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Impact of Bio-fertilizers under Supplementary Irrigation and Rain-fed Conditions on Some Physiological Responses and Forage Quality of Smooth Vetch (*Vicia dasycarpa L.*)

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ABSTRACT

In arid and semi-arid regions, water scarcity and declining soil fertility limit the supply of livestock forage. This study evaluates the use of bio-fertilizers to adjustment water shortage stress and improved smooth vetch (*Vicia dasycarpa* L.) yield under water deficit stress conditions. A 2-year experiment was performed in an agrisilviculture system of plum orchard in 2016 and 2017. In this study, the single, double and triple combined of Arbuscular mycorrhizal fungi (AMF)- *Rhizophagus intraradices*, *Azotobacter chroococcum* (*Az*) and *Thiobacillus* spp. (*Th*) on smooth vetch plants were evaluated under rain-fed and supplemental irrigation. The results indicated that irrigated plants had more Fe and Zn nutrients than rain-fed plants. In comparison with single inoculation, the combined use of AMF + *A. chroococcum* facilitated the forage dry matter digestibility, total digestible nutrient, net energy for lactation, dry matter intake and relative feed value. In irrigated, double and triple combination of AMF with *A. chroococcum* and/or *Th*. improved chlorophyll-a, chlorophyll-b, total chlorophyll, relative water content, total soluble sugar, ascorbic acid, and glutathione while lowering proline and malondialdehyde. The results show that synthesis non-enzymatic antioxidant because of the combined use of bio-fertilizers (AMF, *Az* and *Th*) can reduce reactive oxygen species damage and improve water deficit resistance and yield in smooth vetch rain-fed plants.

Keywords: Chlorophyll, Irrigation, Non-enzymatic antioxidants, Osmolytes adjustment, Yield

1. Introduction

Smooth vetch (*Vicia dasycarpa* L.) is an important annual legume forage plant due to its multiple uses (seed, forage, silage, and green manure), its high nutritional value, and its potential to grow in a wide variety of climatic and soil conditions. Furthermore, vetch plants by fixing atmospheric nitrogen increase soil fertility (Haffani et al. 2017). Smooth vetch can be used in integrated systems such as agroforestry. Agroforestry systems are a sustainable and integrated system that, via some processes such as erosion control, improving water cycle, soil organic matter and nutrient, will lead to soil fertility and conservation improvements that help improve the inhabitant's livelihood (Heydarzadeh et al. 2022). So, in correct management of land-use, it is possible to achieve sustainable development in agriculture (Heydarzadeh et al. 2022).

In arid and semi-arid environments, water is the most limiting factor in reducing agricultural forage production (Saadat et al. 2019). In rain-fed farming, a plant's response to water deficit stress is complicated because of the varying frequency of dry and wet periods, the patterns of soil and atmospheric water deficits, and the degree and timing of drought (Balazadeh et al. 2021). Indeed, global warming, drought, and climate change require new strategies to increase forage cultivation (Tan & Yolcu 2021). Smooth vetch is a rain-fed crop and is moderately drought tolerant, but its yield is radically reduced with increasing drought conditions (Haffani et al. 2017). Consequently, supplemental irrigation is an effective measure that complements crop production and improves the living conditions of a region (Saadat et al. 2021). Under water deficit stress, plants usually respond by osmotic adjustment, stomata regulation, and antioxidant defense to reduce stress-related damages (Habibzadeh et al. 2015). Osmotic adjustment, is defined as a procedure of solute

accumulation in dividing cells when the water potential is decreased, and thereby enables the preservation of the cells turgor. This is considered a kind of adaptation to water scarcity in order to limit the damage of water deficit stress (Rahimzadeh & Pirzad 2017).

Sustainable agricultural systems involve the knowledge of interactions between crops and microorganisms, particularly those that have a direct effect on crop development and stress tolerance (Rahimzadeh & Pirzad 2017). Thus, in agricultural systems, the application of microorganism inoculants is of strategic interest in order to reduce chemical fertilizer usage, and improve environmental sustainability (Saadat et al. 2021). Microorganisms that assist plant growth often include arbuscular mycorrhizal fungi (AMF) and plant growth-promoting rhizobacteria such as *Azotobacter* and *Thiobacillus* spp. (*Th*) that enhance growth by increasing the access to mineral nutrition, drought tolerance, and disease resistance (Heydari & Pirzad 2020; Heydarzadeh et al. 2022). For mycorrhiza and *Azotobacter*, several mechanisms have been proposed to improve water deficit tolerance in plants, the accumulation of osmolytes and antioxidant defense (Mohammadi et al. 2019). Produce various siderophores, plant growth hormones, antifungal, antibiotics and amino acids are the other beneficial of biofertilizers (Heydarzadeh et al. 2022). In fact, mycorrhizal fungi and their coexistence with plants alter water absorption and so improve drought resistance in the host plant (Habibzadeh et al. 2015). It clear that biofertilizers protects plants against the damaging impacts of reactive oxygen species (ROS) produced by water deficit stress. Therefore, biofertilizers improved non-enzymatic antioxidants (mainly proline, soluble carbohydrates, GSH, and AsA) and reduced water stress damage (Sohrabi et al. 2012a; Sohrabi et al. 2012b). The effect of combined inoculation of *Th* and AMF has been stated on dragon's head (Heydari & Pirzad 2020), as well as, the combined use of *Azotobacter* and mycorrhiza on lentils (Amirnia et al. 2019). These effects may be varied in terms of soil water deficit stress. Moreover, the use combined of bio-fertilizers has been proposed as an effective way to increase plant growth, development and production under rain-fed conditions. For this reason, this study assesses the impact of bacteria (*Az* and *Th*) and fungi (AMF) under supplementary irrigation and rain-fed conditions on some physiological responses and the forage quality of smooth vetch in an agrisilviculture system.

2. Material and Methods

In the plum [*Prunus domestica* (L.), Opal] orchard in Urmia, west Azerbaijan, Iran (37°39′24.82″N latitude, 44°58′12.42″ E longitude, 1338 m elevation), these two-years (2016 and 2017) field experiment were conducted based on a randomized complete block design with three replications. The single, double and triple combined of *Rhizophagus intraradices* (AMF), *Azotobacter chroococcum* (*Az*) and *Th*, AMF + *Az*, AMF + *Th*, *Az* + *Th*, AMF + *Az* + *Th* were used as inoculation for smooth vetch seeds, under rain-fed and supplemental irrigation.

Monthly rainfall and air temperature as an average are shown in Table 1.

Table 1- The average monthly temperature and rainfall in the growing seasons of 2016 and 2017 compared to the average of the period from 1987 to 2017 in Urmia

Month	*Jan.*	*Feb.*	*Mar.*	*Apr.*	*May*	*June*	*Jul.*	*Aug.*	*Sep.*	*Oct.*	*Nov.*	*Des.*	*Average/ total*
2015-2016													
Temperature (°C)	-0.4	3.8	7.4	12.2	15.4	20.9	24	22.8	19	11.2	7.3	-5.9	11.47
Rainfall (mm)	44	9.2	19.3	32.9	45.1	6	0	0.1	0	9.2	62	41.8	269.5
2016-2017													
Temperature (°C)	-5.2	-4.3	2.73	9.31	15.9	21.21	25	26	22.87	13.59	7.55	-1.23	11.15
Rainfall (mm)	10.6	43	9.3	69.8	22.3	0.8	0	0.7	0	0	28.2	55.33	240.4
Long term 1987-2017													
Temperature (°C)	-2.1	0.2	5.22	11.26	15.81	20.84	24	23.32	18.9	6.12	5.77	0.35	11.33
Rainfall (mm)	25.4	28.	46.7	54.7	37.1	10	5.6	2.8	4.3	30.5	39.4	28.1	312.8

The mycorrhiza inoculum was composed of sterile sand, mycorrhizal hyphae and spores (10 spores' g^{-1} inoculum) and colonized root fragments of corn crops (*Zea mays* L.) below the seeds. During the sowing time only in the rows below the seeds, 12 g m^{-2} of mycorrhiza inoculum was added to the plots. Prior to sowing, the seeds were inoculated with 10^8 CFU mL^{-1} (Colony-Forming Unit per mL) bacterial population of *A. chroococcum* and *Th*. at a rate of 2 L ha^{-1} in shadow (Wani & Gopalakrishnan 2019).

The following soil physicochemical characteristics were measured: the hydrometer method was used to determine the soil texture (clay, silt 23%, clay 42%, sand 35%) (Day 1965). Soil reaction (pH) was 7.95 that measured by using a pH meter described by Carter and Gregorich (2007), and electrical conductivity (EC) was 0.52 dS m^{-1} that determined in 1:2.5 soil-water suspension (Okalebo et al. 2002). Field capacity (FC=30%) and permanent wilting point (PWP=16%) were determined at soil water potentials of -0.33 bar and -15 bar, respectively, by pressure plates (Richards 1965). Organic matter was 1.35% (multiplying the percent organic carbon value by 1.724) that determined by method as described by Walkley and Black (1934), Soil nitrogen was 1.35 % that analyzed by the Kjeldahl method (Bremner 1996). The available P and K were 10.52 and 488 mg kg^{-1} that determined by the standard Olsen method (Olsen 1954) and flame photometer as described by Rowell (2014), respectively.

The Maragheh Dryland Agricultural Research Institute (MDARI) provided the smooth vetch seeds. On 8th March 2016 and 10th March 2017, the seeds were planted at a depth of 8 cm in plots of 150×200 cm in size with plant spacing between the row and plant (20×2 cm).

To determine the photosynthetic pigments, relative water content (RWC), osmolyte content, ROS, and antioxidants fresh leaves were sampled at the end of the flowering stage (87 and 89 days after sowing in the first and second year, respectively). Fresh leaf samples were held with aluminum foil, frozen in liquid nitrogen, and placed in plastic packets at -80 °C before being stored. Supplemental irrigation was applied at the 10% of flowering stage in both years. The plants were harvested on the 25th June 2016 and 27th June 2017. The water that supply by irrigation were 580 m^3 ha^{-1} in 2016 and 620 m^3 ha^{-1} in 2017 (Richards 1965).

To determine forage quality indices, a Near Infra-Red Spectroscopy (NIR, Inframatic 8600 Perten instruments- with wavelengths ranging from 500 to 2400 nm) was used and calibrated based on Jafari et al. (2003) method. At the end of flowering stage, 100 grams of dried forage (samples were dried in an oven at 68 °C for 72 h) from each plot was ground in order to determine the forage quality. Dry matter digestibility (DMD), acid detergent fiber (ADF), neutral detergent fiber (NDF), total digestible nutrients (TDN), dry matter intake (DMI), net energy for lactation (NEL) and relative feed value (RFV) were the forage quality indices that were measured using NIR (Horrocks & Valentine 1999; Jafari et al. 2003).

To determine the Fe and Zn content, the dried grains of smooth vetch, were milled, and underwent combustion (4 h at 500 °C), after which the grain ashes (5 mg) were digested in 1 mL of 2 N HCl, and the extracts acquired filtered (Whatman filter paper: grade 42).

The Fe and Zn was determined in dry digestion extract using an atomic absorption (2380 Perkin Elmer); they were measured in mg L^{-1}, and expressed as mg kg^{-1} (Houba et al. 1988). The content of chlorophyll-a and b were determined by extracting the fresh leaves using 80% acetone using a spectrophotometer at wavelengths of 646.8 and 663.2 nm, respectively (Lichtenthaler & Wellburn 1983). In addition, the relative content of leaves water (RWC) was measured according to the following equation (Saadat et al. 2021).

$$\% \text{ RWC} = [(\text{fresh weight-dry weight})/(\text{turgid weight-dry weight})] \times 100 \quad \text{(Eq. 1)}$$

For measuring turgid, the leaves were soaking for 16 to 18 h in distilled water, after which the leaves were carefully and quickly blotted dry with tissue paper. The leaf dry weight was determined after drying the leaf sample for 72 h at 70 °C.

The fresh leaf (0.5 *g*) was used to evaluate the concentration of proline by the ninhydrin method (Bates et al. 1973). The absorbance was evaluated at 520 nm using a Spectronic 20 colorimeter (SP 6-200 Unican). A calibration curve was used to determine the concentration of proline. Leaf total soluble sugars (TSS) were determined based on the phenol sulfuric acid method (Dubois et al. 1956). In this method, 0.5 g of fresh leaves were homogenized with ethanol. The extract was filtered and treated with 5% phenol and 98% sulfuric acid. This mixture was left for 1 h and its absorption was measured by spectrophotometer at 485 nm. Malondialdehyde (MDA) was evaluated with minor changes according to the thiobarbituric acid (TBA) reaction (Zhou et al. 2004). One mL of extract was mixed with 0.5% TBA in 2.5 mL and then heated for 20 min at 100 °C. The spectrophotometric absorbance of the supernatant was calculated at 532, 600 and 450 nm (A532, A600 and A450) and the concentration of MDA was calculated using the following formula (Eq. 2):

$$6.45 \times (A532 - A600) - 0.56 \times A450 \quad \text{(Eq. 2)}$$

The ascorbic acid (AsA) measurement was based on creating a purple complex between ferrous ion and bipyridyl generated at 525 nm (reduction of ferric to ferrous ion with ascorbate in acid solution). The AsA content was determined using 0.2 *g* of fresh leaves (Li et al. 2015). The AsA standards were prepared in the range of 0-15 mg L^{-1} in 1 mL of 5% (w/v) trichloroacetic acid (TCA), 1 mL of alcohol, 0.5 mL of 0.4% H_3PO_4 alcohol, 1 mL of 0.5% bathophenanthroline alcohol and 0.5 mL of 0.03% $FeCl_3$ alcohol for 90 min at 30 °C and determined at 534 nm.

The fresh leaves (0.2 *g*) were mixed with 4 mL of 5 mM EDTA-TCA to determine glutathione (GSH) concentration, which was estimated using 5,5′-dithio-bis (2 nitrobenzoic acids) (Khan et al. 2014). Changes in reaction mixture absorbance were reported at 420 nm and GSH concentration was determined from a standard GSH curve. After the flowering stage, 10 plants per plot were randomly harvested from a depth of 10-30 cm. After washing fresh root samples, about 1 *g* of roots were transmitted to the laboratory and cleaned with 10% KOH, marked with 0.05% trypan blue in lactic acid (Phillips & Hayman 1970), and the proportion of root colonization was calculated using the gridline intercept method (McGonigle et al. 1990).

An analysis of variance for the two-year results was performed using the generalized linear model (SAS 9.1.3) combined over the two years. The effects of rain-fed and supplementary irrigation, the application of biofertilizers and the interactions between these two variables were evaluated by ANOVA and distinctions between means were compared using Duncan's multiple range test at $p \leq 0.05$.

3. Results and Discussion

The combined analysis of the 2-year data for the ADF, NDF, DMD, TDN, DMI, RFV and NEL of smooth vetch indicated that the simple effects of year, irrigation conditions and biofertilizers were significant (Table 2). The interaction effect of "irrigation conditions × biofertilizer" was significant on the Fe and Zn of smooth vetch grains (Table 2). According to the combined ANOVA of 2-years, the simple effects of irrigation conditions and biofertilizers on chlorophyll-a, chlorophyll-b and total chlorophyll were significant (Table 2). The interaction effect of "irrigation conditions × biofertilizers" and year had a significant effect on proline, MDA, RWC, GSH and seed yield (Table 2). In addition, the TSS, AsA and root colonization were affected by the interaction of "irrigation conditions × biofertilizer" (Table 2).

Table 2. Variance analysis of *V. dasycarpa* forage quality traits and some physiological traits as affected by irrigation and biofertilizers

Source of variation	***df***	***ADF***	***NDF***	***DMD***	***TDN***	***DMI***	***RFV***	***NEL***	***Fe***	***Zn***	***Chl-a***
Year (Y)	1	124.32**	108.16**	75.38**	207.15**	0.61**	3569.59**	0.08**	3.51^{ns}	4.13^{ns}	0.0008ns
Repeat /Y	4	1.04	0.08	0.63	1.74	0.0009	12.92	0.0007	63.91	3.64	0.001
Irrigation conditions (IC)	1	245.15**	284.48**	148.72**	408.37**	1.62**	8542.26**	0.16**	41293.34**	2990.76**	0.85**
Y × IC	1	0.05^{ns}	0.36^{ns}	0.03^{ns}	0.08^{ns}	0.0006^{ns}	9.03^{ns}	0.00008^{ns}	18.69^{ns}	9.33^{ns}	0.001ns
Biofertilizer (Biof)	7	36.69**	55.33**	22.29**	61.17**	0.29**	1418.25**	0.02**	6924.18**	244.10**	0.51**
Y × Biof	7	0.001^{ns}	0.01^{ns}	0.001^{ns}	0.003^{ns}	0.001^{ns}	6.28^{ns}	0.000003^{ns}	21.76^{ns}	0.62^{ns}	0.01ns
IC × Biof	7	0.08^{ns}	0.01^{ns}	0.05^{ns}	0.14^{ns}	0.002^{ns}	16.69^{ns}	0.00005^{ns}	488.38**	11.43*	0.001ns
Y × IC × Biof	7	0.09^{ns}	0.01^{ns}	0.05^{ns}	0.15^{ns}	0.00009^{ns}	0.79^{ns}	0.00006^{ns}	5.13^{ns}	0.22^{ns}	0.001ns
Error	60	0.11	0.28	0.07	0.19	0.002	7.89	0.00008	20.14	4.73	0.007
Coefficient of variation (%)		1.08	1.32	0.41	0.72	1.49	1.86	0.61	2.89	5.97	3.88
Source of variation	***df***	***Chl-b***	***Chl a+b***	***Proline***	***MDA***	***RWC***	***TSS***	***GSH***	***AsA***	***RC***	***GY***
Year (Y)	1	0.02^{ns}	0.03^{ns}	0.39**	51.84**	159.16**	0.24^{ns}	0.21**	149.37^{ns}	27.53 ns	362070.72**
Repeat /Y	4	0.05	0.04	0.003	0.46	1.47	0.50	0.02	26.78	51.67 ns	9879.19
Irrigation conditions (IC)	1	0.32**	2.26**	4.78**	622.60**	1908.79**	593.16**	3.96**	18903.74**	1118.30**	12838366.91**
Y × IC	1	0.0003^{ns}	0.002^{ns}	0.001^{ns}	0.28^{ns}	0.47^{ns}	0.03^{ns}	0.003^{ns}	1.06^{ns}	0.23 ns	18790.88ns
Biofertilizer (Biof)	7	0.62**	2.23**	0.58**	63.89**	231.23**	76.32**	0.45**	1011.51**	2987.73 **	610997.21**
Y × Biof	7	0.01^{ns}	0.02^{ns}	0.01^{ns}	1.46^{ns}	5.21^{ns}	0.21^{ns}	0.004^{ns}	18.40^{ns}	1.59 ns	633.13ns
IC × Biof	7	0.02^{ns}	0.03^{ns}	0.03*	5.69**	13.98*	7.80**	0.04**	206.71**	100.83 **	163496.57**
Y × IC × Biof	7	0.0009^{ns}	0.003^{ns}	0.004^{ns}	0.56^{ns}	1.80^{ns}	0.28^{ns}	0.002^{ns}	9.27^{ns}	0.003 ns	132.85ns
Error	60	0.02	0.03	0.01	1.54	4.90	0.48	0.014	38.40	32.52	11473.15
Coefficient of variation (%)		13.25	5.76	3.14	7.85	3.47	4.44	9.77	8.81	16.55	9.99

ADF: Acid detergent fiber, NDF: Neutral detergent fiber, DMD: Dry matter digestibility, TDN: Total digestible nutrient, DMI: Dry matter intake, NEL: Net energy for lactation, RFV: Relative feed value. Chl- a: Chlorophyll-a; Chl-b: Chlorophyll-b; Chl a+b: Total Chlorophyll; MDA: Malondialdehyde; RWC: Relative water content; TSS: Total soluble sugar; GSH: Glutathione; AsA: Ascorbic Acid; RC: Root colonization; GY: Grain yield. *,** and ns, significant at 5% and 1% levels of probability, non-significant, respectively

Rain-fed smooth vetch contained the highest of ADF and NDF in forage (Table 3). Double and triple combination use of biofertilizers (AMF, *Az* and *Th*) reduce the smooth vetch ADF and NDF by 17% and 14% compared with plants inoculated with only AMF or bacteria (Table 3). The average ADF and NDF of smooth vetch plants were 30.13% and 39.01% in 2016 and 32.41% and 41.13% in 2017, respectively (Table 3). The mean comparison revealed that in rain-fed conditions, ADF and NDF significantly increased, but irrigated plants had low ADF and NDF content (Table 3). Insoluble fibers increase in cell walls is one of the physiological responses of plants for avoid moisture loss under water deficit stress (Jahanzad et al. 2013). The application of supplementary irrigation seems to slow this process and prevent a remarkable increase in crude fiber growth rate (Jahansouz et al. 2014).

Table 3- Means (± standard deviation) comparison of the effect of year, irrigation conditions and biofertilizer on some *V. dasycarpa* forage quality traits

Treatments	*ADF (%)*	*NDF (%)*	*DMD (%)*	*TDN (%)*	*DMI (%)*	*RFV (%)*	*NEL (%)*
Year							
2016	30.13±2.37^{b}	39.01±2.69^{b}	65.42±1.85^{a}	62.44±3.06^{a}	3.08±0.21^{a}	156.95±15.02^{a}	1.51±0.05^{a}
2017	32.41±2.31^{a}	41.13±2.72^{a}	63.64±1.80^{b}	59.50±2.98^{b}	2.92±0.19^{b}	144.75±13.50^{b}	1.45±0.06^{b}
Irrigation							
Rain-fed	32.87±1.99^{a}	41.79±2.34^{a}	63.29±1.55^{b}	58.90±2.56^{b}	2.87±0.16^{b}	141.42±11.09^{b}	1.43±0.06^{b}
Supplemental irrigation	29.67±2.11^{b}	38.35±2.33^{b}	65.78±1.64^{a}	63.04±2.72^{a}	3.13±0.19^{a}	160.28±13.39^{a}	1.52±0.06^{a}
Biofertilizer							
C	33.83±2.08^{a}	43.80±2.28^{a}	62.53±1.62^{g}	57.66±2.68^{g}	2.74±0.14^{g}	133.18±10.38^{g}	1.41±0.05^{g}
AMF	31.65±2.11^{c}	39.39±2.42^{d}	64.21±1.65^{e}	60.48±2.73^{e}	3.05±0.18^{d}	152.24±12.89^{d}	1.47±0.06^{e}
Az	32.29±2.38^{b}	41.19±2.35^{c}	63.68±1.58^{f}	59.66±3.07^{f}	2.92±0.17^{e}	144.40±12.74^{e}	1.45±0.05^{f}
Th	32.36±2.03^{b}	42.35±2.11^{b}	63.74±1.85^{f}	59.56±2.62^{f}	2.83±0.14^{f}	140.32±10.44^{f}	1.45±0.05^{f}
AMF + *Az*	28.03±2.27^{g}	37.67±2.40^{g}	67.05±1.77^{a}	65.15±2.94^{a}	3.19±0.21^{a}	166.39±15.01^{a}	1.56±0.06^{a}
AMF + *Th*	29.95±2.24^{f}	38.33±2.08^{f}	65.56±1.74^{b}	62.68±2.89^{b}	3.13±0.19^{b}	159.78±13.72^{b}	1.51±0.06^{b}
Az + *Th*	31.29±2.16^{d}	38.97±2.25^{de}	64.52±1.68^{d}	60.94±2.78^{d}	3.08±0.18^{cd}	154.58±12.67^{c}	1.48±0.06^{d}
AMF + *Az* + *Th*	30.77±1.97^{e}	38.87±2.08^{e}	64.92±1.54^{c}	61.61±2.54^{c}	3.09±0.17^{c}	155.90±12.00^{c}	1.49±0.05^{c}

ADF: Acid detergent fiber; NDF: Neutral detergent fiber; DMD: Dry matter digestibility; TDN: Total digestible nutrient; DMI: Dry matter intake; NEL: Net energy for lactation; RFV: Relative feed value. Means with same letters in each column (for single effect of treatments) are not significantly different based on Duncan's multiple range test p≤0.05. C: Control; AMF: Arbuscular mycorrhizal fungi*; Az: Azotobacter chroococcum*; *Th: Thiobacillus* spp.

Combination use of AMF, with *A. chroococcum* and *Th* exhibited a notable impact on ADF and NDF compared to single use biofertilizers (Table 3). It appears that inoculation with microorganism increase plant cytokinin's, leaf growth and carbon allocation from other parts of the leaf, by delaying leaf senescence (Heydari & Pirzad 2020). Therefore, the mycorrhization plays an important role in maintaining the forage quality by decreasing smooth vetch ADF and NDF content, which improves digestibility. The content of insoluble fibers varies according to ecological conditions. Table 3 shows that 2017 had the highest content of insoluble fibers in neutral and acid detergents. In 2016, high rainfall may be the reason for the low ADF and NDF content in smooth vetch forage compared to 2017 (Table 1).

A means comparison revealed that the highest DMD (65.78%), TDN (63.04%), DMI (3.13%), RFV (160.28%) and NEL (1.52%) were obtained from irrigation (Table 3). When compared with the control treatment, the dual- and triple-use of bio-fertilizers improved forage DMD, TDN, DMI, RFV and NEL content (Table 3). The average amounts of DMD, TDN, DMI, RFV and NEL in the first year of the experiment were 65.42, 62.44, 3.08, 156.95, and 1.51% while in the second year they were 63.64, 59.50, 2.92, 144.75, and 1.45%, respectively (Table 3). Dual-inoculation with "AMF + *A. chroococcum*" increased DMD (Table 3). In contrast to ADF and NDF, forage DMD decreased significantly (Table 3). Due to the negative correlation of DMD with NDF and ADF, the significant decrease of NDF and ADF in inoculated plants, caused to more mineral absorption by the plants and increased DMD (Lithourgidis et al. 2006). It has been noted that the mycorrhizal fungus assists in phytohormone production, which stimulates nutrient absorption and photosynthesis process, which in turn increases DMD content (Saadat et al. 2019). TDN refers to nutrients available for livestock and are related to the ADF content in forages. In parallel to ADF increase, TDN content decreases, which decreases the quality of forage plants (Jahanzad et al. 2013). Water stress during the flowering stage may be due to the reduction in photosynthesis process and dry matter accumulation, which consequently decreases TDN in forage (Balazadeh et al. 2021; Jahansouz et al. 2014). It may be that the implemented biofertilizers, either single or combined, produced a significant increase in total TDN either in leaves or in branches of vetch due to the effect of biofertilizers on increasing nutrient uptake, which improved the growth and development of plants for better

components of TND. Thus, in combined fertilizer treatments and supplementary irrigation, due to reduced NDF content (Table 3), the content of DMI forage and consequently forage production increased.

Among the biofertilizer treatments, the combined treatment of AMF with *A. chroococcum* and/or *Th* had the greatest effect on the increase in RFV (Table 3). High contents of NDF and ADF in control plants led to low RFV forage. Therefore, the improvement of RFV forage is due to the increase in smooth vetch DMI and DMD by biofertilizer application. Because DMI and DMD have a negative correlation with forage NDF and ADF respectively, the RFV index can be used to estimate the intake and energy value of forages using DMD and DMI (Balazadeh et al. 2021; Lithourgidis et al. 2006). When the RFV value is higher than 151, the forage is considered prime (Horrocks & Valentine 1999). In our study, under supplementary irrigation, the RFV values corresponded to prime quality. So, the RFV of irrigated smooth vetch plants saw an increase when compared to rain-fed plants (Table 3).

The NEL is an indicator of the quantity of forage energy obtainable for maintenance and milk production after digestive and metabolic losses and it is shown to be inversely correlated with ADF (Charbonneau et al. 2006). A positive synergistic effect of microorganisms increases the growth, development and transfer of nutrients that results in the highest forage NEL in dual- and triple-inoculated plants and supplementary irrigation. Due to the higher ADF content of smooth vetch plants under rain-fed compared to supplementary irrigation (Table 3), an NEL reduction of forage is predictable. In both rain-fed and supplementary irrigation cultivation, the amount of iron (Fe) and zinc (Zn) in plants combined with biofertilizers increased significantly; however, the amount of Fe and Zn in irrigated plants were higher than in rain-fed plants. The highest amounts of Fe (224.67) and Zn (49.14 mg kg^{-1}) have been found in plants AMF + *A. chroococcum* irrigated plants (Figure 1A, B). Combined use of biofertilzers (AMF, *Az* and *Th*) led to improve the accumulation of Fe and Zn in both rain-fed and irrigated plants (Figure 1A, B). Therefore, given the lower Fe and Zn content of the smooth vetch grains under rain-fed situations, it can be concluded that nutrient uptake potential was low in rain-fed plants due to a rise in water deficit (Figure 1A, B). It has been reported that nutrient solubility reduces as soil moisture decreases (Pirzad & Mohammad Zadeh 2018). The combined use of biofertilizer in our study was effective in improving nutrient uptakes such as Fe and Zn (Figure 1A, B). The researchers found that the application of bio fertilization increases plant growth and development, nutrient absorption, and photosynthetic efficiency significantly due to the increased activity of alkaline phosphatase and acid phosphatase, improving soil EC, synthesizing organic acids, and changing the pH or secretion of enzymes (Heydari & Pirzad 2020).

The results showed that smooth vetch leaf in supplemental irrigation conditions had a higher chlorophyll-a (2.42 mg g^{-1} FW), chlorophyll-b (1.51 mg g^{-1} FW) and total chlorophyll content (3.93 mg g^{-1} FW) than rain-fed plants (Figure 2A-C). In addition, the content of chlorophyll-a, chlorophyll-b and total chlorophyll was higher in AMF, dual, and triple-colonized plants than in the control and other individual biofertilizer applications (Figure 2A-C).

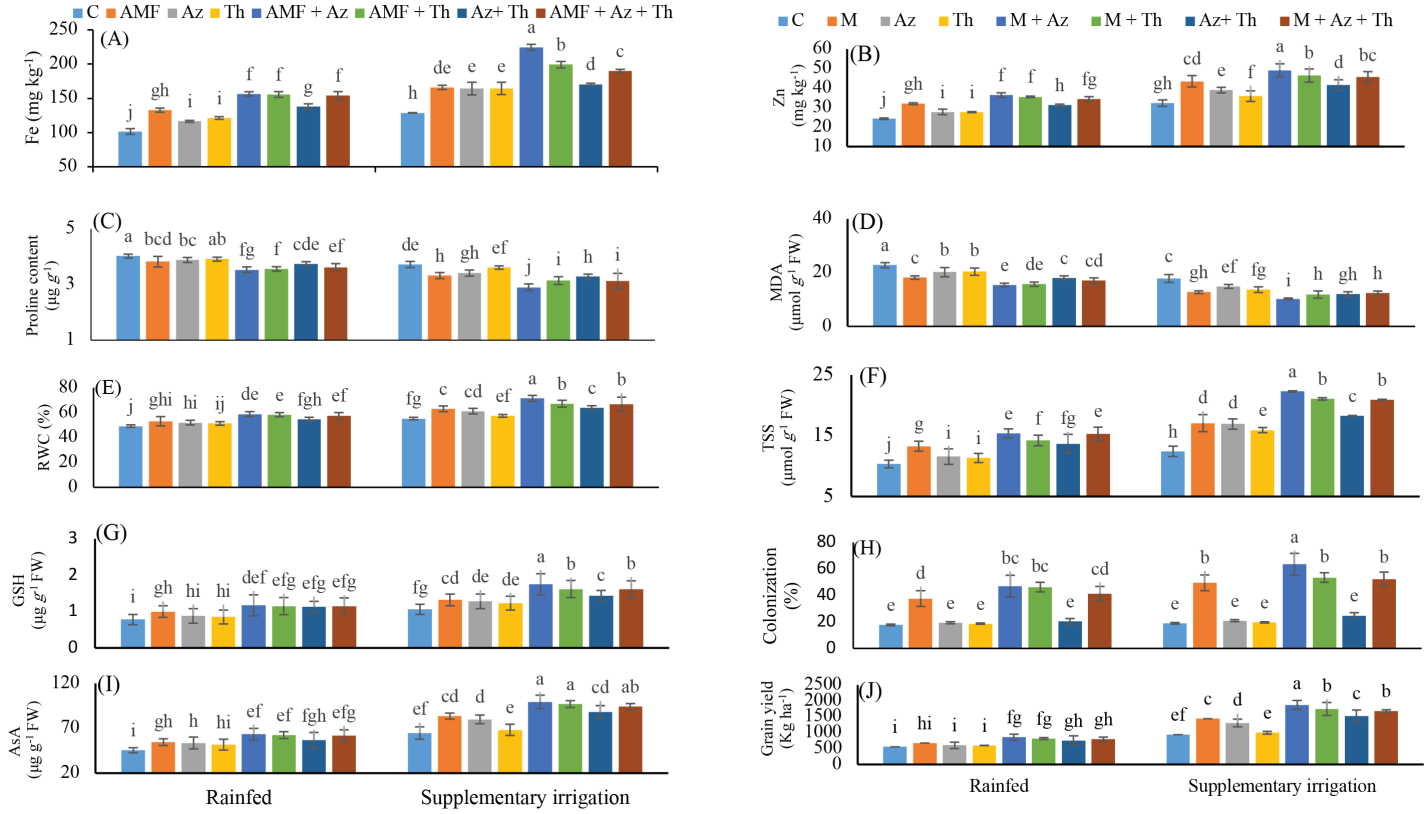

Figure 1- Mean (± standard deviation) comparison of smooth vetch Fe (A), Zn (B), proline (C), MDA (D), RWC (E), TSS (F), GSH (G), root colonization (H), AsA (I) and grain yield (J) as affected by "Irrigation conditions×Biofertilizer". Means with the same letters in each column are not significantly different based on Duncan's multiple range test p≤0.05. C: Control; AMF: Arbuscular mycorrhizal fungi; *Az: Azotobacter chroococcum*; *Th: Thiobacillus* spp

In our experiment, water deficit stress significantly reduced the leaf chlorophyll content (Figure 2 A-C).

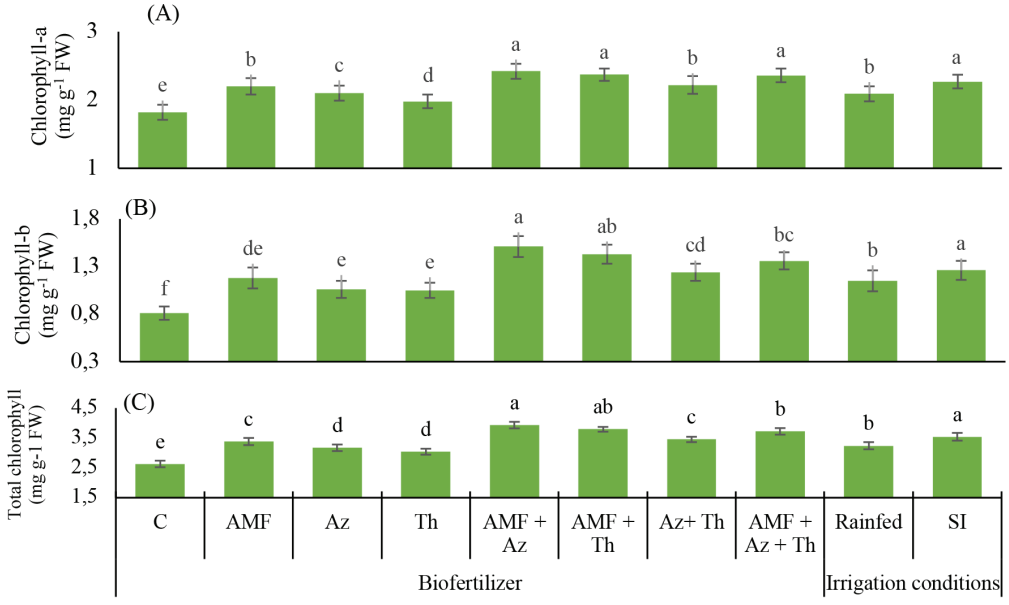

Figure 2- Mean (± standard deviation) comparison of smooth vetch chlorophyll-a (A), chlorophyll-b (B) and total chlorophyll (C) as affected by "Irrigation conditions and Biofertilizer". Means with same the letters in each column are not significantly different based on Duncan's multiple range test p≤0.05. C: Control; AMF: Arbuscular mycorrhizal fungi; *Az: Azotobacter chroococcum*; *Th: Thiobacillus* spp.; SI: Supplementary irrigation

Water is likely to be effective in maintaining active chloroplasts and subsequently performing chlorophyll tasks such as energy absorption and transfer (Saadat et al. 2021). The reduction in chlorophyll content in drought conditions may be due to the impact of water deficiency on the decomposition of chlorophylls and their peroxidation by active oxygen species (Sohrabi et al. 2012b), since the active species of oxygen destroy lipids, proteins and photosynthetic pigments. It has been attributed to a reduction in chlorophyll content in water deficiency conditions to a decreased stability of the chloroplast membrane and its breakdown (Sohrabi et al. 2012a). Under supplementary irrigation conditions, however, the increased chlorophyll content that we observed with the dual- and triple-combination of AMF + *A. chroococcum* and/or *Th* (Figure 2A-C) may have contributed to an increased rate of photosynthesis. Therefore, in explaining the reason for the superiority of the combination of biofertilizers, it can be stated that sufficient nitrogen uptake due to the presence of fungi and nitrogen stabilizing bacteria in biofertilizers caused the plant to have sufficient nitrogen to produce chlorophyll (chlorophyll content has a strong correlation with the amount of nitrogen) (Amirnia et al. 2019).

The concentration of proline and MDA in inoculated plants with combined treatments of AMF plants with *A. chroococcum* and/or *Th* in both rain-fed conditions and supplementary irrigation decreased significantly (Table 4). However, the concentration of proline and MDA was higher in rain-fed plants compared to supplementary irrigation. The lowest amounts of proline (2.9 μg g^{-1} FW) and MDA (10.27±0.28 μmol g^{-1} FW) were obtained in irrigated plants inoculated with "AMF + *A. chroococcum*" (Figure 1C, D). In addition, the concentration of leaf proline and MDA were significantly higher in 2017 than in 2016 (Table 4).

Table 4- Mean (± standard deviation) comparison of the effect of year for some *Vicia dasycarpa* L. traits

Year	*Proline (μg g^{-1} FW)*	*MDA (μmol g^{-1} FW)*	*RWC (%)*	*GSH (μg g^{-1} FW)*	*GY (Kg ha^{-1})*
2016	3.47±0.34[b]	15.10±0.93[b]	60.07±6.77[a]	1.26±0.09[a]	1133.03±88.22[a]
2017	3.60±0.31[a]	16.57±1.02[a]	57.49±6.22[b]	1.17±0.11[b]	1010.20±94.09[b]

MDA: Malondialdehyde, RWC: Relative water content, GSH: Glutathione, GY: Grain yield. Means with the same letters in each column are not significantly different based on Duncan's multiple range test p≤0.05

The plants inoculated with the dual- and triple-combination of "AMF + *A. chroococcum* and/or *Th*", under supplementary irrigation conditions, had the lowest amount of proline prolonged water-deficit stress tolerance (Figure 1C). The accumulation of proline in plants under water shortages has been attributed to various factors, such as the regulatory influence of ABA on light mechanisms (Pirzad & Mohammad Zadeh 2018), and photosynthetic compounds that enhance proline synthesis (Amirnia et al. 2019). It is believed that proline accumulation plays an important function in the stress tolerance of plants as an osmotic adjustment (Mohammadi et al. 2019). The dual- and triple-combined use of AMF, with *Azotobacter* and *Thiobacillus*, exhibited a notable impact on MDA and decrease in both rain-fed and supplemental irrigation conditions (Figure 1D). An increase in MDA, as the main product of lipid peroxidation in the cellular membrane, is associated with a water deficit which leads to excessive oxygen free radicals in the membrane system, so that the membrane lipid is oxidized (Rahimzadeh & Pirzad 2017). The accumulation of MDA may cause damage to the membrane and cells, and MDA level in the plant represents the degree of membrane damage (Mohammadi et al. 2019). However, in all irrigation conditions, the MDA in inoculated plants was lower than in non-inoculated plants, indicating that microorganisms may reduce membrane lipid peroxidation, due to enhanced antioxidant scavenging of ROS (Sohrabi et al. 2012b). AMF + *A. chroococcum* irrigated plants had the highest RWC (71.42%), TSS (22.29 μmol g^{-1} FW), GSH (1.75 μg g^{-1} FW) and AsA (99.34 μg g^{-1} FW) (Figure 1). The combined use of biofertilizers in both irrigated and rain-fed increased RWC, TSS, GSH and AsA than the use of single biofertilizers (Figure 1). In addition, more RWC and GSH were obtained in the first year (Table 4). We found that dual- and triple-inoculation plants of AMF with *A. chroococcum* and/or *Th* had higher RWC, which benefited photosynthetic efficiency (Figure 1E). Reduced growth and root activity, as well as increased evapotranspiration from the plant community, are known to be important factors in RWC reduction (Zhou et al. 2004). It has been stated that the leaf RWC of lentils diminishes as water stress increases (Amirnia et al. 2019). A decrease in the turgor of plant tissues and leaf RWC could be the first effect of water deficit stress, which can have a natural impact on cell growth and size. Biofertilizers enhance water uptake in the host plant by altering root development and spreading the plant's root system (Sohrabi et al. 2012b). Indeed, the use of mycorrhizal fungi and bacteria appears to mitigate the adverse effects of water deficit on plants by increasing leaf water potential, transpiration rate, photosynthetic efficiency, and the rate of CO_2 use in host plants, as well as increasing nutrient absorption, thereby enhancing growth and plant production (Fouad et al. 2014).

When smooth vetch was subjected to rain-fed conditions, the TSS concentration decreased (Figure 1F). It has been reported that limited irrigation causes a reduction in the TSS concentration due to reduced photosynthesis and stomatal closure (Rahimzadeh & Pirzad 2017). Although damage to cell membranes by water deficit stress likely restricts osmotic adjustment, increased leaf water content during water deficit stress may inhibit the formation of osmolytes, such as TSSs (Amirnia et al. 2019).

The higher TSS concentration in biofertilizer-treated plants in both supplementary irrigation and rain-fed conditions (Figure 1F) could be explained by the observed synergic effect of dual- and triple-inoculation on vegetative growth in smooth vetch is likely to be associated with the increased level of photosynthesis induced by these treatments (Fouad et al. 2014). The application of mycorrhizal fungi and bacteria likely improved growth and led to a higher concentration of TSS by supplying water and nutrients.

The decrease in GSH and AsA concentration in rain-fed plants, resulted in enhanced lipid peroxidation (Sohrabi et al. 2012a). To protect the antioxidant system that protects plants from oxidative destruction owing to drought stress, an excessive level of inhibitory AsA is significantly more efficient (Mohammadi et al. 2019). Both AMF and bacteria inoculation significantly increased GSH and AsA concentration compared to the control treatment (Figure 1G, I). It seems that inoculation AMF plants with *A. chroococcum* and/or *Th* have enhanced the accumulation of AsA and GSH as protective compounds to cope with the detrimental effects of water deficiency stress.

Root colonization was the minimum in the rain-fed and irrigated control plants. An increase in root colonization was only observed in the AMF plants. The AMF + *A. chroococcum* irrigated plants had the highest root colonization (63.45%). The combined use of AMF with other biofertilizers improved fungal root colonization in rain-fed plants (Figure 1H). The growth and root colonization decrease under water stress may be caused by changes in the hyphae's morphological features or by reduced spore development and density (Saadat et al. 2021). In addition, water deficit hurts spore germination, hyphal growth, and proliferation in soil, resulting in a reduction in mycorrhizal colonization, suppression of arbuscular formation, and reduced photosynthetic availability, all of which indirectly causes a decrease in colonization under water deficit stress (Heydari & Pirzad 2020). The highest AMF colonization was determined in dual- and triple-inoculation of AMF with *A. chroococcum* and/or *Th* inoculated plants under rain-fed and supplementary irrigation (Figure 1H), The effect of microorganisms on increasing water stress tolerance procedures appears to be connected more to the roots colonized as previously mentioned (Habibzadeh et al. 2015).

The AMF + *A. chroococcum* irrigated plants had the highest grain yield (1873.29 kg ha^{-1}). Grain yield in both rain-fed and irrigated plants demonstrated an effective increase in inoculated plants with dual and triple biofertilizers than in plants only inoculated with AMF or bacteria. The lowest amount of grain yield (555.05 kg ha^{-1}) was obtained under rain-fed conditions and non-inoculated control plants. However, the individual application of biofertilizers had no significant difference when compared to the control in rain-fed conditions (Figure 1J). Furthermore, an increase by 11% in smooth vetch grain yield was observed in the first year compared to the second year (Table 4). The co-inoculation of AMF + *A. chroococcum* enhanced seed yield by 35% and 49 %, respectively, when compared with the control plants (Figure 1J). A reduced grain yield in rain-fed conditions when compared to supplemental irrigation may have arisen from the decrease of pure photosynthesis and nutrients transmitted from leaves to seeds (Pirzad & Mohammad Zadeh 2018). The results show that in combination biofertilizer treatments, due to the mutual interaction of bacteria-fungi, biological nitrogen fixation, increased solubility of non-mobile phosphate, and the production of various plant growth stimulants, stimulate nutrient absorption and, by impacts on photosynthesis processes, improve seed yield components and eventually increase grain yield (Amirnia et al. 2019; Heydari & Pirzad 2021).

4. Conclusions

This study shows that supplemental irrigation improved the forage DMD, TDN, NEL, DMI and RFV content, while higher ADF and NDF, were achieved under rain-fed conditions. Double and triple use of AMF with *A. chroococcum and/or Th* were more efficient for improving DMD, TDN, NEL, DMI and RFV content than the control plants. In both rain-fed and irrigated plants, Fe and Zn accumulated in greater quantities in dual and triple biofertilizers treatments, compared with singly inoculated plants. Dual- and triple-use of AMF with *A. chroococcum* and/or *Th* This resulted in an increase in chlorophyll, RWC, TSS, AsA, and GSH in irrigated plants and reducing proline and MDA. Moreover, in inoculated plants with biofertilizers, the tolerance through boosting non-enzymatic antioxidant synthesis increased, which protects against ROS under rain-fed conditions. Overall, the combined use of AMF + *A. chroococcum* under supplemental irrigation conditions enhanced smooth vetch fodder quality and several physiological characteristics in rain-fed conditions by reducing the adverse effects of ROS production and increasing grain yields.

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