

Amelioration of water deficiency stress in roselle (*Hibiscus sabdariffa*) by arbuscular mycorrhizal fungi and plant growth-promoting rhizobacteria

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Abstract

Belowground interactions between plant roots, arbuscular mycorrhizal fungi (AMFs), and plant growth-promoting rhizobacteria (PGPR) can improve growth and yield under abiotic stress conditions. A pot factorial experiment based on completely randomized design with three replications was conducted to investigate the effects of AMFs (without inoculation as control, inoculation with *Funneliformis mosseae* and *Funneliformis intraradices*) and PGPRs (without inoculation as control *Pseudomonas fluorescens* p-169 inoculation) on roselle (*Hibiscus sabdariffa* L.) grown under water deficiency stress (WDS) [90% (I1), 75% (I2), 50% (I3), and 25% (I4) of field capacity as well-watered, mild, moderate, and severe stress, respectively]. The results showed that by applying WDS, the plant growth properties such as root and sepals' dry weight, 1000-seed weight, seed yield, chlorophyll a, b, and total, carotenoids, and leaf water content was significantly reduced. The application of AMFs and PGPR under WDS conditions increased 1000-seed weight, seed yield. In response to WDS osmotic adjustment were provided in Roselle and under stress conditions. The highest seed yield was found under well-watered treatment by inoculation of *F. mosseae* without PGPR and the application of *Pseudomonas fluorescens* (6.37 and 6.51 g/plant, respectively). These results suggesting the antagonistic effects of AMFs and PGPR. AMFs inoculation under severe stress increased sepals dry weight compared to the non-inoculation. In conclusion, increased activity of enzymatic antioxidants and higher production of non-enzymatic antioxidant compounds, as well as photosynthetic pigments in symbiotic association with AMFs, can alleviate reactive oxygen species damage resulting in increased growth and yield parameters and improve water stress tolerance.

Keywords: antioxidant enzyme activities; limited irrigation; osmotic adjustment; photosynthetic pigments; proline content; root colonization; Roselle; sepals' dry weight

Abbreviations: AMFs, arbuscular mycorrhizal fungi; PGPR, plant growth-promoting rhizobacteria; WDS, water deficiency stress; CAT, catalase activity; POD, peroxidase activity; ROS, reactive oxygen species; RWC, relative water content; Chl, chlorophyll content

Introduction

Roselle or Mackay tea (*Hibiscus sabdariffa* L.) belonging to Malvaceae family is an annual industrial and medicinal herb, that widely grown in many countries. This crop probably originated from West Africa or India but is currently grown in many regions of the world, such as parts of Asia, Central America, and Australia (Fallahi *et al.*, 2017). In Iran, roselle is mainly produced in Sistan and Balouchestan province on about 300 ha with a mean dry calyx yield of 700-900 kg/ha (Fallahi *et al.*, 2017). This plant is often used in traditional medicine being rich in phytochemicals like polyphenols especially anthocyanins, polysaccharides, and organic acids thus having enormous prospective in modern therapeutic uses (Riaz and Chopra, 2018). Traditionally, roselle is cultivated for its stem, leaves, calyces, and seeds as all parts have industrial, medicinal and other applications. Roselle fruits (calyx) are containing many essential nutrients such as vitamin A, vitamin C, minerals, polysaccharide, pectin, β -carotene, anthocyanin, and dietary fiber, also contains alkaloids, ascorbic acid, anisaldehyde, β -sitosterol, citric-acid, cyanidin-3-rutinoside, delphinidin, galactose, gossypetin, hibiscetin, mucopolysaccharide, protocatechuic acid, quercetin, stearic acid and wax (Ahmad *et al.*, 2010; Fallahi *et al.*, 2017). The plant products (*viz.*, calyx, leaves, oil extracted from seeds) are also reported to be antiseptic, aphrodisiac, astringent, cholagogue, demulcent, digestive, purgative, and resolving. Besides, it is used as a folk remedy in the treatment of abscesses, bilious conditions, cancer, cough, debility, dyspepsia, fever, hangover, heart ailments, hypertension, and neurosis (Da-Costa-Rocha *et al.*, 2014).

Water deficiency stress (WDS) is likely to become more frequent and intense as a result of global climate change, which may severely impact on growth and yield of agricultural, horticultural, and medicinal plants especially in arid and semi-arid parts of the world (Qiao *et al.*, 2020). Hence, due to limited irrigation sources, employing sustainable irrigation methods and systems, including deficit irrigation systems that save water and secure yield, are vital (Amiri *et al.*, 2016). The drought and osmotic stress-induced damage in plants are mainly due to the water shortage or the imbalance of plant water absorption and loss. Therefore, maintaining plant water balance by reducing water loss or increasing root water absorption is an essential way of improving plant tolerance to WDS (Gleason, 2015). In the case of WDS, plants cope with stress by producing and storing osmolytes such as amino acids, sugars, certain minerals, hormones, and proteins. Among organic compositions, amino acid-like proline is one of the most important osmosis regulators that accumulated in all organs of a plant, it has the fastest accumulation in leaves (Sharma *et al.*, 2019).

Water deficiency stress leads to a series of modifications in physiological attributes of a plant such as antioxidant enzymes activity, photosynthesis, photorespiration, and synthesis of amino acid, protein, and carbohydrate (Ahanger *et al.*, 2017). It is generally accepted that stress-induced deregulation of plant metabolism leads to the enhanced production of reactive oxygen species (ROS). To avoid cellular damage due to ROS generation, plants produce some antioxidant enzymes which induce and provide secondary protection against oxidative stress. ROS are generated from abiotic stresses such as salt stress that must be scavenged for the security of essential macromolecules from the destructive effects of ROS and maintenance of normal growth. Moreover, it is the other enzyme that converts two molecules of H_2O_2 to water and oxygen. Changes in activities of different antioxidant enzymes under stress conditions were reported in corn (Carrasco-Ríos and Pinto, 2014), stevia (Aghighi Shahverdi *et al.*, 2019), and *Brassica oleracea* (Soengas *et al.*, 2018). These reports show that the induction of ROS-scavenging enzymes, such as SOD, POD, and CAT, is the most general defence mechanism of WDS or salinity tolerance for detoxifying ROS (Ghahremani *et al.*, 2019).

Under WDS conditions, high fertilizer input does not always guarantee the highest levels of quality and quantity traits of plants (Sbrana *et al.*, 2014). Microorganisms, specifically arbuscular mycorrhizal fungi (AMFs), have already been shown to benefit the host plant through a symbiotic association with the roots (Amiri *et al.*, 2017). The probable mechanisms of increasing tolerance to drought in the plant include increasing hydraulic conductivity of roots, increasing water uptake under low moisture conditions due to the expansion of fungal roots, creating osmotic balance, maintaining Turgor pressure, increasing photosynthetic activity, aggregating carbohydrates and proline and increasing nutrient absorption (Rouphael *et al.*, 2015). The

mechanisms involved in enhancing the effects of AMFs inoculation have been investigated by several researchers. Improvement of plant nutrient concentration through the inoculation of mycorrhizae when water is limited has been thought to ameliorate water status (Amiri *et al.*, 2017). Additionally, mycorrhizal hyphae have been shown to penetrate the soil pores and absorb water that is normally unavailable for a non-mycorrhizal plant. AMFs inoculation increases the exploratory area for phosphorus and water absorption by expanding the surface area of the plant root (Amiri *et al.*, 2017). These AMFs coexist with the roots of plants and grow inside the cortex cells while increasing roots into the soil, increase the uptake of nutrients, especially phosphorus, which is slightly motile. Usually inoculated plants using water relations and better nutrition compared to others can escape WDS temporally, less damaged, and thus proline and soluble sugar increased less (Al-Arjani *et al.*, 2020). The results of the study have shown that soil fungal growth-promoting bacteria with AMFs increased biomass and mineral uptake even under stressful conditions (Ombódi *et al.*, 2019). According to results and available reports, the use of fluorescent *Pseudomonads* may be a tool to moderate the effects of drought stress on *Hibiscus sabdariffa* L. In addition to inducing carbohydrate and protein metabolism related to photosynthesis by *Fluorescent Pseudomonads*, it has been reported that the soluble sugars of maize inoculated under drought stress are higher than non-inoculated plants (Shoresh *et al.*, 2010).

Plant growth-promoting rhizobacteria (PGPR) of the genus *Azotobacter*, *Azospirillum*, and *Pseudomonas* are among the most important drivers of growth of plants that in addition to nitrogen bio-stabilization, soil phosphorus solubilizing, and production of significant amounts of growth hormones especially of auxin, gibberellin, and cytokinin affect growth and yield of crops (Gouda *et al.*, 2018). Today, several crops and variety-specific PGPR fertilizers are available in the market and being used by the farmers successfully. Promising results have been reported by the researchers on improvement in growth, yield, pest/disease defence mechanisms, and stress survivability features of the crop plants (Kumar *et al.*, 2020). These bacteria by a variety of ways, including dissolution of low soluble and insoluble compositions, thereby increasing their availability, nitrogen fixation, controlling plant diseases by producing antimicrobial compositions and competition for nutrient uptake, siderophore and plant hormone production such as indole acetic acid (IAA) increase plant tolerance to salinity, drought, and toxicity of the elements (Narula *et al.*, 2000; Awasthi *et al.*, 2011). Soil fluorescent soil pseudomonads especially due to catabolic adaptation, excellent root colonization and production potential of a wide range of enzymes and metabolites that live under various biotic and abiotic stress conditions can be useful, are taken into consideration (Vurukonda *et al.*, 2016). It has been reported that treating plants with plant PGPRs increased proline levels. For example, the treatment of cucumber with a mixture of PGPR strains resulted in an increase in proline content of leaves 3 to 4 times as compared to the control plant (Wang *et al.*, 2012).

Although many studies have been conducted to evaluate WDS tolerance of different medicinal species, so far, no study has been conducted to evaluate the effects of AMFs and PGPR inoculations under WDS on roselle. Therefore, our experiment was performed to determine the effects of AMFs and PGPR inoculation under WDS on morpho-physiological attributes of roselle grown in Iran.

Materials and Methods

Plant material and experimental design

A factorial experiment based on randomized complete design (CRD) with three replications of pots was conducted in the greenhouse of the Faculty of Agriculture and Natural Resources, Mohaghegh Ardabili University, Ardabil, Iran in 2016. Experimental factors included WDS [90% (I1), 75% (I2), 50% (I3), and 25% (I4) of field capacity as well-watered, mild, moderate, and severe stress, respectively], AMFs (non-inoculation, *F. mosseae*, and *F. intraradices*), and PGPR (control and *Pseudomonas fluorescens*). The seeds of roselle (*Hibiscus sabdariffa* L.) were prepared from medicinal herbs Research Institute of Shahid Beheshti University, Iran, two species of AMFs were collected from Soil Biology Laboratory of Tabriz University, Iran

and we prepared *Pseudomonas* bacteria from Karaj Soil and Water Research Institute, Iran. The ground used had a 2:1 soil-sand ratio with a loamy clay texture. First, we prepared soil used to disinfect for 1 h at 100 °C and then prepared inoculated seedlings with AMFs and PGPR. To inoculate 1 kg of soil, 50 g of AMFs will be used (Esmailpour *et al.*, 2013). After 20 days, the seedlings were transferred to pots. Air temperature ranged from 25 °C to 27 °C during the day and 18-21 °C during the night. Humidity ranged from 60% to 65%. The greenhouse was lighted using regular and florescence lamps 15-16 h, daily. After the growth of seedlings, we thinned seedlings at several stages and finally, in each pot, we kept 2 plants. WDS treatments were imposed at the flowering stage.

Seed yield components and root characteristics

At maturity, plants of each pot were harvested. The harvested material such as root volume, root dry weight, sepal dry weight, number of sepals per plant, 1000-seed weight, and seed yield per plant was weighed and counted for these traits. Roots were rinsed free of soil, cut into 1-2 cm fragments and thoroughly mixed, and subsamples (1 g) saved for determination of root colonization with AMF. These samples were cleared with 10% (w/v) KOH and stained with 0.03% (v/v) trypan blue in lactoglycerol according to the method of (Phillips and Hayman, 1970), and microscopically examined for colonization by determining percentage root segments containing arbuscules and vesicles using a gridline intercept method (Giovannetti and Mosse, 1980).

Measurement of stomatal conductance

Rates of stomatal conductance (mol H₂O/m².sec) in primary leaves were measured in the growth chamber using a portable photosynthesis measurement system (LI-6400, LI-COR, Inc.). As mentioned above, leaves were irradiated using a metal halide lamp in the growth chamber.

Osmotic potential

Osmotic potential (MPa) was determined according to (Kusvuran, 2012), 1 g of fresh leaves from the fourth leaf of the plants was weighed and homogenized with 19 ml distilled water. The homogenized leaf samples were kept at -20 °C. The homogenized samples were passed from the 0.45 µm precision filters. These samples were measured with the freezing point osmometer with Knauer mark and 7400 model instruments (Berlin, Germany). The osmotic potential was calculated according to the Van't Hoff equation.

Relative water content (RWC)

0.2 g fresh leaf was weighed and placed in 50 ml distilled water for 4 h, and then the turgid leaves were weighed. Then, leaf samples were oven-dried for calculating the dry weight at 70 °C for 48 h. The RWC was determined by the following equation [1] (Ahmadi *et al.*, 2015).

$$\text{Equation [1]} \quad \text{RWC} = (\text{FW}-\text{DW})/(\text{SW}-\text{DW}) \times 100$$

whereas, FW: fresh weight, DW: dry weight and SW: saturated weight

Photosynthetic pigments

Fresh leaf tissue was used for chlorophyll assay. 0.2 g of leaf tissue was grinded with 80% acetone gradually to give chlorophyll into acetone solution and finally, the volume of 80% acetone solution reached 20 ml. The resulting solution was centrifuged at 400 rpm for 10 minutes and then the absorbance of the supernatant was read by spectrophotometer at 470, 645, and 663 nm. The amount of chlorophylls and carotenoids were calculated according to the following equations [2-5] (Arnon, 1967).

$$\text{Equation [2]} \quad \text{Chlorophyll a} = (19.3 \times A_{663} - 0.86 \times A_{645}) V/100W$$

$$\text{Equation [3]} \quad \text{Chlorophyll b} = (19.3 \times A_{645} - 3.6 \times A_{663}) V/100W$$

Equation [4]	Total chlorophyll = chlorophyll a + chlorophyll b
Equation [5]	Carotenoids = $100 (A_{470}) + 3.27 (\text{mg Chl a}) - 104 (\text{mg Chl b})/227$

Peroxidase (POD) enzyme assay

Peroxidase enzyme activity was measured by (Kar and Mishra, 1976) method. So that 50 μL of the enzyme extract was added to 2.5 ml of reaction solution containing 100 mM of Tris-HCL, 5 mM of oxygenated water and 10 mM pyrogallol in an ice bath and the absorbance changes were read at the wavelength of 425 nm. To compare the activity of the enzyme, a control sample (BLANK) was used. In this sample, 0.05 mM of Tris-HCL buffer was used instead of 50 μL of the enzyme extract. The enzyme activity was calculated based on the change in uptake in mg of protein per minute.

Catalase (CAT) enzyme assay

To measure catalase enzyme activity, 2.5 ml of Tris buffer (50 mM, pH = 7) and 0.3 ml of oxygenated water (5 mM) were prepared, then 60 μL of enzyme extract was added in an ice bath and the absorbance was read at the wavelength of 240 nm. To compare the activity of the enzyme, a control sample (BLANK) was also used. In this sample, 0.05 mM of Tris-HCL buffer was used instead of 60 μL of enzyme extract. The enzyme activity was calculated based on the change in uptake in mg of protein per minute (Kar and Mishra, 1976).

Measurement of free proline

For extraction of proline, 0.5 g of the leaf was grinded using 5 ml of 95% ethanol in porcelain mortar and the supernatant was removed. The extraction procedure was repeated twice and each time with 5 ml of 70% ethanol. The solution was centrifuged for 10 minutes at 3500 rpm. After separating the liquid phase from the solid phase, the liquid phase was used to extract proline. To determine the concentration of proline, 1 ml of alcoholic extract was diluted with 10 ml distilled water, and 5 ml of ninhydrin reagent was added (125 mg of ninhydrin + 2 ml of phosphoric acid 6 ml + 3 ml glacial acetic acid). For the preparation of phosphoric acid 6 molar, 40.45 ml of 85% phosphoric acid (v/v) with a density of 1.71 kg was added to distilled water and then made to 100 ml volume. Then, 5 ml of glacial acetic acid was added, stirred, and put in boiling water for 45 min. After removing the samples from the boiling water and cooling, add 10 ml of benzene was added and mixed with a mechanical stirrer to allow proline to enter benzene phase. The samples were left for 30 minutes and then using proline we provided calibration curve and the absorbance was measured with a spectrophotometer at 515 nm (Irigoyen *et al.*, 1992).

Statistical analysis

Distribution normality of achieved data was done according to the Kolmogorov-Smirnov and Shapiro-Wilk test. Then the studied traits were statistically analysed by the Statistical Analysis System software (SAS Institute, Cary, NC, USA, and Version 9.2). The differences among means were separated using LSD test (least significant difference) at 0.05 statistical probability level. The Pearson correlation coefficient was used to measure relationships between growth, morph-physiological, and biochemical traits by using SAS software vr.9.2.

Results

Root colonization, volume, and dry weight

Results indicated that the effects of WDS, AMFs, and PGPR were significant on root colonization, volume, and dry weight. Also, comparisons of means showed a significant interaction between WDS \times AMF \times PGPR for root volume and root dry weight ($p \leq 0.01$). WSD induced different changes in root colonization, volume, and dry weight (Table 1). The highest root colonization (65.8%) was observed under well-watered (I_1)

by *F. intraradices*, and the lowest colonization by all WDS levels under non-inoculation. Also, the co-application of *F. mosseae* and *Pseudomonas fluorescens* increased (93.4%) root colonization compared to the control treatment (data not shown). WSD significantly decreased root volume and dry weight (by 45.1% and 55.4% in I₄, respectively) while inoculation with AMFs and PGPR increased these traits (Table 1). The highest root volume and dry weight (20.66 ml and 5.47 g, respectively) were observed under normal irrigation (I₁) by the co-application of *F. intraradices* and *Pseudomonas fluorescens* and the lowest means of these traits (8.33 ml and 1.23 g, respectively) in the non-inoculation under severe stress (I₄) (Figure 1A and B).

Table 1. Comparisons of means and analysis of variance indicating the effects of WDS, arbuscular mycorrhizal fungi and plant growth promoting rhizobacteria on roselle yield components and physiological properties

S.O.V	Root colonization (%)	Root volume (ml)	Root dry weight (g)	Sepals dry weight (g)	Number of sepals per plant	1000-seed weight (g)	Seed yield (g/plant)
WDS							
I1	43.72 a	19.33 a	4.44 a	2.99 a	11.22 a	31.81 a	5.55 a
I2	39.86 a	16.38 b	3.72 b	2.27 b	9.33 b	26.57 b	4.62 b
I3	34.27 b	12.05 c	2.45 c	1.17 c	7.00 c	23.25 c	2.85 c
I4	29.19 c	10.61 d	1.98 d	0.70 d	5.56 d	19.58 d	1.54 d
AMFs							
Non-inoculation	6.1 c	13.21 b	2.53 b	1.6 c	7.58 c	24.64 b	3.12 c
<i>F. mosseae</i>	49.83 b	15.17 a	3.41 a	1.82 b	9.00 a	25.57 a	4.33 a
<i>F. intraradices</i>	54.36 a	15.42 a	3.51 a	1.94 a	8.25 b	25.70 a	3.46 b
PGPR							
Control	32.81 b	14.42 b	3.0 b	1.85 a	8.14 b	22.95 b	3.48 b
<i>Pseudomonas fluorescens</i>	40.72 a	14.78 a	3.3 a	1.72 b	8.42 a	27.65 a	3.80 a
Statistics							
WDS × AMF	*	**	**	**	**	**	**
WDS × PGPR	ns	ns	**	**	ns	**	**
AMF × PGPR	**	**	**	**	**	**	**
WDS × AMF × PGPR	ns	**	**	**	*	**	**

S.O.V	Stomatal conductivity (mol/m ² .sec)	Osmotic potential (MPa)	RWC (%)	Chlorophyll a (mg/g FW)	Chlorophyll b (mg/g FW)	Total chlorophyll (mg/g FW)	Carotenoids (mg/g FW)	CAT activity (U/mg protein. min)	POD activity (U/mg protein. min)	Proline content (μmol/g FW)
WDS										
I1	16.87 a	11.01 d	86.49 a	5.85 a	2.14 a	7.99 a	1.94 a	5.32 d	1.06 d	5.73 d
I2	13.4 b	11.64 c	77.61 b	5.17 b	1.7 b	6.87 b	1.48 b	5.96 c	1.36 c	6.9 c
I3	12.17 c	14.5 b	64.52 c	4.34 c	1.28 c	5.61 c	1.03 c	7.64 b	1.88 b	8.07 b
I4	11.14 d	15.73 a	58.22 d	3.67 d	1.04 d	4.71 d	0.85 d	8.92 a	2.33 a	9.74 a
AMFs										
Non-inoculation	13.01 c	13.68 a	68.19 b	4.24 b	1.46 c	5.7 b	1.15 c	5.42 c	1.47 c	6.71 b
<i>F. mosseae</i>	13.47 b	12.84 b	73.2 a	5.06 a	1.52 b	6.57 a	1.39 b	8.23 a	1.65 b	8.18 a
<i>F. intraradices</i>	13.71 a	13.13 b	73.74 a	4.97 a	1.63 a	6.61 a	1.44 a	7.23 b	1.86 a	7.95 a
PGPR										
Control	13.03 b	13.55 a	71.18 b	4.59 b	1.54 a	6.12 b	1.31 b	6.61 b	1.65 a	6.88 b
<i>Pseudomonas fluorescens</i>	13.76 a	12.89 b	72.24 a	4.93 a	1.54 a	6.46 a	1.34 a	7.31 a	1.67 a	8.34 a
Statistics										
WDS × AMF	**	ns	ns	**	**	**	**	**	*	**
WDS × PGPR	ns	*	*	**	**	**	**	ns	**	ns
AMF × PGPR	**	**	**	**	**	**	**	**	**	**
WDS × AMF × PGPR	**	**	**	**	**	**	**	**	**	**

I1, I2, I3, and I4 indicate irrigation levels (WDS treatment) at 90%, 75%, 50% and 25% of field capacity, respectively. Means followed by different letters, in the same columns, are significantly different at $p \leq 0.05$ by LSD test. ns, and *, ** show no significant differences at 0.05, and 0.01 level of probability, respectively

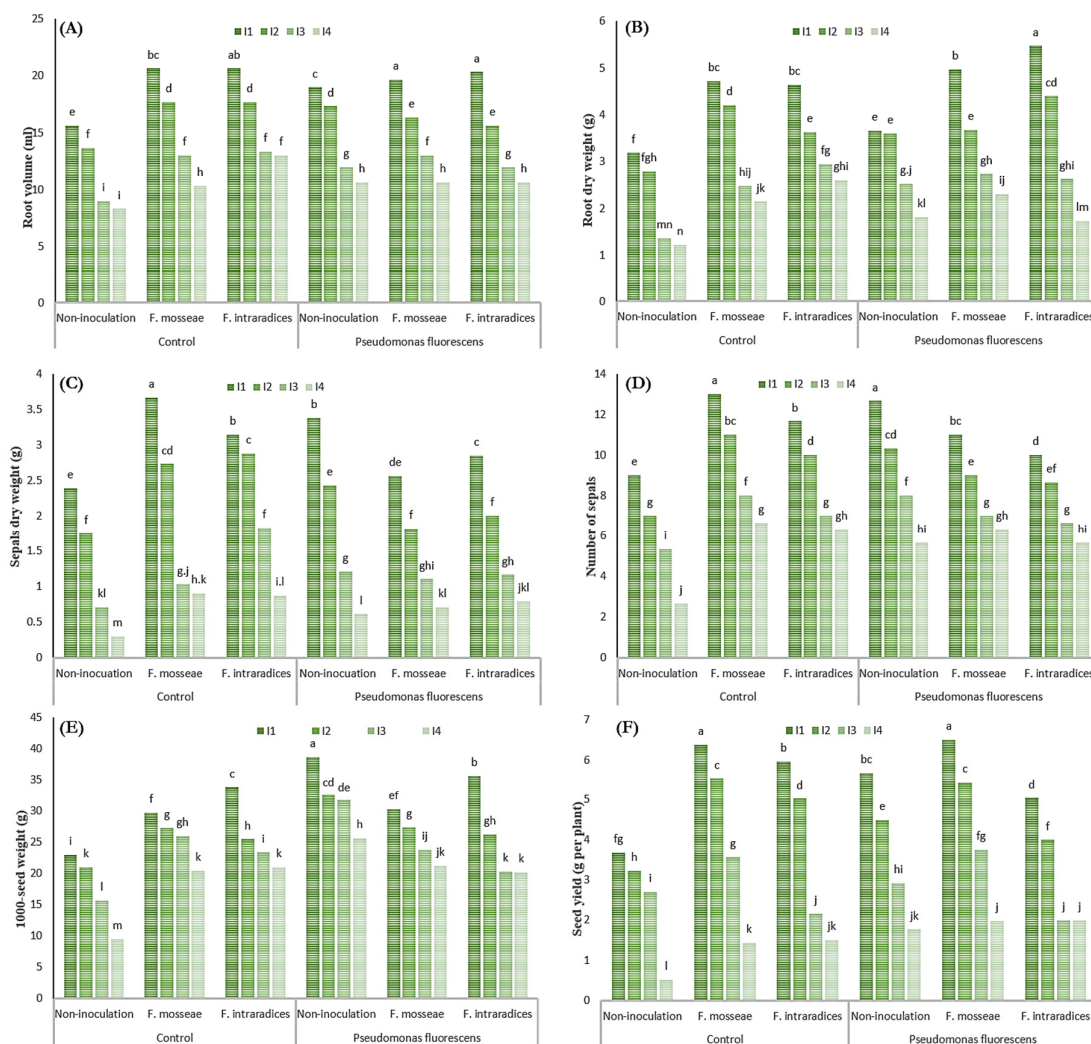


Figure 1. The interaction effects of WDS × AMFs × PGPR on root volume (A), root dry weight (B), sepals' dry weight (C), number of sepals (D), 1000-seed weight (E), and seed yield of roselle (F)

Number and dry weight of sepals

The effects of WDS, AMFs, and PGPR treatments as well as the interaction WDS × AMFs × PGPR were significant on the number and dry weight of sepals of roselle. Drought stress decreased the number and dry weight of sepals while inoculation by AMFs increased. The highest sepal's dry weight (3.66 g) was related to the *F. intraradices* inoculation under well-watered (I₁) and the lowest (0.3 g) to non-inoculation under severe stress (I₄). The results indicated that the AMFs inoculation under severe stress increased sepals dry weight compared to the non-inoculation (Figure 1C).

The number of sepals per plants that had been subjected to the severe water stress was significantly lower compared to the well-watered plants (Table 1). Plants cultivated under severe stress conditions that had received inoculation, especially AMFs (*F. mosseae* and *F. intraradices*) plants, attained a higher number of sepals per plants than non-inoculated plants. The highest number of sepals per plant was achieved in the *F. mosseae* inoculation with non-inoculation of PGPR under well-watered (13.0 number) and the application of *Pseudomonas fluorescens* with AMFs inoculation under well-watered treatment (12.6 number). The lowest this trait (2.6 number) was found non-inoculation treatment (control) under severe water stress (I₄). In well-

watered plants, number of sepals was increased by single AMFs and PGPR inoculation similarly to dual colonization (Figure 1D).

1000-seed weight

1000-seed weight significantly influenced by WDS, AMFs, and PGPR as well as the interaction of WDS × AMFs × PGPR ($p \leq 0.01$) (Table 1). In well-watered conditions (I_1), the highest 1000-seed weight (38.6 g) was observed in non-inoculation with the application of *Pseudomonas fluorescens* and the lowest (9.43 g) was achieved in control treatments (non-inoculation) under severe stress condition (I_4) (Figure 1E).

Seed yield

Seed yield significantly influenced by interaction of WDS, AMFs, and PGPR ($p \leq 0.01$). The severe stress (I_4) decreased seed yield by 72.25% compared to the well-watered (I_1). Also, the *F. mosseae* inoculation and the application of PGPR increased by 27.94% and 8.42% compared to the control treatment, respectively (Table 1). The highest seed yield was found under well-watered treatment by inoculation of *F. mosseae* without PGPR and the application of *Pseudomonas fluorescens* (6.37 and 6.51 g/plant). The lowest seed yield (0.51 g/plant) was achieved non-inoculation treatments under severe stress (I_4). Dual inoculated plants with AMFs and PGPR showed the decreasing seed yield under stress conditions, suggesting the antagonistic effects of AMFs and PGPR (Figure 1F).

Stomatal conductivity

As shown in Table 2, the stomatal conductivity significantly influenced by WDS, AMFs, and PGPR as well as the interaction of WDS × AMFs × PGPR ($p \leq 0.01$). Results indicated that the WSD decreased the stomatal conductivity while the inoculation of AMFs and PGPR increased (Table 2). The highest stomatal conductivity was found under well-watered treatment by inoculation of *F. mosseae* without PGPR (17.83 mol/m².sec) and the application of *Pseudomonas fluorescens* without AMFs (17.86 mol/m².sec). The lowest stomatal conductivity (9.33 mol/m².sec) was achieved non-inoculation treatments under severe stress (I_4). Dual inoculated plants with AMFs and PGPR, under stress conditions (I_3 and I_4), showed the increasing stomatal conductivity, suggesting the synergistic effects of AMFs and PGPR (Figure 2A).

Table 2. Correlation coefficients among yield components and physiological traits of roselle under WDS, AMFs, and PGPR treatments

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17				
1	1																				
2	0.44	1																			
3	0.55	0.93	1																		
4	0.29	0.94	0.85	1																	
5	0.32	0.93	0.85	0.92	1																
6	0.25	0.8	0.74	0.74	0.84	1															
7	0.4	0.92	0.85	0.87	0.91	0.75	1														
8	0.33	0.9	0.81	0.88	0.87	0.77	0.84	1													
9	-0.35	-0.89	-0.86	-0.86	-0.88	-0.76	-0.89	-0.82	1												
10	0.4	0.97	0.92	0.94	0.94	0.81	0.92	0.9	-0.89	1											
11	0.5	0.88	0.89	0.84	0.86	0.74	0.82	0.81	-0.85	0.88	1										
12	0.25	0.9	0.83	0.92	0.91	0.81	0.82	0.86	-0.82	0.91	0.83	1									
13	0.44	0.92	0.91	0.9	0.91	0.79	0.85	0.86	-0.88	0.93	0.98	0.92	1								
14	0.47	0.94	0.88	0.92	0.89	0.73	0.89	0.92	-0.86	0.94	0.81	0.85	0.86	1							
15	0.36	-0.48	-0.38	-0.6	-0.44	-0.34	-0.47	-0.48	0.49	-0.5	-0.32	-0.57	-0.42	-0.44	1						
16	-0.01	-0.64	-0.61	-0.66	-0.6	-0.45	-0.71	-0.64	0.69	-0.67	-0.57	-0.59	-0.6	-0.66	0.71	1					
17	0.45	0.66	0.7	0.58	0.63	0.58	0.73	0.69	-0.61	0.7	0.61	0.66	0.65	0.69	-0.24	-0.49	1				
	-1	-0.9	-0.8	-0.7	-0.6	-0.5	-0.4	-0.3	-0.2	-0.1	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1

1. Root colonization, 2. Root volume, 3. Root dry weight, 4. Sepals dry weight, 5. Number of sepals, 6. 1000-seed weight, 7. Seed yield, 8. Stomatal conductivity, 9. Osmotic potential, 10. RWC, 11. Chlorophyll a content, 12. Chlorophyll b content, 13. Total chlorophyll content, 14. Carotenoids content, 15. CAT activity, 16. POD activity, 17. Proline content.

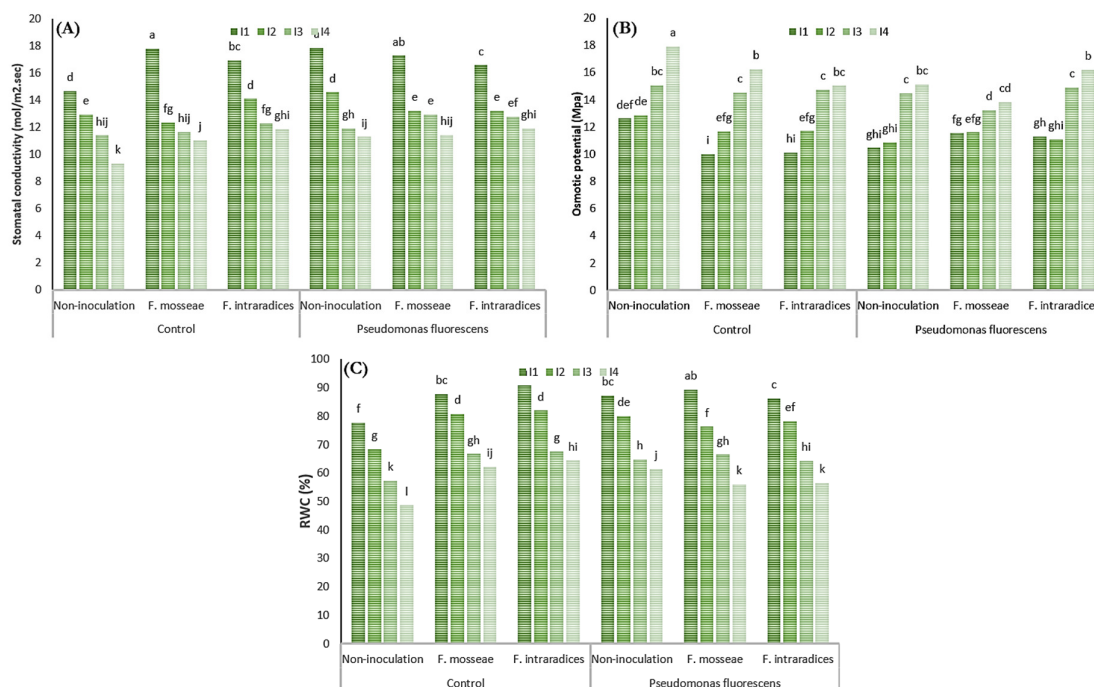


Figure 2. The interaction effects of WDS × AMFs × PGPR on stomatal conductivity (A), osmotic potential (B), and RWC of roselle(C)

Osmotic potential

The effects of WDS, AMFs, and PGPR treatments as well as the interaction WDS × AMFs × PGPR were significant on the osmotic potential (Table 1). Water deficit stress was caused by increasing the osmotic potential while the application of AMFs and PGPR decreased. The highest osmotic potential (17.93 MPa) was observed under severe stress (I₄) by non-inoculation with AMFs and PGPR and the lowest (9.99 MPa) under well-watered treatment by *F. mosseae* without PGPR (Figure 2B).

Relative water content

As shown in Table 1, RWC significantly influenced by the interaction effects of WDS, AMFs, and PGPR ($p \leq 0.01$). The severe stress (I₄) decreased RWC by 32.68% compared to the well-watered (I₁). Also, the *F. intraradices* inoculation increased by 7.52% compared to the control treatment (Table 2). The highest RWC (90.6%) was observed under well-watered by *F. intraradices* without PGPR and the lowest (48.6%) under severe stress (I₄) treatment by non-inoculation with AMFs and PGPR (Figure 2C).

Photosynthesis pigments

Photosynthesis pigments such as Chl a, b, and total Chl as well as carotenoids significantly influenced by interaction effects of WDS, AMFs, and PGPR ($p \leq 0.01$). Contents of photosynthesis pigments decreased as a result of WDS while inoculated plants by AMFs and PGPR showed increased (Table 1). The highest Chl a, b, and total Chl were observed in inoculated plants by *F. mosseae* without PGPR under well-watered (6.23, 2.53, and 8.76 mg/g FW, respectively) and the lowest these traits (2.61, 0.74, and 3.35 mg/g FW) in non-inoculation treatment under severe water stress (Figure 3A, B, and C). As shown Figure 3D, the application of AMFs and PGPR increased carotenoids content under water stress conditions (I₃ and I₄) compared to the non-inoculated plants. The highest and lowest carotenoids content (2.21 and 0.58 mg/g FW, respectively) was observed in the application of *F. intraradices* under well-watered (I₁) and the non-inoculation treatment under severe stress (I₄), respectively.

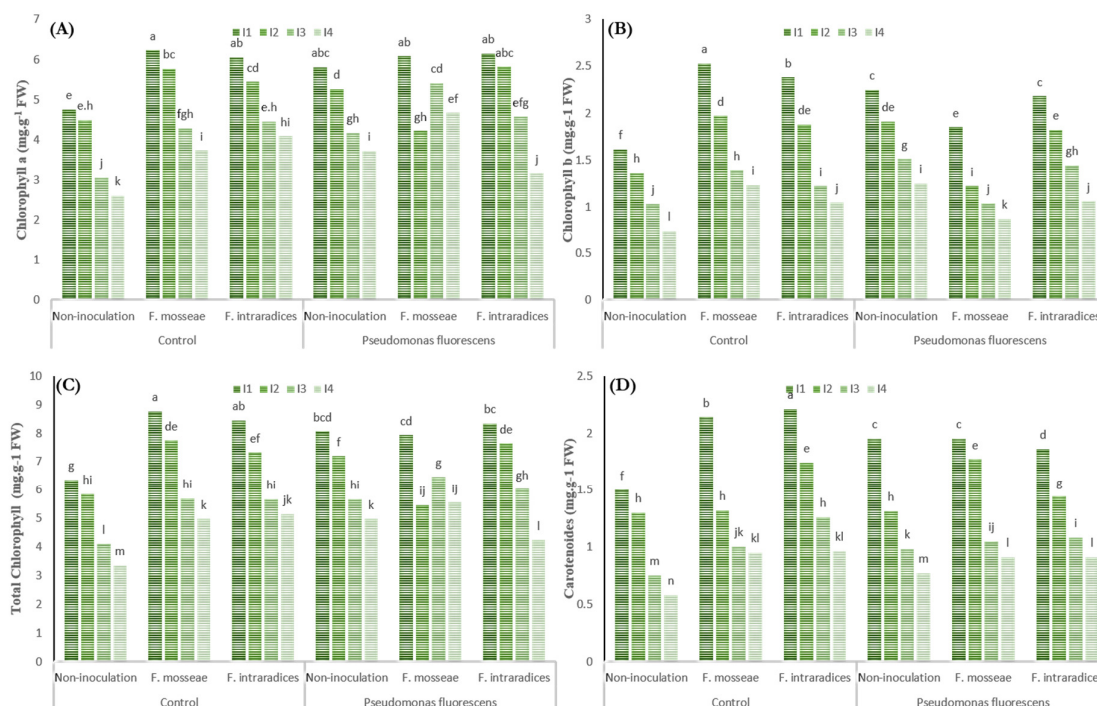


Figure 3. The interaction effects of WDS × AMFs × PGPR on chlorophyll a content (A), chlorophyll b content (B), total chlorophyll content (C), and carotenoids content of roselle (D)

Catalase and peroxidase activity

The interaction effects of WDS, AMFs, and PGPR were significant in the activity of CAT and POD enzymes ($p \leq 0.01$). The results showed WSD and the application of AMFs and PGPR enhanced the activity of CAT and POD enzymes (Table 1). As shown in Figure 4, dual inoculated plants with *F. mosseae* and *Pseudomonas fluorescens* under severe stress (I4) showed the highest CAT activity (10.71 U/mg protein.min) and the lowest activity (3.73 U/mg protein.min) related to non-inoculated plants under well-watered. In severe stress conditions (I4), POD activity was increased by single *F. intraradices* and *Pseudomonas fluorescens* inoculation similarly to dual colonization. POD activity ranged from 0.78 U/mg protein.min (control treatment) to 2.66 U/mg protein.min (*Pseudomonas fluorescens* inoculation without AMFs under severe stress) (Figure 4B).

Proline content

As shown in Table 1, proline content significantly influenced by WDS, AMFs, PGPR, as well as interaction effects of WDS × AMFs × PGPR. By increasing the intensity of water stress as well as the application of AMFs and PGPR, an increase in proline content was observed. Dual application of *Pseudomonas fluorescens* and *F. mosseae* under severe stress (I4) showed the highest proline content (12.55 $\mu\text{mol/g FW}$) and the lowest proline content related to the non-inoculation treatment under well-watered plants as well as *F. intraradices* inoculation without PGPR under non-stress conditions (4.45 and 4.44 $\mu\text{mol/g FW}$) (Figure 4C).

Correlation coefficients

The results of correlation coefficients are presented in Table 2. According to the results, there were significantly negative and positive correlations among yield components and physiological properties. For example, the morphological and seed yield components (such as root colonization, root volume, and dry weight, sepals' dry weight, number of sepals, 1000-seed weight, and seed yield, were significantly and positively correlated among themselves and with RWC, Chl a, b, and total Chl contents, as well as carotenoids content.

However, the sepals' dry weight, seed yield, and pigment contents were negatively and significantly correlated with the enzymatic activities (CAT and POD) and with the osmotic potential.

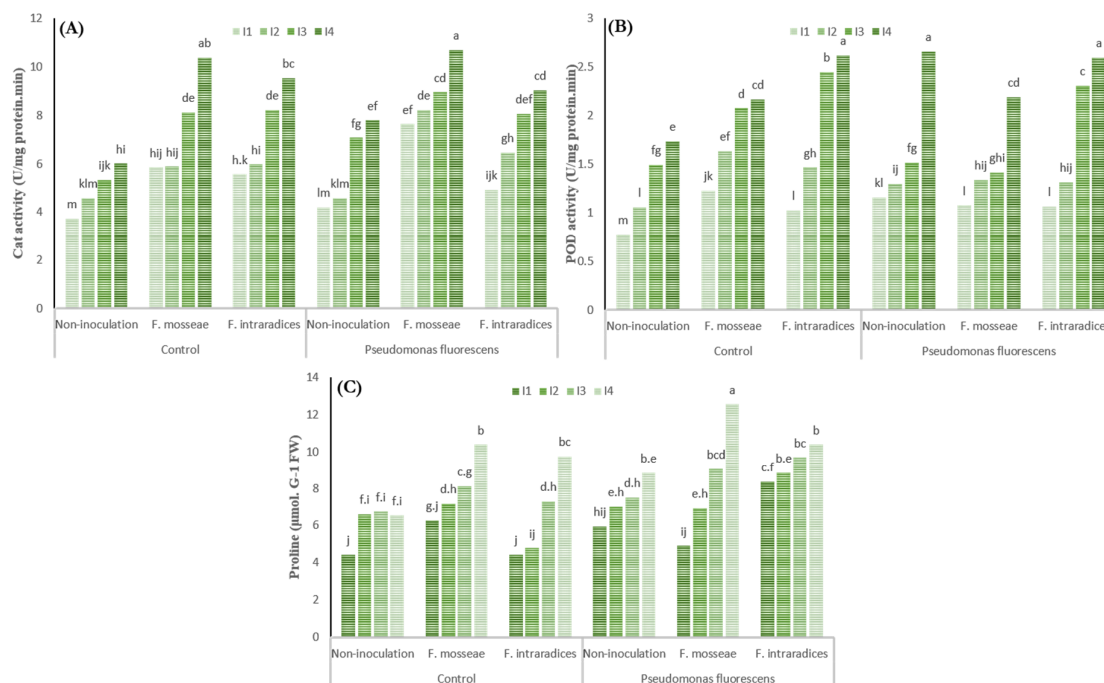


Figure 4. The interaction effects of WDS x AMFs x PGPR on CAT activity (A), POD activity (B), and proline content (C)

Discussion

The experiment aimed to determine the effects of AMFs and PGPR inoculation under WDS on morpho-physiological attributes of roselle grown in Iran. Results showed that the effects of WDS, AMFs, and PGPR, as well as the interaction of WDS x AMFs x PGPR, were significant on morphological traits and yield component (root volume and dry weight, sepals dry weight, number of sepals, 1000-seed weight, and seed yield) and physiological traits (stomatal conductance, osmotic potential, RWC, Chl a, Chl b, total Chl, carotenoids, the activity of CAT and POD enzymes, and proline content).

The effects of AMFs and PGPR on the physiological traits and yield components of roselle under WDS indicated that the microorganisms in the soil can alleviate the stress by regulating different plant morphological and physiological mechanisms. The stress significantly affected roselle physiological properties and decreased plant yield components. The AMFs improved plant physiology, growth, and yield under WDS by affecting the activity of antioxidant enzymes, (scavenging reactive oxygen species) and increasing plant pigments including Chl a, Chl b, and total Chl, and carotenoids. Accordingly, our results for the first time indicate the alleviating effects of AMFs on roselle physiology, growth, and yield components under WDS.

Plant water status can affect different physiological properties such as leaf turgor potential, plant growth, stomatal conductance, transpiration rate, photosynthesis, and respiration. Maintenance of plant water status is a fundamental phenomenon for the maintenance of normal growth of plants under stressful environments (Kusvuran, 2012). In this study, WDS adversely affected different plant-water relation parameters such as leaf water potential, osmotic potential, and RWC. The effects of each of the mycorrhizal and bacterial inoculants on the RWC of plants were obvious, whereas a maximized positive effect on water content was observed in single-inoculated plants by *F. intraradices* (Rahimzadeh and Pirzad, 2017). According to the previous research,

increase plant tolerance to WDS by improving plant water relations, osmotic adjustment, increasing leaf gas exchange, photosynthesis, protection against oxidative damage caused by drought stress, and increasing nitrate reductase activity (Dutta *et al.*, 2015). The accumulation of soluble matter led to osmosis regulation that is one of the mechanisms of adaptation under water shortage conditions.

Mycelium of mycorrhizal arbuscular fungus plays an important role in the effect of the fungus on the host plant water relations and absorbs water from very fine pores of the soil. Under WSD conditions, the water potential of AMFs inoculated plants were 21% more than non-inoculated plants. (Rouphael *et al.*, 2015) reported that AMFs likely absorbed more water by changing root morphology, developing the host plant root, and increasing absorption surface through hyphae and improved the host plant water relations. The interaction between the plants and soil micro-organisms provided good conditions for water shortage stress (Verma *et al.*, 2016). According to the experiment results, it seems that WDS can reduce RWC percentage, and using AMFs single or dual can play a role in reducing the adverse effects better than non-application. According to the experiment, with increasing proline content the leaf RWC was also reduced, and increasing proline indicated increasing drought stress severity and reducing water of plants which resulted in reduced leaf RWC.

In our experiment, WSD tended to diminish photosynthetic pigments such as Chl and carotenoids. Under well-watered conditions, however, the increased Chl content that we observed with single inoculation of AMFs by *F. mosseae* might have contributed to an increased rate of photosynthesis possibly related to a large number of chloroplasts in bundle sheaths in the leaves (Rahimzadeh and Pirzad, 2017). During stress, chloroplast decomposed and Thylakoid structures disappeared. On the other hand, drought stress disturbed enzyme systems reducing reactive oxygen activity and increasing lipid peroxidation and as a result damaged the cell membrane and pigment degradation (Ruíz-Sánchez *et al.*, 2011). The reduction in Chl content under water shortage stress conditions observed in this study has been also reported by Misra and Srivastava (2000) in Japanese peppermint. Chlorophyll content in the mycorrhizal plants was increased due to improved phosphorus absorption. In pepper inoculated with *G. intrsrtdices*, chlorophyll a and b significantly increased compared to non-mycorrhizal plants (Demir, 2005). AMFs can increase stomatal conductivity, photosynthesis, and finally chlorophyll of plants after water shortage stress (Selvaraj and Chellappan, 2006). The PGPR and AMFs have the potential to modulate and regulate plant physiological and biochemical responses to drought stress and therefore increase plant survival under severe and diverse environmental conditions (Marasco *et al.*, 2012).

On the other hand, carotenoids act as pigments taking light in photosynthesis and could be classified as antioxidants removing free radicals in the plant used and thereby improving the stress oxidative incurred by the plant during adaptation (Kapoor *et al.*, 2008). The increase in the content of photosynthetic pigments during the adaptation process by AMFs was previously studied by (Rahimzadeh and Pirzad, 2017). Misra and Srivastava (2000) in a study on Japanese peppermint (*Mentha arvensis*) observed that water shortage stress significantly reduced Chl content. PGPR and AMFs have the potential to modulate and regulate plant physiological and biochemical responses to drought stress, thereby enhancing plant survival under bad and diverse environmental conditions (Marasco *et al.*, 2012).

In our study, all enzyme activities (CAT and POD enzymes) enhanced with increasing water stress intensity. Also, increased enzyme activities in inoculated plants by AMFs and PGPR indicated that the microorganisms alleviated the oxidative damage from water shortage. Similar to our results, (Rahimzadeh and Pirzad, 2017) found that inoculation with *Pseudomonas* augmented antioxidants under severe drought stress, suggesting that they can alleviate the drought-induced oxidative damage. Additionally, symbiosis with AMFs helps plants to cope with drought stress, probably by maintaining photosynthetic processes intact or little altered as a result of a rise in antioxidant activities (Rouphael *et al.*, 2015). The plants have different mechanisms to reduce the adverse effects of reactive oxygen species, including enzymatic and non-enzymatic antioxidant defence system. Antioxidant enzymes include CAT, superoxide dismutase, POD, polyphenol oxidase, and ascorbate peroxidase, which are involved in removing oxygen free radicals in the cell, as well as the most important constituents and the first defence against damage (Pan *et al.*, 2005). Under severe WDS

conditions, the increase of leaf CAT activity in *Pseudomonas fluorescens*-treated and mycorrhizal plants that we observed suggests that inoculated plants might have the potential to activate this enzyme to counteract oxidative, water deficit-induced damage (Ghorbanpour *et al.*, 2013). Thus, the inoculants were able to regulate oxidative reactions and antioxidant defence (Ortiz *et al.*, 2015).

Increasing drought stress increased CAT enzyme activity that increased plant tolerance to drought stress. Superoxide dismutase enzyme that is increased under stress conditions is the first enzyme that is activated in the antioxidant cycle (Chakraborty and Pradhan, 2012). It acts as the first defence antioxidant system against reactive oxygen molecular forms and converts free radicals into H₂O₂ created by water and oxygen molecules by CAT and POD (Gratão *et al.*, 2005). It has been shown that PGPR by producing antioxidant factors or modulating photosynthesis reduces the damage of reactive oxygen species and can protect the plant against the presence of reactive oxygen species and prevent damage to the plant (Young *et al.*, 2013). Peroxidase as a catalyst converts glutathione to glutathione disulfide and is involved in the conversion of hydrogen peroxide to water. Glutathione affects the activity of di-ascorbate reductase enzyme that converts di-hydro-ascorbate to ascorbate (Hossain and Fujita, 2012). Under stress, all major plant processes such as energy production, lipid metabolism, and antioxidant enzyme activity are affected (Weisany *et al.*, 2012). Erdogan *et al.* (2016) concluded that plants inoculated with PGPR had higher antioxidant enzyme activity such as CAT, POD, and APX).

The reported results increase in leaf proline content in consequence of increasing WSD suggest that the proline accumulation probably is a common response of roselle under water deficit as an osmotic adjustment. The lower content of proline may be attributed to either greater drought resistance or less injury of colonized plants under drought stress conditions, and proline content was lower in single-inoculant treatments than in dual-inoculation treatments (Rahimzadeh and Pirzad, 2017). Although damage to cell membranes by water stress probably limits osmotic adjustment, high leaf water content under drought stress may prevent the accumulation of osmolytes such as proline content. Pirzad *et al.* (2011) reported an increase in proline content due to water shortage stress in *Matricaria chamomilla* L. It has also been reported that the application of PGPR by removing oxygen free radicals reduces the damage to fatty acids and proteins, and thus reduces the destructive effect of stress, thereby reducing proline synthesis and accumulation as a plant reaction to stress (Agami *et al.*, 2016). AMFs were more effective in increasing proline content at high levels of severe stress and had a higher leaf proline accumulation compared to non-inoculated plants. Usually, plants inoculated with AMFs using water and better nutrition to the plants without AMFs are capable to tolerate drought conditions temporarily and are less damaged, and therefore levels of proline in plants without mycorrhiza increased less. Based on the experiment, increased proline content showed the highest intensity of water stress and reduced RWC of the leaf.

Conclusions

According to previous studies on AMFs and plants symbiosis and the study results, we concluded that mycorrhizal plants for the advantages of symbiosis with AMFs through increasing the uptake of minimum water available in the soil by fungal hyphae as well as reducing the amount of water loss by reducing leaf area, Chl content and finally photosynthesis rate during the stress period have shown greater resistance to non-mycorrhizal plants, thereby improving the recovery of this plant in warm and arid areas. The study results showed that WDS significantly reduced all yield components. The application of PGPR or AMFs (especially) is important for emphasizing reducing the negative environmental impacts of chemical fertilizers, which can be a good alternative for them. According to the results obtained from this experiment, it can be stated that although with reducing water use and consequently WSD occurrence, the plant dry matter yield is reduced, using bio-fertilizers especially at higher levels of drought stress to some extent we can reduce the adverse effects on the plant productivity which can be attributed to the positive effect of bio-fertilizers on improving the plant nutrient conditions under WSD.

Authors' Contributions

MB, AEKG and MTG supervised and designed the experiment. SS performed the experiment, analysed the parameters, SS and MTG analysed the data. SS wrote the paper. MB and AEKG improved the language and English writing of the entire manuscript. All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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