigation (Fig. 2E). Ascorbate peroxidase enzyme activity was increased with the use of vermicompost as compared to the control. Although, the activity of APX in all inoculated plants (except for individual inoculation with *Thiobacillus*) was higher than that non-inoculated control, the activity of APX was upraised by the simultaneous application of bio-inoculants and vermicompost. The maximum activity of APX was recorded in mycorrhiza+*Thiobacillus*+vermicompost, followed by mycorrhiza+vermicompost, respectively (Table 5).

Catalase: At the flowering stage, the activity of the catalase (CAT) enzyme was decreased by performing supplemental irrigation (Fig. 2F). Catalase activity was increased with the application of mycorrhiza, alone and/or in combination with *Thiobacillus* and vermicompost compared with untreated control plants. Besides, the activity of the CAT enzyme was upraised under individual inoculation with mycorrhiza as compared to single inoculation with *Thiobacillus*, regardless of using vermicompost (Table 5).

Guaiacol peroxidase: The activity of the guaiacol

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Table 3. ANOVA for chicory yield, biochemical and physiological traits affected by vermicompost, mycorrhiza, *Thiobacillu thiooxidans*, growth stage and supplemental irrigation

Source of variations	df	Biological yield	Root colonization	Soluble proteins	Anthocyanin	Total phenolics
Year (Y)	1	34.236**	44.673^{ns}	112.28^{ns}	0.000016 ^{ns}	2.9524^{ns}
Block (year)	4	0.0156^{ns}	11.568^{ns}	91.016**	0.002449^{ns}	0.8488*
Irrigation (I)	1	132.23**	483.24**	145.79**	0.037352**	1.2369*
Growing stage (G)	1	3001.2**	1413.6**	5.1778^{ns}	0.004680*	3.5341**
Treatments (T)	7	153.03**	6697.5**	2514.2**	0.022489**	18.487**
I × G	1	83.714**	240.39**	875.99**	0.05943**	11.736**
I × T	7	3.3287**	10.741^{ns}	9.3483^{ns}	0.00031^{ns}	0.0547^{ns}
G × T	7	17.146**	52.145**	12.629^{ns}	0.00038^{ns}	0.1003^{ns}
I × G ×T	7	2.7478^{ns}	2.8830^{ns}	14.278^{ns}	0.00022^{ns}	0.0489^{ns}
Y×I	1	1.9248^{ns}	1.7801^{ns}	92.724*	0.00119^{ns}	1.5749*
Y×G	1	80.287**	186.23**	300.48**	0.00100^{ns}	2.0318**
Y×T	7	3.5165**	18.579**	14.571^{ns}	0.00123^{ns}	0.0397^{ns}
Y×I×G	1	1.7203^{ns}	3.3478^{ns}	95.926*	0.00016^{ns}	1.5572*
Y×I×T	7	0.8610^{ns}	0.4063^{ns}	5.7359^{ns}	0.00018^{ns}	0.0937^{ns}
Y×G×T	7	4.2509**	17.260**	14.877^{ns}	0.00018^{ns}	0.1268^{ns}
Y×I×G×T	7	0.8651^{ns}	0.5281^{ns}	6.3027^{ns}	0.00021^{ns}	0.0977^{ns}
Error	124	1.0845	6.1535	16.829	0.00069	0.2581
CV (%)		9.55	11.34	7.89	12.74	7.58

ns = not significant; * and ** significant in the 5% and 1% level, respectively.

Continu of table 3.

Source of Variations	df	APX	CAT	G-POD	SOD	H_2O_2	MDA	EL
Year (Y)	1	0.668^{ns}	1.837**	4.0959*	2.7564^{ns}	8.5227*	155.89^{ns}	723.66**
Block (year)	4	0.965**	0.075^{ns}	0.3067*	0.4749^{ns}	0.6640^{ns}	20.813^{ns}	2.1253**
Irrigation (I)	1	1.089**	1.695**	0.9437**	0.5139^{ns}	1.6639 *	492.73**	1578.5**
Growing stage (G)	1	0.073^{ns}	0.392^{ns}	0.75488*	0.0302^{ns}	4.7836**	126.92**	145.97**
Treatments (T)	7	2.092**	6.097**	0.6399**	8.0414**	41.446**	2994.2**	2119.7**
I × G	1	3.213**	5.083**	3.0701**	9.8586**	11.216**	1704.9**	1511.9**
I × T	7	0.047^{ns}	0.035^{ns}	0.0316^{ns}	0.1985^{ns}	0.1656^{ns}	10.873^{ns}	8.9539**
G × T	7	0.124^{ns}	0.035^{ns}	0.0247^{ns}	0.9719**	0.1155^{ns}	38.891*	5.7197**
I × G ×T	7	0.041^{ns}	0.058^{ns}	0.0317^{ns}	0.2594^{ns}	0.1418^{ns}	18.783^{ns}	8.1967**
Y×I	1	0.033^{ns}	0.283^{ns}	0.2763^{ns}	0.0087^{ns}	0.0282^{ns}	0.6722^{ns}	70.603**
Y×G	1	0.133^{ns}	0.044^{ns}	0.4310^{ns}	0.0012^{ns}	0.4852^{ns}	127.59**	27.945**
Y×T	7	0.118^{ns}	0.235^{ns}	0.0735^{ns}	1.7599**	0.1305^{ns}	83.718**	26.598**
Y×I×G	1	0.029^{ns}	0.254^{ns}	0.2420^{ns}	0.0181^{ns}	0.0657^{ns}	0.6255^{ns}	15.634**
Y×I×T	7	0.009^{ns}	0.067^{ns}	0.0026^{ns}	0.0857^{ns}	0.0511^{ns}	4.8203^{ns}	0.4926 ^{ns}
Y×G×T	7	0.051^{ns}	0.022^{ns}	0.0101^{ns}	0.1211^{ns}	0.0297^{ns}	1.7009^{ns}	5.0710**
Y×I×G×T	7	0.010^{ns}	0.064^{ns}	0.0026^{ns}	0.0995^{ns}	0.0528^{ns}	5.4403^{ns}	0.7639 *
Error	124	0.087	0.121	0.1241	0.2387	0.2901	17.188	0.2782
CV (%)		13.41	12.57	15.69	6.83	9.08	7.45	10.52

Abbreviations were: Ascorbate peroxidase (APX), Catalase (CAT), Guaiacol peroxidase (G-POD), Superoxide dismutase (SOD), Hydrogen peroxide (H_2O_2), Malondialdehyde (MDA) and Electrolyte Leakage (EL). ns = not significant; * and ** significant in the 5% and 1% level, respectively.

Table 4. Mean comparisons of root colonization of *Cichorium intybus* affected by "year× growing stage× fertilizer."

Treatment	Root colonization (%)			
	2016		2017	
	Vegetative	Flowering	Vegetative	Flowering
Control	1.24^{m}	4.31^{klm}	3.13^{lm}	4.69^{kl}
Mycorrhiza (M)	31.88^{e}	41.96^{bc}	32.28^{e}	37.13^{d}
T. thiooxidans (T)	5.18^{jkl}	8.11^{hij}	7.45^{hijk}	10.25^{gh}
M+T	28.68^{f}	39.81^{cd}	33.91^{e}	39.51^{cd}
Vermicompost (V)	2.92^{lm}	6.05^{jkl}	4.22^{klm}	5.93^{jkl}
M+V	33.98^{e}	46.37^{a}	37.07^{d}	41.19^{bc}
T+V	6.70^{ijk}	10.32^{gh}	9.56^{ghi}	12.31^{g}
M+T+V	30.97^{ef}	43.81^{ab}	37.42^{d}	41.68^{bc}

Means followed by different letter(s) differ significantly at P<0.05 by Duncan's multiple range test

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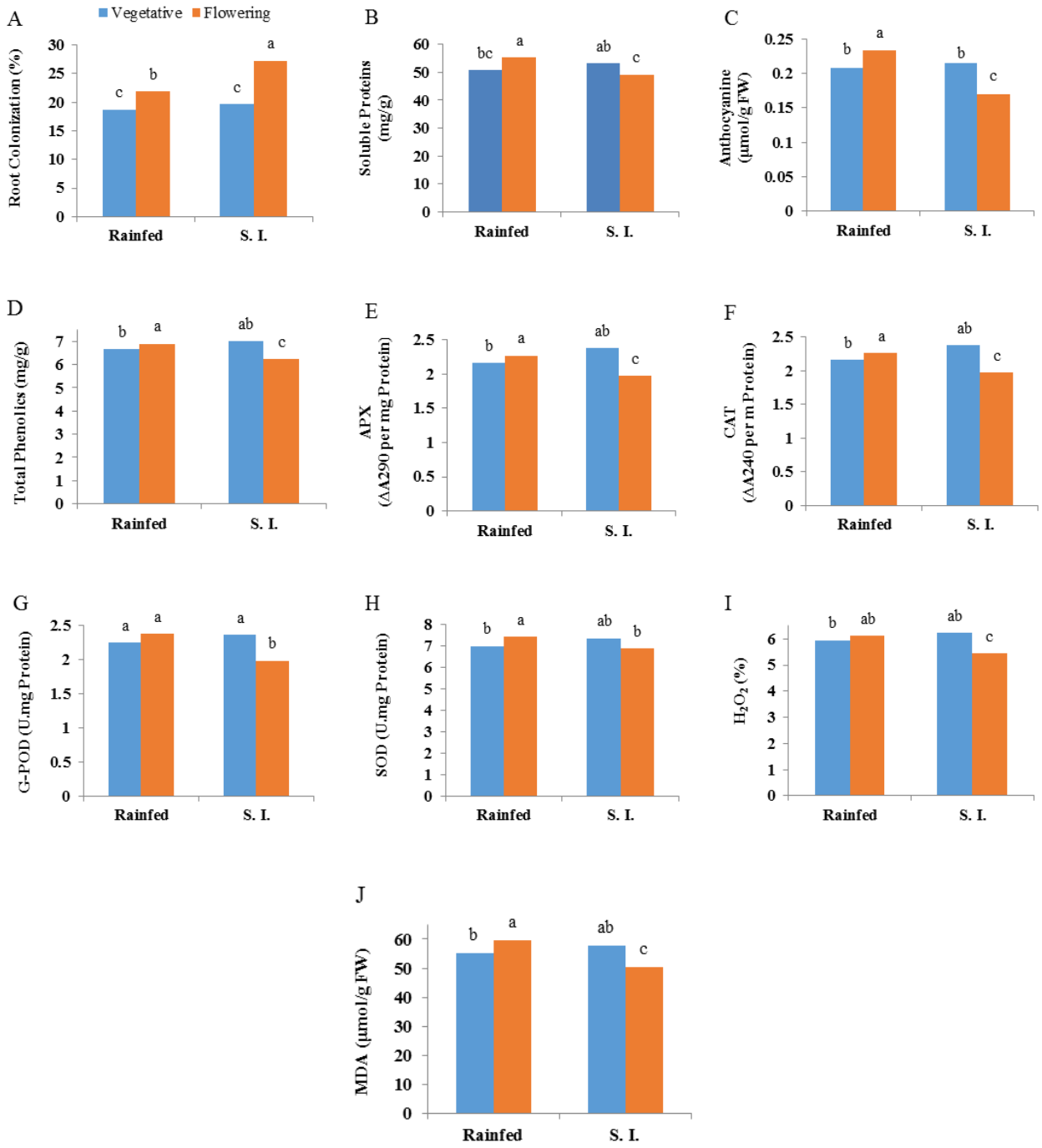

Figure 2. Interaction effects of supplemental irrigation and growth stage on root colonization (A), soluble proteins (B), anthocyanin (C), total phenolics (D), ascorbate peroxidase activity (E), catalase activity (F), guaiacole peroxidase activity (G), superoxide dismutase activity (H), H_2O_2 content (I), and malondialdehyde content (J). S. I. = Supplemental Irrigation. Bars with the same letter are not significantly different according to Duncan's multiple range test at P<0.05.

peroxidase (G-POD) enzyme was decreased with the application of supplemental irrigation at the flowering stage (Fig. 2G). The activity of the guaiacol peroxidase enzyme was enhanced in plants singly inoculated with *Thiobacillus* as well as vermicompost. Also, in presence of vermicompost, the G-POD activity was improved in plants singly inoculated with *Thiobacillus* as well as mycorrhiza (Table 5).

Superoxide dismutase: The activity of superoxide dismutase (SOD) enzyme was diminished in plants subjected to supplemental irrigation (Fig. 2H). As compared to control, the use of vermicompost was able to increase the activity of SOD only at vegetative stage. At both growth stages, co-inoculated plants had more SOD activity than singly inoculated plants, regardless using vermicompost. At vegetative stage, SOD activity was decreased in mycorrhiza, *Thiobacillus*, mycorrhiza + vermicompost, and *Thiobacillus* + vermicompost

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Table 5. Means comparison for chicory yield, biochemical and physiological traits affected by vermicompost, mycorrhiza, *Thiobacillu thiooxidans*, growth stage and supplemental irrigation

Source of variations	Biological yield (g/plant)	Root colonization (%)	Soluble Proteins (mg/g fresh weight)	Anthocyanin (µmol/g FW)	Total phenolics (mg/g fresh weight)
2016	11.329 ^{a}	21.393 ^{a}	51.243 ^{a}	0.20702 ^{a}	6.5813 ^{a}
2017	10.487 ^{b}	22.358^{a}	52.772 ^{a}	0.20644 ^{a}	6.8293 ^{a}
Rainfed	10.076^{b}	20.289^{b}	52.879 ^{a}	0.22067^{a}	6.7855 ^{a}
Supplemental	11.736^{a}	23.462^{a}	51.136 ^{b}	0.19278^{b}	6.6249 ^{b}
Vegetative	6.9523 ^{b}	19.162 ^{b}	51.843 ^{a}	0.21166^{a}	6.8409 ^{a}
Flowering	14.859 ^{a}	24.589 ^{a}	52.172 ^{a}	0.20179^{b}	6.5695 ^{b}
Control	7.4500^{g}	3.3443^{f}	37.105^{g}	0.163750^{d}	5.2064^{f}
Mycorrhiza (M)	11.055^{d}	35.813^{b}	56.511^{c}	0.214125^{b}	7.3034^{bc}
T. thiooxidans (T)	9.1648^{f}	7.7471^{d}	44.795^{e}	0.179958^{c}	6.4011^{d}
M+T	13.206^{b}	38.468^{b}	61.634^{b}	0.225208^{b}	7.0994^{c}
Vermicompost (V)	8.6061^{f}	4.7832^{e}	41.865^{f}	0.187125^{c}	5.7600^{e}
M+V	12.554^{c}	39.653^{a}	60.976^{b}	0.240292^{a}	7.7175^{a}
T+V	10.307^{e}	9.7222^{c}	48.809^{d}	0.193042^{c}	6.6617^{d}
M+T+V	14.904^{a}	38.468^{a}	64.366^{a}	0.250333^{a}	7.4925^{ab}

Same letters in each column show no significant differences in the 5% according to the Duncan's multiple range test.

Continue of table 5.

Source of variations	APX	CAT	G-POD	SOD	H_2O_2	MDA	EL
2016	2.133^{a}	2.671^{b}	2.0984^{b}	7.2736^{a}	6.1406 ^{a}	54.776 ^{a}	32.796 ^{b}
2017	2.251 ^{a}	2.866 ^{a}	2.3905^{a}	7.0339^{a}	5.7192 ^{b}	56.578 ^{a}	36.679 ^{a}
Rainfed	2.267 ^{a}	2.863 ^{a}	2.3145 ^{a}	7.2055^{a}	6.0230^{a}	57.279 ^{a}	37.605^{a}
Supplemental	2.116 ^{b}	2.675^{b}	2.1743 ^{b}	7.1020^{a}	5.8368^{b}	54.075 ^{b}	31.870 ^{b}
Vegetative	2.211 ^{a}	2.814 ^{a}	2.3071 ^{a}	7.1663 ^{a}	6.0878^{a}	55.490 ^{a}	35.609 ^{a}
Flowering	2.172 ^{a}	2.723 ^{a}	2.1817 ^{b}	7.1412 ^{a}	5.7721 ^{b}	54.864 ^{b}	33.865 ^{b}
Control	1.896^{de}	2.350^{c}	1.9736^{d}	7.3154^{c}	8.1441^{a}	71.499^{a}	48.492^{a}
Mycorrhiza (M)	2.133^{bc}	3.067^{b}	2.0803^{cd}	6.2859^{e}	4.8366^{e}	52.785^{e}	29.752^{e}
T. thiooxidans (T)	1.795 ^{e}	2.072^{d}	2.2296^{abc}	6.7103^{d}	6.3330^{c}	62.803^{c}	41.113^{c}
M+T	2.282 ^{b}	3.049^{b}	2.2617^{abc}	7.6010^{b}	5.2145^{d}	44.103^{g}	28.124^{f}
Vermicompost (V)	2.166 ^{bc}	2.547^{c}	2.2003^{bc}	7.7323^{ab}	7.4330^{b}	67.079^{b}	44.317^{b}
M+V	2.565^{a}	3.375^{a}	2.3313^{ab}	6.6408^{d}	4.5946^{e}	48.400^{f}	26.239^{g}
T+V	2.061^{cd}	2.336^{c}	2.4285^{a}	7.0431^{c}	6.0733^{c}	58.523^{d}	37.334^{d}
M+T+V	2.636^{a}	3.357^{a}	2.4505^{a}	7.9016^{a}	4.8103^{e}	40.224^{h}	22.527^{h}

Abbreviations were; Ascorbate peroxidase (APX, ΔA290 per mg Protein), Catalase (CAT, ΔA240 per m Protein), Guaiacol peroxidase (G-POD, U.mg Protein), Superoxide dismutase (SOD, U.mg Protein), Hydrogen peroxide (H_2O_2, %), Malondialdehyde (MDA, µmol/g fresh weight) and Electrolyte Leakage (EL, %). Same letters in each column show no significant differences in the 5% according to the Duncan's multiple range test.

Table 6. Means comparisons of malondialdehyde of *Cichorium intybus* affected by "year×growing stage", and soluble proteins and total phenolics content affected by "year×growing stage×irrigation".

Year	Growing stage	Malondialdehyde (µmol/g fresh weight)	Soluble proteins (mg/g fresh weight)		Total phenolics (mg/g fresh weight)	
			Rainfed	Supplemental irrigation	Rainfed	Supplemental irrigation
2016	Vegetative	56.41^{a}	51.06^{c}	51.61^{c}	6.65^{b}	6.69^{b}
	Flowering	53.14^{b}	54.57^{ab}	45.75^{d}	6.95^{a}	5.84^{c}
2017	Vegetative	56.57^{a}	50.11^{c}	52.70^{bc}	6.99^{a}	7.03^{a}
	Flowering	56.58^{a}	55.79^{a}	52.58^{bc}	6.94^{a}	6.65^{b}

Means followed by different letter (s) differ significantly at P<0.05 by Duncan's multiple range test

treatments compared to control. Also, individual inoculation with mycorrhiza and *Thiobacillus* resulted in an increase in the activity of SOD in comparison with control at flowering stage (Fig. 3B). The activity of superoxide dismutase was higher in vermicompost, mycorrhiza + *Thiobacillus* and mycorrhiza + *Thiobacillus* + vermicompost than control treatment. It was higher in first year (2016) than that was in 2017 (Table 7).

Hydrogen peroxide: The amount of Hydrogen peroxide (H_2O_2) at flowering was diminished by performing supplemental irrigation (Fig. 2I). The H_2O_2 content was decreased by the application of vermicompost alone or along with mycorrhiza and/or *Thiobacillus* as compared to the control. In general, the content of H_2O_2 was decreased in the presence

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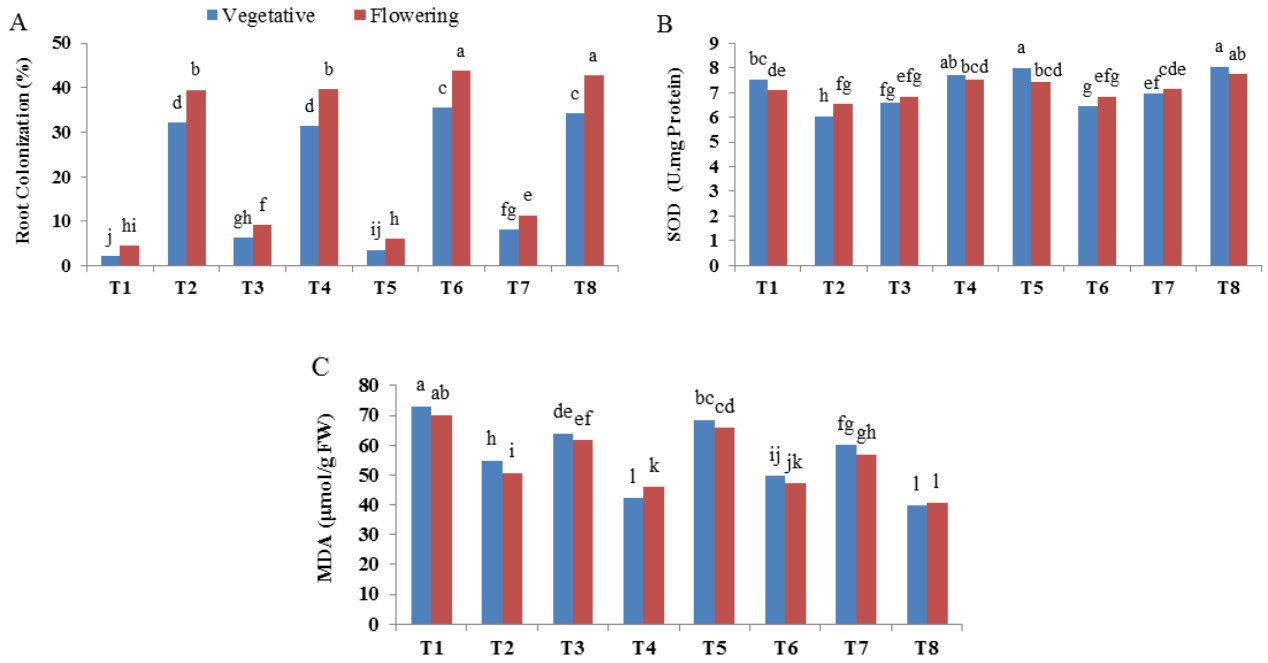

Figure 3. Means comparisons of root colonization (A), superoxide dismutase activity (B), and malondialdehyde content (C) of *Cichorium intybus* affected by "growth stage×fertilizer". T_1= Ctrl, T_2= Mycorrhiza (M), T_3= *Thiobacillus* (T), T_4= M+T, T_5= Vermicompost (V), T_6= M+V, T_7= T+V, T_8= M+T+V.

Table 7. Means comparison for superoxide dismutase activity and malondialdehyde affected by "year×fertilizer".

Year	Treatment	Superoxide dismutase (U.mg Protein)	Malondialdehyde (µmol/g FW)
2016	Control	7.5492^{b}	72.024^{a}
	Mycorrhiza (M)	6.0253^{h}	50.967^{h}
	T. thiooxidans (T)	6.773^{efg}	62.951^{de}
	M+T	7.9758^{a}	40.716^{k}
	Vermicompost (V)	8.0535^{a}	68.234^{bc}
	M+V	6.3935^{gh}	46.564^{ij}
	T+V	7.0741^{cde}	59.664^{ef}
	M+T+V	8.3441^{a}	37.087^{l}
2017	Control	7.0815^{cde}	70.974^{ab}
	Mycorrhiza (M)	6.5464^{efg}	64.602^{g}
	T. thiooxidans (T)	6.6475^{efg}	62.655^{de}
	M+T	7.2260^{bcd}	47.489^{hi}
	Vermicompost (V)	7.4116^{bc}	65.924^{cd}
	M+V	6.8880^{def}	50.237^{h}
	T+V	7.0120^{cde}	57.383^{fg}
	M+T+V	7.4590^{bc}	43.359^{jk}

Means followed by different letter(s) differ significantly at P<0.05 by Duncan's multiple range test.

of mycorrhiza, regardless of using vermicompost (Table 5).

Malondialdehyde: Supplemental irrigation caused a significant reduction of drought-induced high concentrations of malondialdehyde (MDA) (Fig. 2J). At both growth stages, the control plants registered higher MDA content than the plants treated with vermicompost and/or mycorrhiza and *Thiobacillus*. Of course, the MDA content of plants inoculated with mycorrhiza+ *Thiobacillus* was significantly lower than that of the single inoculated plants as well as the control. Totally, the concentration of MDA was decreased in the presence of mycorrhiza as compared to other treatments (Fig. 3C). In mycorrhiza+*Thiobacillus*+vermicompost treatment, the maximum reduction of malondialdehyde was observed. Despite the same order, it was more reduction in the first year. The highest malondialdehyde in control shows the effectiveness of treatments reducing membrane injuries as malondialdehyde (Table 7).

Biological yield: At the flowering stage, supplemental irrigation caused an increase in the biological yield of rainfed plants. The application of vermicompost alone caused an increase in the biological yield of the plant compared to the control at the flowering stage. The biological yield of all inoculated

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Table 8. Mean comparisons of biological yield of *Cichorium intybus* affected by "irrigation× growing stage× fertilizer."

Treatment	Biological yield (g/plant)			
	Rainfed		Supplemental irrigation	
	Vegetative	Flowering	Vegetative	Flowering
Control	4.680 ^{r}	9.370 ^{ij}	4.915 ^{r}	10.83 ^{gh}
Mycorrhiza (M)	6.746 $^{n-p}$	14.32 ^{e}	7.083 $^{m-o}$	16.08 ^{cd}
T. thiooxidans (T)	5.665 $^{p-r}$	11.30 ^{g}	5.948 $^{o-r}$	13.75 ^{ef}
M+T	8.714 $^{j-l}$	15.70 ^{d}	9.150 ^{jk}	19.26 ^{b}
Vermicompost (V)	5.323 ^{qr}	10.55 $^{g-i}$	5.589 $^{p-r}$	12.96 ^{f}
M+V	7.685 $^{l-n}$	15.79 ^{d}	8.069 $^{k-m}$	18.66 ^{b}
T+V	6.001 $^{o-r}$	12.81 ^{f}	6.302 $^{o-q}$	16.12 ^{cd}
M+T+V	9.446 ^{ij}	17.11 ^{c}	9.918 $^{h-j}$	23.14 ^{a}

Means followed by different letter(s) differ significantly at P<0.05 by Duncan's multiple range test.

Table 9. Mean comparisons of biological yield of plant and electrolyte leakage of *Cichorium intybus* affected by "irrigation× growing stage× fertilizer."

Year	Treatment	Electrolyte Leakage (%)			
		Rainfed		Supplemental irrigation	
		Vegetative	Flowering	Vegetative	Flowering
2016	Control	47.53b	50.48a	49.91ab	40.99e
	Mycorrhiza (M)	28.89k	30.45j	30.34j	21.12m
	T. thiooxidans (T)	36.86gh	43.31d	38.71fg	31.22j
	M+T	27.78k	31.53j	29.17k	19.22mn
	Vermicompost (V)	43.48d	45.94c	45.65c	37.67g
	M+V	25.49l	28.63k	26.76l	16.25
	T+V	34.05i	39.73f	35.75h	25.99l
	M+T+V	21.64m	25.06l	22.72m	17.12n
2017	Control	51.57a	53.68a	49.16ab	44.52d
	Mycorrhiza (M)	33.48i	36.62gh	31.81j	25.28l
	T. thiooxidans (T)	47.03b	50.84a	44.68c	36.24g
	M+T	31.04j	35.31h	29.49jk	21.44m
	Vermicompost (V)	45.52c	51.19a	43.25d	41.81de
	M+V	29.77jk	33.97i	28.28k	20.72m
	T+V	42.52d	47.85b	40.41e	32.36i
	M+T+V	40.84e	27.98k	22.65m	19.17mn

Means followed by different letter(s) differ significantly at P<0.05 by Duncan's multiple range test.

plants (single and co-inoculated) was increased as compared to non-inoculated controls. The maximum biological yield of the plant was recorded in M+T+V at the flowering stage. Also, the biological yield of single inoculated plants with mycorrhiza was higher than that of those singly inoculated with *T. thiooxidans* (Table 8).

EL: Despite increasing in rainfed systems, the level of electrolyte leakage (EL) was decreased with performing supplemental irrigation at the flowering stage. The use of vermicompost alone caused a little reduction in EL level at both growth stages. While the maximum reduction of EL belongs to "mycorrhiza+ *Thiobacillus*+vermicompost" for two years and however growth stages (Tables 5 and 9).

Discussion

Adopting management strategies, such as supplemental irrigation, is vital for alleviating the detrimental effects of drought stress on crop productivity under rainfed conditions. The aim of supplemental irrigation is to improve crop performance by consuming small amounts of water by rainfed crops during the times when precipitations are not enough for normal plant growth (Tadayon *et al.*, 2012). We observed that chicory's biological yield was significantly increased by performing supplemental irrigation under rainfed conditions. Similar results have also been reported (Kumar Jha *et al.*, 2018). Dual-inoculated plants with Mycorrhiza and *Thiobacillus* significantly increased the biological yield of plants compared to control. It can be said that there was a synergistic effect between mycorrhiza and *Thiobacillus* on chicory growth in this study. The increased biological yield of the inoculated plants is probably related to increased absorptive surface area by mycorrhizal hyphae, to provide more access to soil volume for absorption of water and nutrients (Symanczik *et al.*, 2018), to improve mineral nutrition and increase photosynthesis (Wang, 2011), and thus increase growth and development of plants.

Root colonization depends on several factors, such as rhizosphere conditions and plant species (Koide, 1991). Intense drying and rewetting cycles in rhizosphere soil (0–30 cm) may have limited root mycorrhizal colonization of rainfed plans (Bowles *et al.*, 2016). Drought stress decreases two phases of mycorrhizal spore germination (hydration of spore and hyphal growth), so it can be said that performing supplementary irrigation results in an increase in the mycorrhizal spore germination and root colonization rate. Besides, mycorrhizal colonization rate decreases

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with changing the quantity and quality of host root exudates under rainfed conditions, because these exudates are stimulators for mycorrhizal symbiosis (Mahdi Dar *et al.,* 2018; Zarei *et al.,* 2016; Wu, 2017).

The evaluation of electrolyte leakage from a plant's cell membrane is a classical method to determine membrane integrity in response to drought stress (Beltrano *et al.,* 2013). Our data showed that inoculation with mycorrhiza and *Thiobacillus* along with performing supplemental irrigation reduced the level of EL over control (Table 9). It has also been reported that application of vermicompost decreases EL in plants subjected to drought stress (Kiran, 2019).

Our study revealed that drought caused an increase in SOD, APX, G-POD and CAT activity, while the activity of these enzymes decreased with performing supplemental irrigation. In general, inoculation with Mycorrhiza and *Thiobacillus* (singly or in combination with each other), along with using vermicompost, led to an increase in the activity of all enzymes over control. It has been reported that the activity of antioxidant enzymes increases in plants subjected to drought stress (Mohammadi *et al.,* 2011; Malik *et al.,* 2015). Similar to the results of this study, Rahimzadeh and Pirzad (2017) concluded that under drought stress, the activity of antioxidant enzymes in mycorrhizal plants was higher than control ones. Vermicompost application under drought conditions may enhance the activity of antioxidant enzymes by increasing the uptake of ions such as Ca, which acts as an activator of the enzymes (Kiran, 2019).

Anthocyanins, a group of water-soluble flavonoids, have substantial roles in maintaining water homeostasis and in scavenging ROSs produced through abiotic stresses such as drought in various plant species (Razavizadeh *et al.,* 2017). Similar to our results, increasing anthocyanin content under drought stress has been reported (Caser *et al.,* 2016). In this experiment, a reduction of anthocyanin content through performing supplemental irrigation was seen. Lower anthocyanin content in supplementally irrigated plants indicates that supplemental irrigation per se does not reduce the content of the anthocyanins; rather, it is the dilution effect due to the larger size and higher biological yield of those plants. Findings of Cecatto *et al.* (2016) and Saini *et al.* (2019) are in line with our results, as the inoculated plants (singly or in combination) showed high levels of anthocyanins as compared to the control. Similarly, the anthocyanin content in vermicompost-treated plants was higher than that of the controls.

Under drought conditions, plants accumulate proteins to retain the water status of their leaves, and diminish the detrimental effects of ROSs (Moradtalab *et al.,* 2019). We found that performing supplemental irrigation caused a reduction in soluble protein content as compared to the rainfed plants. Also, enhanced soluble protein content was seen in plants inoculated with mycorrhiza and/or *Thiobacillus* with or without using vermicompost as compared to the controls. Abdel-Fattah *et al.* (2014) reported that mycorrhization increased the protein content of the inoculated plants. Probably, inoculation with mycorrhiza and/or *Thiobacillus* enhanced the content of soluble proteins through lessening RNA decomposition and stimulating new proteins synthesis (Tuo *et al.,* 2017). Similar to our results, Song *et al.,* (2015) found that the application of vermicompost and/or bio-inoculants increased the content of soluble proteins in spinach. The use of vermicompost increases the availability and uptake of macro and micro elements, especially nitrogen, needed by plants, which leads to enhanced protein levels.

Hydrogen peroxide (H_2O_2), one of the reactive oxygen species, is generated in large quantities during drought stress. It is a toxic molecule capable of causing oxidative damage to membrane lipids, proteins and nucleic acids (Ebrahimian and Bybordi, 2012). Because of high antioxidant enzyme activity in all inoculated and vermicompost-treated plants, the content of H_2O_2 was lower in these plants in comparison with the control. It was also observed that supplemental irrigation significantly lessened the content of H_2O_2 in chicory plants. It has been reported that with the use of vermicompost and/or mycorrhizal inoculants, the content of H_2O_2 was decreased in comparison with the untreated controls under water stress (Liu *et al.,* 2015; Moradtalab *et al.,* 2019).

Phenolics, an important group of non-enzymatic antioxidants, can protect plants against the detrimental effects of oxidative stress (Baslam *et al.,* 2013). In this study, the phenolics content was decreased with performing supplemental irrigation. Moreover, the inoculated and/or vermicompost-treated plants had higher levels of phenolics in comparison with the controls. It has been reported that the activity of the phenolylalanine ammonia-lyase enzyme, which is involved in the biosynthesis of phenolics, increases through mycorrhization (Bagheri *et al.,* 2014). Since vermicompost contains phenylalanine, the precursor for phenolic compounds, this would have contributed to the increase in phenolic contents (Kumar and Ponnuswami, 2013).

Malondialdehyde (MAD), as a product of lipid peroxidation, is an effective indicator for assessing the detrimental effects of oxidative stress in plants (Saeedfar *et al.,* 2015). At both growth stages, the content of MDA in inoculated (either single or combined) and/or vermicompost-treated plants diminished compared to the control. This result is in agreement with the findings of other studies (Garcia-Sanchez *et al.,* 2014; Kiran, 2019). Besides, supplementary irrigated plants had a lower content of MDA in comparison with rainfed ones. Use of vermicompost with and without using bio-inoculants increased the activity of antioxidant enzymes, scavenged the ROSs in plant cells, and lessened lipid peroxidation.

Conclusion

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On the basis of our results, drought stress increased the content of anthocyanin, H_2O_2, MDA, phenolics, and soluble proteins; decreased plant biological yield, and the rate of root colonization; and induced the activity of antioxidant enzymes (SOD, CAT, G-POD, and APX). Also, the electrolyte leakage increased under drought conditions. However, supplemental irrigation significantly increased the biological yield of the plant and root colonization rate, diminished the activity of the antioxidant enzymes, and decreased the amount of MDA, H_2O_2, anthocyanin, phenolics, and soluble proteins. Besides, with performing supplemental irrigation, the EL was decreased in irrigated plants. Dual inoculation with mycorrhiza and *Thiobacillus* reduced the drought stress damaging effects by decreasing the level of MDA, H_2O_2, and EL and increasing the antioxidant enzymes' activity, the content of phenolics, soluble proteins, anthocyanin, root colonization rate, and biological yield of the plant. Also, vermicompost application (alone or in combination with microorganisms) was a beneficial tool to cope with the detrimental effects of drought stress. So, this study has revealed that inoculation with beneficial microorganisms (mycorrhiza and *Thiobacillus*) and/or the application of vermicompost can diminish the damaging effects of drought stress through scavenging the ROSs and improving the plant's water status. There was a synergistic effect between mycorrhiza and *Thiobacillus* in most of the studied traits. In general, performing supplemental irrigation as well as using beneficial microorganisms and vermicompost would increase chicory yield under rainfed conditions and improve its tolerance to drought stress.

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Declaration of interest statement

The authors have no conflicts of interest to declare.

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