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ORIGINAL ARTICLE

Arbuscular mycorrhizal fungi and *Pseudomonas* in reduce drought stress damage in flax (*Linum usitatissimum* L.): a field study

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Abstract Drought stress, which is one of the most serious world environmental threats to crop production, might be compensated by some free living and symbiotic soil microorganisms. The physiological response of flax plants to inoculation with two species of arbuscular mycorrhizal (AM) fungi (*Funneliformis mosseae* or *Rhizophagus intraradices*) and a phosphate solubilizing bacterium (*Pseudomonas putida* P13; PSB) was evaluated under different irrigation regimes (irrigation after 60, 120, and 180 mm of evaporation from Class A pan as well-watered, mild, and severe stress, respectively). A factorial (three factors) experiment was conducted for 2 years (2014–2015) based on a randomized complete block design with three replications at Urmia University, Urmia, located at North-West of Iran (37° 39′ 24.82″ N44° 58′ 12.42″ E). Water deficit decreased biomass, showing that flax was sensitive to drought, and AM root colonization improved the performance of the plant within irrigation levels. In all inoculated and non-inoculated control plants, leaf chlorophyll decreased with increasing irrigation intervals. Water deficit-induced oxidative damage (hydrogen peroxide, malondialdehyde, and electrolyte leakage) were significantly reduced in dual colonized plants. All enzymatic (catalase, superoxide dismutase, glutathione reductase, and ascorbate peroxidase) and non-enzymatic (glutathione, ascorbic acid, total carotenoids) antioxidants were reduced by water-limiting irrigation. Dual inoculated plants with AM plus *Pseudomonas* accumulated more enzymatic and non-enzymatic antioxidants than plants with bacterial or fungal inoculation singly. Dual colonized plants significantly decreased the water deficit-induced glycine betaine and proline in flax leaves. These bacterial-fungal interactions in enzymatic and non-enzymatic defense of flax plants demonstrated equal synergism with both AM fungi species. In conclusion, increased activity of enzymatic antioxidants and higher production of non-enzymatic antioxidant compounds in symbiotic association with bacteria and mycorrhiza can alleviate reactive oxygen species damage resulting in improve water stress tolerance.

Keywords Antioxidants · Flax · Mycorrhiza · *Pseudomonas putida* · Oxidative damage · Water deficit

Introduction

Plants growing in a detrimental environment, such as those occurring in arid and semiarid soils, undergo water shortage which is the most common stress affecting plant growth in such regions (Marulanda et al. 2008). With severe drought an all-too-common occurrence, some farmers turn to irrigation for a solution, but it may not be feasible or even desirable. Plants have evolved complex physiological and biochemical adaptations in order to adjust and adapt to drought stress. The physiological mechanisms (accumulation of compatible osmolytes like proline and soluble carbohydrates, overproduction of reactive oxygen species [ROS] and formation of free-radical scavenging compounds such as ascorbate and glutathione) associated with water-stress tolerance have been extensively studied (Gill and Tuteja 2010; Osakabe et al. 2014). Osmolytes contribute to the lowering of the osmotic potential and, in turn, of the leaf water potential which allow the plants to maintain high organ hydration and turgor (Ashraf and Foolad 2007). Several factors activate the resistance response

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of plants to water stress so that they can alleviate detrimental effects of drought (Ramoliya et al. 2004).

Sustainable systems require an understanding of interactions between plants and microorganisms (e.g., arbuscular mycorrhizal fungi [AMF] and phosphate solubilizing bacteria [PSB]), with a direct influence on plant growth and water deficit stress tolerance (Habibzadeh et al. 2015; Marulanda et al. 2009). Mycorrhizal plants often have greater tolerance to drought than non-mycorrhizal plants (Al-Karaki et al. 2004). Several non-nutritional mechanisms have been proposed for explaining host plant protection by AM symbiosis against drought-induced detrimental effects (Ruiz-Lozano et al. 2001). The osmotic stress normally caused by drought is counteracted by mycorrhizal plants through biochemical changes that mostly include increased biosynthesis of osmolytes (mainly proline, soluble carbohydrates and glycine betaine) (Ashraf and Foolad 2007). Furthermore, mycorrhizal plants withstand drought-induced oxidative stress by increased production of enzymatic (catalase, superoxide dismutase, glutathione reductase and ascorbate peroxidase) and non-enzymatic (ascorbic acid, total carotenoids and glutathione) antioxidant compounds (Pedranzani et al. 2016). Zhu et al. (2010) reported that the AM fungus (*Glomus etunicatum*) is capable of alleviating the damage caused by stress by reducing membrane lipid peroxidation and membrane permeability and increasing the accumulation of osmotic adjustment compounds and antioxidant enzyme activity.

Mycorrhizal colonized plants can interact with several soil microorganisms including phosphate solubilizing bacteria that are capable of making the plant more tolerant to drought stress conditions (Dimkpa et al. 2009). Inoculated seedlings with the exopolysaccharide (EPS) producing strain *Pseudomonas putida* showed improved soil aggregation, root-adhering soil, and higher relative water content (RWC) of leaves that imparted tolerance against drought stress to plants (Bensalim et al. 1998; Sandhya et al. 2009). Different arbuscular mycorrhizal fungi species differentially can affect both the populations and activity of *Pseudomonas* (Marschner and Crowley 1996), and bacteria (especially those producing plant growth regulators) can affect the development of mycorrhizas (Azcon et al. 1978). *Pseudomonas putida* strains, gram-negative rhizobacteria, have the capacity to adapt to diverse niches in the soil (Wu et al. 2010) and also can colonize a wide range of plants (Fernandez-Pinar et al. 2012). *P. putida* exhibited a high osmotic tolerance and also showed increased proline concentration, involved in osmotic cellular adaptation (Marulanda et al. 2009). Moreover, co-inoculation with PSB and mycorrhizal fungi has been proposed as an efficient procedure for increasing plant growth. Assessing the effects of plant-beneficial microorganisms (two *Pseudomonas* strains and a mixed mycorrhizal inoculum, alone or in combination) on the quality of tomato fruits of plants cultivated in the field, showed the benefit of dual colonization (Bona et al. 2017).

Flax or linseed (*Linum usitatissimum*, Linaceae), native of Europe and South Asia, is an economically important oilseed and a multi-purpose crop (the third largest natural fiber crop and one of the five major oil crops in the world), and its importance is shown by all parts of the plant having specific economic uses. The quality and utilization of flax oil is determined by its fatty acid composition (FAOSTAT 2014; Muir and Westcott 2003). Flax is highly dependent on AM fungi in order to meet its early phosphorus requirements (Thingstrup et al. 1998). It has been reported that inoculation of the AM fungus *F. mosseae* led to various increased morphological and physiological parameters of flax (Neetu et al. 2011).

Over the last two decades, the area of flax cultivation has decreased, resulting in a worldwide gap between production and consumption. It is necessary to increase flax productivity per unit area in water deficit conditions, because drought has played a significant role in this reduction. One possible way to enhance production is tolerance to stresses by means of rhizosphere microbial manipulation. This study aimed at investigating directly in the field, the interaction effects of two AMF species, and a *Pseudomans* strain on the water deficit-induced physiological responses of flax plants including antioxidant defense machinery (enzymatic and non-enzymatic) against drought stress damage.

Materials and Methods

Experimental design and plant culture

A 2-year field experiment was conducted in the research field of Urmia University, Urmia, Iran, located in the northwest of Iran (37° 39′ 24.82″ N latitude, 44° 58′ 12.42″ E longitude, 1338 m altitude). Environmental conditions of the experimental site from April to September for the 2 years include the monthly highest (27.6 °C) and lowest (11.3 °C) temperature, sum of sunny hours (323.8 h) and rainfall (0.5 mm) during 2014 and 2015. For each year, the experiment was laid out in a factorial arrangement based on a randomized complete block design with three replications. Factors were various irrigation regimes (irrigation after 60 (I_1), 120 (I_2), or 180 (I_3) mm of evaporation from Class A pan), AMF inocula (non-inoculated, *F. mosseae*, or *R. intraradices*) and bacterial strain (non-inoculated or *P. putida* strain P13).

Irrigation water needed before irrigation (VN) is the amount of water needed during irrigation to replenish the soil moisture deficit, thereby restoring the soil to field capacity. The value of VN was calculated according to Benami and Ofen (1984):

$$VN = \frac{[(FC - WP) \times BD \times D \times (1 - ASM) \times A]}{100}$$

where *VN* is the irrigation water needed before irrigation (m^3), *FC* is the field capacity (%), *WP* is the wilting point (%), *BD* is the bulk density (g cm^{-3}), *D* is the root zone depth (m), *ASM* is the available soil moisture before irrigation (a fraction), and *A* is the area of the field (m^2).

The mycorrhizal inoculum (initially isolated from the endemic AMF community of a maize farm) was a mixture of sterile sand, mycorrhizal hyphae, and spores (20 spores g^{-1} inoculum), and colonized root fragments which were produced on maize (*Zea mays* L.) host plants by Dr. Younes Rezaee Danesh at Urmia University. Inoculum (250 g m^{-2}) was banded in the rows below the flax seeds and lightly covered with soil. For the non-mycorrhizal control treatment, seeds were sown without inoculation. Inoculum potentials were 3400 and 3360 propagules per plant for *F. mosseae* and *R. intraradices*, respectively.

Pseudomonas putida strain P13 (abbreviated *P. putida*), was provided by Dr. Malboobi, Green Biotech, Iran. This strain, isolated by screening soil samples collected from different region of Iran, was able to withstand temperature as high as 42 °C, high concentrations of NaCl up to 5%, and a broad range of initial pH from 5 to 11. Such criteria make it a superior candidate for biofertilizers that are able to utilize both organic and mineral phosphate substrates to release absorbable phosphate ion for plants (Malboobi et al. 2009a). Seeds were inoculated with the microbial inoculum (*P. putida*) before sowing. Wet seeds were rolled into the bacterial suspension (10^8 cfu ml^{-1}) until they were uniformly coated. For the non-bacterial control plants, seeds were sown without inoculation.

Prior to sowing, the land was harrowed, plowed and rolled. Seeds (brown flax seeds with 99% germination and 5 g 1000-seed weight) were provided by Agricultural Research, Education and Extension Organization (AREEO) West Azarbaijan, Urmia, Iran. They were sown on 19 April 2014 and 14 April 2015 into a loamy soil by hand at a depth of 2 cm in plots of 1.5-by-2 m size, with plant spacing of 20 by 2 cm. Then they were covered with only a thin layer of soil and the soil was kept moist until they sprouted. After sowing, all plots were given a pre-emergence irrigation. Irrigation treatments were applied after 5 May for 2 years. By this time, flax plants were at the 4-leaf stage.

The soil texture was loamy (32% silt, 24% clay, and 44% sand) with 24% field capacity, 1.2 g cm^{-3} soil density, 1.34% organic matter (OM), 0.9 dS m^{-1} electrical conductivity (EC), 8.1 pH, 0.09% nitrogen, 25 mg kg^{-1} available phosphorus, and 166 mg kg^{-1} available potassium. Soil pH, EC, and concentration of K (using atomic absorption spectrometry), were determined in saturated soil extracts (Tandon 1993), total organic matter by di-chromate oxidation (Nelson and Sommers 1986), and available phosphorus (P_{ava}) by sodium bicarbonate extraction (Olsen and Sommers 1986). Soil texture was determined according to Gee and Bauder (1986). The number of native AMF spores (30 per 10 g soil) (Gerdemann and Nicolson 1963) and *P. putida* strain P13 population (2×10^2 cfu g^{-1} soil) (Minaxi et al. 2013) were determined in the field soil before planting.

Parameters measured

Evaluation of AM fungal colonization

The percentage of flax root length colonized was determined for four plants per treatment. Root colonization was measured in fresh roots (washed with distilled water over a sieve to remove soil) cleared by 10% KOH for 10 min at 90 °C and stained in 0.05% lactic acid-glycerol-Trypan Blue (Phillips and Hayman 1970). The gridline intersection method (Giovannetti and Mosse 1980) was used. Root samples from each plant were evaluated for 100 intersections per sample at ×100 magnification. Total colonization was the number of intersections assessed minus intersections lacking fungal structures divided by the total number of intersections assessed.

Biomass

At the end of the growing season, when the plants had produced mature seeds (on 6, 20, and 27 August in both years for I_3, I_2, and I_1, respectively, with different times of harvesting because of different dates of seed maturity under the three irrigation regimes), all the plants were harvested and the root systems were separated from the shoots. Aerial parts of the plants were dried in a forced-air oven at 70 °C for 2 days and their dry weights were obtained as biomass.

Leaf phosphorus

To measure leaf P, dried leaves were milled, digested, and analyzed as described by Watanabe and Olsen (1965) and Ohnishi et al. (1975) with combustion (4 h at 500 °C) of the leaf sample. The plant ashes (5 mg) were digested in 1 ml of concentrated HCl. The samples were then filtered, and total P was quantified as PO_4^- using the ascorbic acid method (Watanabe and Olsen, 1965). The amount of PO_4^- in solution was determined colorimetrically at 882 nm.

Physiological parameters

Ten plants were used from each replicate plot, and samples of leaves were collected on 6 July in each of the 2 years. Fresh leaf samples were covered with aluminum foil and frozen in liquid nitrogen before being stored in plastic envelopes at −80 °C. These samples were used for determinations of physiological parameters. Fresh leaves were used to measure electrolyte leakage (EL).

Total soluble sugars (TSS)

Leaf total soluble sugars were determined based on the phenol sulfuric acid method (Dubois et al. 1956). In this method, 0.5 g of fresh leaves was 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.

Proline

Leaf proline colorimetric determination proceeded according to Bates et al. (1973) based on proline's reaction with ninhydrin. Leaf tissue (0.5 g) was crushed in a mortar and was homogenized in 10 ml 3% sulfosalicylic acid and the extract was centrifuged to eliminate the leaf tissue. For proline colorimetric determinations, a 1:1:1 solution of proline, ninhydrin acid, and glacial acetic acid was incubated at 100 °C for 1 h. The reaction was arrested in an ice bath, the chromophore was extracted with 4 ml of toluene, and its absorbance was determined in a spectrophotometer at 520 nm. The proline concentration was determined by a standard curve prepared with proline.

Glycine betaine (GB)

The amount of GB was assessed based on the method of Grieve and Grattan (1983). Dry plant material (0.5 g) was mechanically shaken with 20 ml of deionized water for 48 h at 25 °C before filtering. Thawed extracts were diluted 1:1 with 2 N sulfuric acid. The aliquots were kept in centrifuge tubes and cooled in ice water for 1 h. Cold potassium iodide-iodine reagent (0.2 ml) was added. The samples were stored at 0–4 °C for 16 h and were centrifuged at 10,000*g* for 15 min. The supernatant was aspirated. Periodite crystals were dissolved in 9 ml of 1, 2-dichloroethane (reagent grade). After 2–2.5 h, the absorbance was measured at 365 nm with a spectrophotometer.

Evaluation of enzymatic defense activity (catalase, CAT; superoxide dismutase, SOD; ascorbate peroxidase, APX; glutathione reductase, GR)

Frozen leaf samples (0.25 g) were crushed with liquid nitrogen and extracted with a pestle in an ice-cold mortar with 4 ml of 0.05 M Na_2HPO_4/NaH_2PO_4 (pH 7.0) buffer containing 0.2 mM ethylene-diamine tetracetic acid (EDTA) and 1% polyvinylpyrrolidone (PVP). The homogenates were centrifuged at 4 °C for 20 min at 15000 g. The supernatants were collected and used for enzyme activity assays.

CAT activity was assayed by measuring the rate of disappearance of hydrogen peroxide using the method of Maehly and Chance (1959). The reaction mixture contained 2.5 ml of 50 mM phosphate buffer (pH 7.4), 0.1 ml of 1% hydrogen peroxide, and 50 ml enzyme extract diluted to keep measurements within the linear range of the analysis. The decrease in hydrogen peroxide was followed by a decline in absorbance at 240 nm.

APX activity was determined according to the method of Chen and Asada (1989) with minor modifications. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM ascorbate, 1.54 mM hydrogen peroxide, and 50 ml enzyme extract. The oxidation of ascorbate was followed by decrease in the absorbance at 240 nm.

Superoxide dismutase activity was measured using the method described by Dhindsa et al. (1981). SOD activity was assayed by its ability to inhibit photochemical reduction of nitroblue tetrazolium. The test tubes containing the assay mixture (1.5 ml reaction buffer, 0.2 ml of methionine, 0.1 ml enzyme extract with an equal amount of $NaCO_3$, NBT solution, riboflavin, EDTA and 1.0 ml DDW) were incubated in light under 15 W fluorescent lamps for 15 min. Illuminated and non-illuminated reactions without supernatant served as calibration standards. Absorbance of samples and controls (blank) was read at 560 nm wavelength.

Glutathione reductase activity was determined according to Sgherri et al. (1994). Extraction of GR was performed in 1.5 ml of solution containing 100 mM potassium phosphate buffer (pH 7.0), 1 mM Na_2EDTA, and 2% PVP. The homogenates were filtered and centrifuged at 18,000 g for 20 min at 4 °C. The assay mixture comprised 200 mM potassium phosphate buffer (pH 7.5), 0.2 mM Na_2EDTA, 1.5 mM $MgCl_2$, 0.5 mM GSSG, 50 μM NADPH, and enzyme extract containing 100 μg protein in a final volume of 1 ml. Correction was made for the non-enzymatic oxidation of NADPH by recording the decrease in absorbance at 340 nm.

Chlorophylls and carotenoids

Chlorophyll a, chlorophyll b, and carotenoids were estimated by extracting the leaves using 80% acetone as explained by Lichtenthaler (1987). The chlorophyll concentration was calculated based on the following formulas:

$$Ca = 12.25\ A663.2 - 2.79\ A646.8$$
$$Cb = 21.50\ A646.8 - 5.10\ A663.2$$
$$Cx + c = (1000\ \text{A470-1.8Ca-85.02Cb}) \Big/ 198$$

In this formula, C_a, C_b, and C_{X+C} indicate the concentrations of chlorophyll a, chlorophyll b, and carotenoids, respectively. "A" indicates the absorbance at the designated wavelength.

Glutathione (GSH) and ascorbic acid (AsA)

Glutathione concentration was measured as described by Smith (1985). Five hundred milligrams of the leaves was homogenized in a cold mortar with 5 ml 5% (*w*/*v*) sulfosalicylic

acid and the homogenate was filtered and centrifuged at 1000*g* for 10 min. One milliliter of supernatant was neutralized by 1.5 ml of 0.5 mM K-phosphate buffer (pH 7.5). The standard incubation medium was a mixture of: 0.5 ml 0.1 M sodium phosphate buffer (pH 7.5) containing 5 mM EDTA, 0.2 ml 6 mM 5, 5-dithiobis-(2-nitrobenzoic acid), 0.1 ml of 2 mM NADPH, and 0.1 ml (1 unit) glutathione reductase. The reaction was initiated by adding 0.1 ml glutathione standard or extract. The change in absorbance was recorded at 412 nm for 9 min.

Ascorbate measurement was based on the reduction of ferric to ferrous ion with ascorbate in acid solution followed by the formation of a pink complex between ferrous ion and bipyridyl that absorbs at 525 nm. Fresh tissue (0.1 g) was homogenized in 1.5 ml of 5% ice-cold metaphosphoric acid and centrifuged at 10,000*g* for 10 min. Supernatant was collected and used for the estimation of ascorbate according to Law et al. (1983).

Electrolyte leakage (EL)

Leaf samples (freshly harvested, not frozen) were washed with three changes of deionized water to eliminate surface-adhered electrolytes. Leaf samples (0.1 g) were cut into discs of uniform size and incubated in deionized water at room temperature. After 1 h, the electrical conductivity (L1) of the immersion solution was measured by using a conductivity meter. The immersion solution was then placed in a boiling water bath at 100 °C for 10 min, and the electrical conductivity (L2) was measured after cooling. Membrane relative permeability was calculated by the formula L1/L2 × 100% (Bai et al. 1996).

Malondialdehyde (MDA)

Malondialdehyde was measured according to the thiobarbituric acid (TBA) reaction as described by Zhang and Qu (2004). Fresh leaf tissues (0.5 g) were frozen in liquid nitrogen and homogenized in 1 ml of 5% trichloroacetic acid (TCA). The homogenates were transferred into tubes and centrifuged at 4000 g for 10 min at room temperature. Two milliliters of extract was added to 2 ml 0.6% TBA and placed in a boiling water bath for 10 min. Absorbance of the supernatant was determined spectrophotometrically at 532, 600, and 450 nm (A532, A600, and A450). The concentration of MDA was calculated based on the formula:

$$6.45 \times (A532 - A600) - 0.56 \times A450$$

Hydrogen peroxide (H_2O_2)

The concentrations of H_2O_2 were assayed by using 0.5 g of leaf tissue which was homogenized in an ice bath with 5 ml 0.1% (*w*/*v*) TCA. The homogenate was centrifuged at 12,000 g for 15 min, and then 0.5 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI (potassium iodide). The absorbance was measured at 390 nm. H_2O_2 was quantified versus a calibration curve using solutions with known H_2O_2 concentrations (Velikova et al. 2000).

Statistical analysis

To determine the individual and interactive effects, four-way, factorial analysis of variance (ANOVA) including the data collected for both years (with year as a factor) was performed using SAS software. All data were analyzed using a general linear model (GLM). The model was subsequently validated by checking normality and homoscedasticity of residuals. No transformations were used. The mean values of levels within factors were compared by Student–Neuman-Keul (SNK) test at ($P \leq 0.05$).

Results

The combined ANOVA of 2-year data revealed a significant interaction effect of irrigation regimes × bacteria × mycorrhiza on dry weight, total soluble sugars, glycine betaine, glutathione reductase, total chlorophyll, carotenoids, glutathione, ascorbic acid, H_2O_2 ($P \leq 0.01$), root colonization, catalase, malondialdehyde, protein and leaf phosphorus concentration ($P \leq 0.05$; Table 1). Furthermore, there were significant interactions of irrigation regimes × mycorrhiza on electrolyte leakage ($P \leq 0.05$), year × irrigation regimes × bacteria × mycorrhiza on proline and ascorbate peroxidase ($P \leq 0.01$), irrigation regimes × mycorrhiza, irrigation regimes × bacteria and bacteria × mycorrhiza on superoxide dismutase (Table 1).

Biomass

The dry weight of the plants that had been subjected to drought treatment was significantly lower compared to the well-watered plants. Plants cultivated under mild stress conditions that had received inoculation, especially dual-colonized (PSB and AMF) plants, attained a higher dry weight than non-inoculated plants. The great reduction of biomass (33%) in severe stress could not be compensated by any single or dual inoculation treatment. In well-watered plants, biomass was increased by single AMF and PSB inoculation similarly to dual colonization (Table 2).

AM fungal root colonization

The highest root colonization belonged to *R. intraradices* (83%) and *F. mosseae* (77%) in dual-colonized plants irrigated after 120 mm of pan evaporation. The synergistic effect of *P. putida*

Table 1 Combined (2-year data) analysis of variance of some physiological responses to irrigation under phosphate solubilizer and mycorrhizal inoculation of flax

Source of variation	df	Mean Squares											
		Dry weight[a]	Colonization[a]	Leaf phosphorus[a]	df	Proline	Total soluble sugars	Glycine betaine	Catalase	Superoxide dismutase	Glutathione reductase	Ascorbate peroxidases	Total chlorophyll
Year (Y)	1	1.26^{**}	104.03^{ns}	0.00049^{ns}	1	183.11^{**}	56.447^{**}	16.64^{*}	0.95^{**}	10.88^{**}	0.093^{ns}	0.260^{*}	0.464^{ns}
Replicate/Y	4	0.41	31.85	0.00245	3	7.68	2.779	0.75	0.23	0.12	0.073	0.168	0.444
Irrigation(A)	2	2.20^{**}	186.19^{**}	0.37112^{**}	2	3104.39^{**}	979.707^{**}	1089.85^{**}	21.76^{**}	22.87^{**}	5.863^{**}	19.326^{**}	21.615^{**}
Y × A	2	0.42^{**}	3.009^{ns}	0.00028^{ns}	2	208.45^{**}	4.359^{ns}	4.34^{ns}	0.80^{**}	1.01^{**}	0.0001^{ns}	0.357^{**}	1.029^{**}
Pseudomonas(B)	1	0.08^{ns}	21.33^{ns}	0.12335^{**}	1	1598.05^{**}	392.789^{**}	504.56^{**}	14.37^{**}	11.10^{**}	7.185^{**}	10.195^{**}	30.716^{**}
Y × B	1	0.03^{ns}	104.03^{ns}	0.00004^{ns}	1	11.92^{ns}	0.534^{ns}	3.43^{ns}	0.004^{ns}	0.0003^{ns}	0.032^{ns}	1.080^{**}	0.185^{ns}
Mycorrhiza(C)	2	0.23^{**}	$32{,}110.19^{**}$	0.07127^{**}	2	398.87^{**}	181.828^{**}	154.51^{**}	3.15^{**}	4.54^{**}	3.872^{**}	1.794^{**}	14.383^{**}
Y × C	2	0.21^{**}	915.89^{**}	0.00047^{ns}	2	31.73^{**}	2.047^{ns}	0.47^{ns}	0.05^{ns}	0.27^{ns}	0.006^{ns}	0.127^{*}	0.515^{*}
A × B	2	0.02^{ns}	487.86^{**}	0.00031^{ns}	2	1.92^{ns}	10.285^{*}	8.55^{*}	0.24^{*}	0.44^{**}	0.578^{**}	0.755^{**}	6.701^{**}
Y × A × B	2	0.03^{ns}	64.12^{ns}	0.00031^{ns}	2	5.43^{ns}	0.076^{ns}	1.43^{ns}	0.12^{ns}	0.19^{ns}	0.001^{ns}	0.092^{ns}	0.339^{ns}
A × C	4	0.16^{**}	98.26^{*}	0.00700^{**}	4	2.62^{ns}	27.017^{**}	9.91^{*}	0.41^{**}	0.22^{*}	0.189^{**}	1.003^{**}	0.433^{*}
Y × A × C	4	0.17^{**}	35.24^{ns}	0.00009^{ns}	4	13.56^{**}	0.581^{ns}	5.42^{ns}	0.007^{ns}	0.05^{ns}	0.002^{ns}	0.296^{**}	0.071^{ns}
B × C	2	0.06^{ns}	50.69^{ns}	0.00527^{**}	2	18.09^{**}	26.516^{**}	4.24^{ns}	0.40^{**}	0.82^{**}	1.474^{**}	0.072^{ns}	1.815^{**}
Y × B × C	2	0.03^{ns}	19.62^{ns}	0.00028^{ns}	2	8.26^{ns}	4.259^{ns}	0.57^{ns}	0.04^{ns}	0.14^{ns}	0.005^{ns}	0.097^{ns}	0.054^{ns}
A × B × C	4	0.09^{**}	91.76^{*}	0.00160^{*}	4	7.37^{ns}	18.084^{**}	12.02^{**}	0.22^{*}	0.13^{ns}	0.255^{**}	0.444^{**}	0.894^{**}
Y × A × B × C	4	0.03^{ns}	65.74^{ns}	0.00011^{ns}	4	14.59^{**}	3.815^{ns}	1.17^{ns}	0.006^{ns}	0.12^{ns}	0.001^{ns}	0.359^{**}	0.115^{ns}
Error	68	0.02	32.59	0.00064	51	3.18	2.278	2.68	0.07	0.08	0.028	0.042	0.123
Coefficient of variation (%)	13.03	10.93	4.84		6.85	8.35	8.47	6.93	9.49	7.24	8.90	7.74	

Source of variation	df	Mean Squares						
		Total carotenoids[a]	Glutathione	Ascorbic acid	Malondyaldehid	Leaf protein	Electrolyte leakage	H_2O_2
Year (Y)	1	13.792^{*}	0.380^{ns}	1.650^{**}	191.48^{**}	2.60^{**}	0.873^{**}	1.7922^{**}
Replicate/Y	3	0.415	5.915	0.092	2.29	0.35	0.083	0.0361
Irrigation(A)	2	2538.830^{**}	1539.984^{**}	13.489^{**}	5992.32^{**}	6.42^{**}	3.578^{**}	51.1919^{**}
Y × A	2	5.297^{ns}	3.178^{ns}	0.543^{*}	130.97^{**}	0.01^{ns}	0.093^{ns}	0.2849^{ns}
Pseudomonas(B)	1	1209.306^{**}	1109.514^{**}	1.201^{**}	2499.76^{**}	1.23^{**}	3.406^{**}	38.7903^{**}
Y × B	1	1.166^{ns}	0.003^{ns}	0.763^{*}	6.46^{ns}	0.02^{ns}	0.016^{ns}	0.2045^{ns}
Mycorrhiza(C)	2	239.792^{**}	247.047^{**}	12.801^{**}	757.16^{**}	1.12^{**}	0.933^{**}	2.7547^{**}
Y × C	2	1.722^{ns}	0.632^{ns}	0.182^{ns}	29.25^{ns}	0.06^{ns}	0.049^{ns}	0.0313^{ns}
A × B	2	48.297^{**}	56.235^{**}	6.063^{**}	112.91^{**}	0.13^{ns}	0.075^{ns}	0.6935^{*}
Y × A × B	2	0.511^{ns}	1.866^{ns}	0.499^{ns}	11.55^{ns}	0.01^{ns}	0.062^{ns}	0.0003^{ns}
A × C	4	82.932^{**}	50.102^{**}	0.933^{**}	47.30^{**}	0.12^{ns}	0.100^{*}	1.1054^{**}
Y × A × C	4	3.096^{ns}	0.718^{ns}	0.124^{ns}	34.51^{*}	0.05^{ns}	0.039^{ns}	0.1357^{ns}

Table 1 (continued)

B × C	2	49.474**	23.674**	1.316**	37.81*	0.04^{ns}	0.080^{ns}	4.5215**
Y × B × C	2	1.577^{ns}	1.539^{ns}	0.230^{ns}	23.66^{ns}	0.0005^{ns}	0.104^{ns}	0.0361^{ns}
A × B × C	4	62.405**	26.147**	1.363**	38.60*	0.16*	0.064^{ns}	1.4891**
Y × A × B × C	4	0.647^{ns}	2.379^{ns}	0.060^{ns}	22.43^{ns}	0.02^{ns}	0.010^{ns}	0.1729^{ns}
Error	51	2.768	2.523	0.152	9.96	0.05	0.034	0.1489
Coefficient of variation (%)	7.83	7.30	7.39	6.16	8.07	10.97	8.61	

[a] These traits (dry weight, colonization, leaf phosphorus) were measured in 3 replications for each year, while other physiological parameters were measured in two replications for 2014 and three replications for 2015 (Because we lost one replication of leave samples stored in −80 °C in 2014)

*Significant at the 5% probability level; ns, not significant; **Significant at the 1% probability level

on root colonization was observed only in the mild-stress condition, but there was no superiority of dual colonization on root colonization in well-irrigated or severe-stressed plants. Non-inoculated plants (control) had the lowest colonization for all irrigation regimes similar to PSB-only plants (Table 2).

Leaf phosphorus concentration

Leaf phosphorus was diluted by limited irrigation, so the minimum leaf P was observed in severe stressed plants (irrigated after 180 mm of evaporation). Ion phosphorus accumulation in leaves of inoculated plants of both fungal species was higher than those of the leaves of the control and bacteria-only inoculated plants. A positive synergistic effect of test organisms resulted in the highest leaf P in dual-colonized plants in all irrigation regimes (Table 2).

Proline

Leaf proline concentration decreased in all mycorrhizal (single and dual inoculation) plants as a result of water stress. The maximum leaf proline concentration (41.50 and 45.30 mg/ g FW in 2014 and 2015, respectively) was obtained under the most restrictive irrigation regime (I_3) with a decreasing trend in well-watered conditions (I_1). The reduction was more pronounced in co-inoculated plants than non-inoculated control plants or plants singly inoculated with PSB or AMF. Leaf proline concentration in mycorrhizal plants (single and dual colonization) was respectively reduced by 20, 25, and 38% for I_3, I_2, and I_1 (Table 3).

Total soluble sugars

Well-watered plants contained higher total soluble sugars (18.92 μmol/g FW) than those with limited irrigation. The lowest leaf TSS (9.93 μmol/g FW) was obtained in non-inoculated control plants under severe drought conditions being 50% less than well-watered plants. In dual colonization, TSS accumulated in high amounts for all irrigation regimes followed by AMF- and PSB-alone treatments. The superiority of well-watered AMF plants in terms of TSS accumulation changed to similarity with PSB in limited irrigation regimes (Table 2). Leaf TSS was higher in the second than in the first year (Table 4).

Glycine betaine

Glycine betaine was in higher concentration in 2014 than in 2015 (Table 4). In all treatments, accumulation of GB was significantly increased (from 17.84 to 30.21 μmol/g) with drought caused by increasing irrigation intervals. In mycorrhizal plants inoculated with *F. mosseae* or *R. intraradices*, GB accumulation was significantly reduced similarly to bacterial colonized plants. Simultaneous use of fungi and bacteria

Table 2 Comparison of 2-year means of flax traits by irrigation regimes, bacteria, and mycorrhizal fungi species

Irrigation regime[a]	Bacteria	Mycorrhizal Fungi Species	Dry weight (g/plant)	Colonization (%)	Total soluble sugars	Glycine betaine (μmol/g FW)	Catalase (μmol/g FW)	Glutathione reductase (Unit/mg protein)
60		Non-AMF-inoculated	1.24^{bc}	19^{d}	18.92^{e}	17.84 ^{de}	3.77^{cd}	2.23^{ef}
	Non-bacterial	*F. mosseae*	1.52^{a}	61^{c}	19.52^{e}	17.82 ^{de}	3.89^{c}	1.97^{gh}
		R. intraradices	1.65^{a}	68^{bc}	25.90^{b}	13.63^{fg}	4.69^{b}	2.81^{b}
		Non-AMF-inoculated	1.40^{ab}	17^{d}	22.94 ^{cd}	11.86^{g}	4.76^{b}	2.52^{bcd}
	Pseudononas putida P13	*F. mosseae*	1.49^{a}	62^{c}	28.40^{a}	9.36^{h}	5.25^{a}	3.64^{a}
		R. intraradices	1.50^{a}	71^{bc}	27.90^{a}	9.53^{h}	5.29^{a}	3.50^{a}
120		Non-AMF-inoculated	0.96^{cd}	17^{d}	13.38 ^{fg}	24.06^{c}	3.16^{ef}	2.11^{fg}
	Non-bacterial	*F. mosseae*	1.05^{cd}	64^{c}	17.67^{e}	20.22^{d}	3.81^{cd}	1.88^{gh}
		R. intraradices	1.12^{cd}	70^{bc}	15.28^{f}	19.51^{d}	3.54^{cde}	2.31^{def}
		Non-AMF-inoculated	0.96^{cd}	16^{d}	14.59^{f}	20.13^{d}	3.69^{cd}	1.90^{gh}
	Pseudononas putida P13	*F. mosseae*	1.05^{cd}	76^{b}	24.48 ^{bc}	14.61^{fg}	4.72^{b}	2.59^{bc}
		R. intraradices	1.43^{ab}	83^{a}	21.83^{d}	15.55^{ef}	4.60^{b}	2.78^{b}
180		Non-AMF-inoculated	0.89^{d}	19^{d}	9.93^{i}	30.21^{a}	2.61^{g}	1.25^{i}
	Non-bacterial	*F. mosseae*	1.07^{cd}	70^{bc}	12.33 ^{gh}	25.52 ^{bc}	2.46^{g}	1.80^{h}
		R. intraradices	0.93^{d}	75^{b}	10.69 ^{hi}	27.13^{b}	2.84^{fg}	1.78^{h}
		Non-AMF-inoculated	1.14^{cd}	17^{d}	10.56 ^{hi}	27.66^{b}	2.82^{fg}	1.44^{i}
	Pseudononas putida P13	*F. mosseae*	1.00^{cd}	60^{c}	14.46^{f}	22.83^{c}	3.30^{de}	2.40^{cde}
		R. intraradices	0.93^{d}	68^{bc}	15.56 ^{f}	20.23^{d}	3.64^{cd}	2.62^{bc}

Irrigation regime[a]	Total chlorophyll (Unit/mg protein)	Total carotenoids (mg/g FW)	Glutathione (mg/g FW)	Ascorbic acid (μmol/g FW)	Malondyaldehid (μmol/g FW)	Leaf Protein (μmol/g FW)	H_2O_2 (%)	Leaf phosphorus (μmol/g FW) (%)
60	3.64^{efg}	21.90^{cd}	18.37^{f}	5.12^{bcd}	50.52^{fg}	3.01^{bc}	3.99^{de}	0.54^{c}
	5.26^{c}	32.92^{ab}	24.14^{e}	6.33^{b}	42.60^{h}	3.13^{bc}	4.30^{d}	0.59^{b}
	5.50^{c}	34.02^{a}	30.78^{c}	7.53^{a}	37.86^{h}	3.68^{a}	2.75^{gh}	0.62^{b}
	5.59^{c}	34.88^{a}	30.18^{cd}	5.42^{bcd}	37.54^{h}	3.31^{b}	2.53^{h}	0.58^{b}
	7.00^{a}	38.16^{a}	34.71^{b}	5.41^{bcd}	25.02^{i}	3.83^{a}	2.29^{h}	0.69^{a}
	7.08^{a}	35.36^{a}	37.69^{a}	6.16^{b}	24.08^{i}	3.85^{a}	2.70^{gh}	0.70^{a}
120	3.48^{efg}	11.82^{fgh}	16.29^{fg}	3.94^{ef}	63.40^{bc}	2.67^{cd}	6.22^{b}	0.43^{efg}
	3.78^{defg}	18.22^{de}	18.15^{f}	5.95^{bc}	54.64^{efg}	2.78^{cd}	5.56^{bc}	0.52^{c}
	4.03^{def}	18.44^{de}	18.02^{f}	5.83^{bcd}	58.18^{cdf}	3.01^{bc}	4.43^{d}	0.51^{c}
	3.63^{efg}	15.32^{ef}	18.96^{f}	4.72^{de}	55.92^{def}	2.79^{cd}	4.26^{d}	0.46^{de}
	5.22^{c}	29.60^{b}	28.76^{cd}	5.38^{bcd}	48.80^{g}	3.12^{bc}	3.23^{fg}	0.60^{b}
	6.09^{b}	27.42^{b}	27.66^{d}	5.81^{bcd}	39.34^{h}	3.05^{bc}	3.57^{ef}	0.59^{b}
180	3.45^{fg}	9.86^{h}	12.99^{h}	3.76^{f}	70.72^{a}	2.24^{e}	7.22^{a}	0.37^{h}

Table 2 (continued)

Irrigation regime[a]	Total chlorophyll (Unit/mg protein)	Total carotenoids (mg/g FW)	Glutathione (mg/g FW)	Ascorbic acid (μmol/g FW)	Malondyaldehid (μmol/g FW)	Leaf Protein (μmol/g FW)	H_2O_2 (%)	Leaf phosphorus (μmol/g FW) (%)
	3.55^{efg}	9.88^{h}	11.75^{h}	4.20^{ef}	65.94^{ab}	2.74^{cd}	5.83^{bc}	0.41^{g}
	3.86^{defg}	10.20^{gh}	12.84^{h}	3.94^{ef}	65.16^{ab}	2.38^{de}	6.12^{bc}	0.40^{g}
	3.10^{g}	14.38^{efg}	14.40^{gh}	4.58^{ef}	63.54^{bc}	2.36^{de}	4.59^{d}	0.42^{fg}
	3.98^{def}	14.40^{efg}	17.09^{fg}	4.92^{ef}	61.98^{bcd}	2.66^{cd}	5.40^{c}	0.45^{def}
	4.43^{d}	15.50^{ef}	18.38^{f}	6.03^{bc}	56.96^{cdef}	2.76^{cd}	5.62^{bc}	0.48^{d}

Means followed by the same letter in each column are not significantly different

[a] Irrigation after evaporation from Class A pan

showed the highest reduction in glycine betaine concentration (Table 2).

Catalase

In both 2014 and 2015, catalase activity was significantly decreased by extensive irrigation intervals, so the highest and lowest catalase activity belonged to irrigation after 60 and 180 mm of evaporation, respectively. CAT activity of AM and PSB inoculations was higher in I_1 and I_2 than for the control plants. At all irrigation levels, the CAT activity increases were higher for dual colonized plants than those of other treatments (Table 2).

Superoxide dismutase

Increasing irrigation intervals led to a reduction of SOD activity in non-inoculated control flax to less than that in PSB and AMF inoculated plants (Fig. 1a, b). The decreasing trend of SOD activity with increasing irrigation interval was steeper in 2015 than in 2014 (not illustrated). The SOD activity in co-inoculated plants was greater than in plants singly inoculated with an AM fungus or *P. putida* (Fig. 1c).

Glutathione reductase

Co-inoculated plants (AMF + PSB) showed the highest activity of glutathione reductase under all irrigation regimes. We observed a descending trend for GR activity by severity of water deficit stress from 60 to 180 mm of evaporation, although, these reductions were different for AMF and PSB colonized plants (Table 2).

Ascorbate peroxidase

The activity of APX in well-watered plants demonstrated a significant increase in dual-inoculated similar to PSB- and AMF- (*R. intraradices*, only) inoculated plants. There were no significant differences, however, in APX activity of severely stressed plants (I_3). But, APX activity was higher in dual-colonized plants irrigated after 120 mm of evaporation. All changes in APX activity were similar between the 2 years (Table 3).

Glutathione and ascorbic acid

Mycorrhizal plants cultivated under well-watered conditions contained the highest concentrations of non-enzymatic defense compounds (glutathione and ascorbic acid) in their leaves. When the plants were subjected to drought stress (I_2 and I_3), the concentrations of glutathione and ascorbic acid were reduced in all treatments. Although, the leaf glutathione and ascorbic acid

Table 3 Comparison of 2-year means of flax proline and ascorbate peroxidase concentration affected by year × irrigation × bacteria ×mycorrhizae

	Irrigation regime[a]	Bacteria	Mycorrhizal Fungi Species	Proline (μmol/g FW)	Ascorbate peroxidase (Unit/mg protein)
2014			Non-AMF-inoculated	19.25^{i}	$2.43^{f–i}$
		Non-bacterial	*F. mosseae*	16.60^{ij}	$2.38^{f–i}$
			R. intraradices	19.15^{i}	3.16^{cde}
	60		Non-AMF-inoculated	19.00^{i}	3.17^{cde}
		Pseudononas putida P13	*F. mosseae*	10.95^{kl}	3.2^{cd}
			R. intraradices	10.70^{kl}	3.78^{ab}
			Non-AMF-inoculated	37.35^{cd}	$1.81^{i–l}$
		Non-bacterial	*F. mosseae*	32.10^{e}	$2.50^{e–i}$
			R. intraradices	32.70^{e}	$2.43^{f–i}$
	120		Non-AMF-inoculated	31.20^{ef}	$1.99^{h–l}$
		Pseudononas putida P13	*F. mosseae*	24.55^{h}	$2.89^{d–g}$
			R. intraradices	26.02^{gh}	$2.79^{d–j}$
			Non-AMF-inoculated	41.50^{b}	1.45^{lmn}
		Non-bacterial	*F. mosseae*	38.10^{bcd}	$1.65^{j–m}$
			R. intraradices	38.60^{bcd}	1.55^{lmn}
	180		Non-AMF-inoculated	36.10^{d}	1.35^{lmn}
		Pseudononas putida P13	*F. mosseae*	26.90^{gh}	$1.82^{i–l}$
			R. intraradices	29.20^{efg}	$2.03^{h–l}$
2015			Non-AMF-inoculated	18.90^{i}	$2.34^{f–j}$
		Non-bacterial	*F. mosseae*	18.20^{i}	$2.27^{g–k}$
			R. intraradices	14.06^{jk}	3.31^{bcd}
	60		Non-AMF-inoculated	14.00^{jk}	3.64^{abc}
		Pseudononas putida P13	*F. mosseae*	7.80^{l}	3.91^{a}
			R. intraradices	7.53^{l}	4.02^{a}
			Non-AMF-inoculated	30.90^{ef}	1.49^{lmn}
		Non-bacterial	*F. mosseae*	25.43^{gh}	$2.07^{h–l}$
			R. intraradices	27.46^{fgh}	$1.86^{i–l}$
	120		Non-AMF-inoculated	25.06^{gh}	1.37^{lmn}
		Pseudononas putida P13	*F. mosseae*	16.50^{ij}	$3.02^{c–f}$
			R. intraradices	11.60^{k}	$2.90^{d–g}$
			Non-AMF-inoculated	45.30^{a}	0.91^{n}
		Non-bacterial	*F. mosseae*	40.53^{bc}	1.06^{mn}
			R. intraradices	39.36^{bcd}	1.04^{mn}
	180		Non-AMF-inoculated	37.86^{bcd}	$2.69^{d–h}$
		Pseudononas putida P13	*F. mosseae*	32.43^{e}	1.39^{lmn}
			R. intraradices	28.93^{efg}	$1.60^{k–n}$

Means followed by the same letter in each column are not significantly different

[a] Irrigation after evaporation from a Class A pan

concentrations were higher in AM-colonized plants (alone or in combination with *P. putida*) than in the controls, that was to a greater extent in dual colonized plants with *R. intraradices* than *F. mosseae* (Table 2).

Photosynthetic pigments

The highest leaf chlorophyll concentration was obtained in dual-inoculated plants under well-watered conditions, and it was significantly reduced under stress conditions (I_2 and I_3). This declining trend of chlorophyll was observed for PSB-inoculated plants as well as mycorrhizal flax. The superiority of dual colonization in higher leaf chlorophyll was not noticeable under mild stress (Table 2).

Carotenoid, a non-enzymatic defense of plants against water deficit stress, increased in dual-inoculated plants as much as for single-inoculated mycorrhizal plants and PSB inoculation alone. This effectiveness of co-inoculation was in the

Table 4 Comparison means between 2 years for flax traits

Year	Total soluble sugars	Glycine betaine (μmol/g FW)	Total carotenoids (μmol/g FW)	Leaf protein (mg/g FW)	Electrolyte leakage (%)	Hydrogen peroxide (%) (μmol/g FW)
2014	17.10[b]	19.84[a]	20.76[b]	2.76[b]	1.56[b]	4.30[b]
2015	18.72[a]	18.96[b]	21.56[a]	3.11[a]	1.76[a]	4.59[a]

Means followed by the same letter in each column are not significantly different

order of $I_2 > I_3 > I_1$ (Table 2). Total carotenoids were higher in 2015 than in 2014 (Table 4).

Malondialdehyde

As an indicator of lipid peroxidation, the concentration of malondialdehyde was higher in the leaves of plants exposed to drought stress versus those of the controls. The concentration of MDA in I_2 and I_3 increased 25 and 42% more than in plants that were well irrigated (I_1), respectively (Table 2). The use of microorganisms had a significant role in modifying this increase. Therefore, in I_2 and I_3, concentrations of MDA in mycorrhizal plants were lower than in control plants. The combination of *P. putida* with two types of AM fungi resulted in 50, 38, and 14% reductions in MDA for I_1, I_2, and I_3, respectively. Between AMF species, there was no superiority with respect to MDA concentration reductions (Table 2).

Electrolyte leakage

In flax leaves, increasing irrigation intervals caused major damage to the cell membrane in non-mycorrhizal control

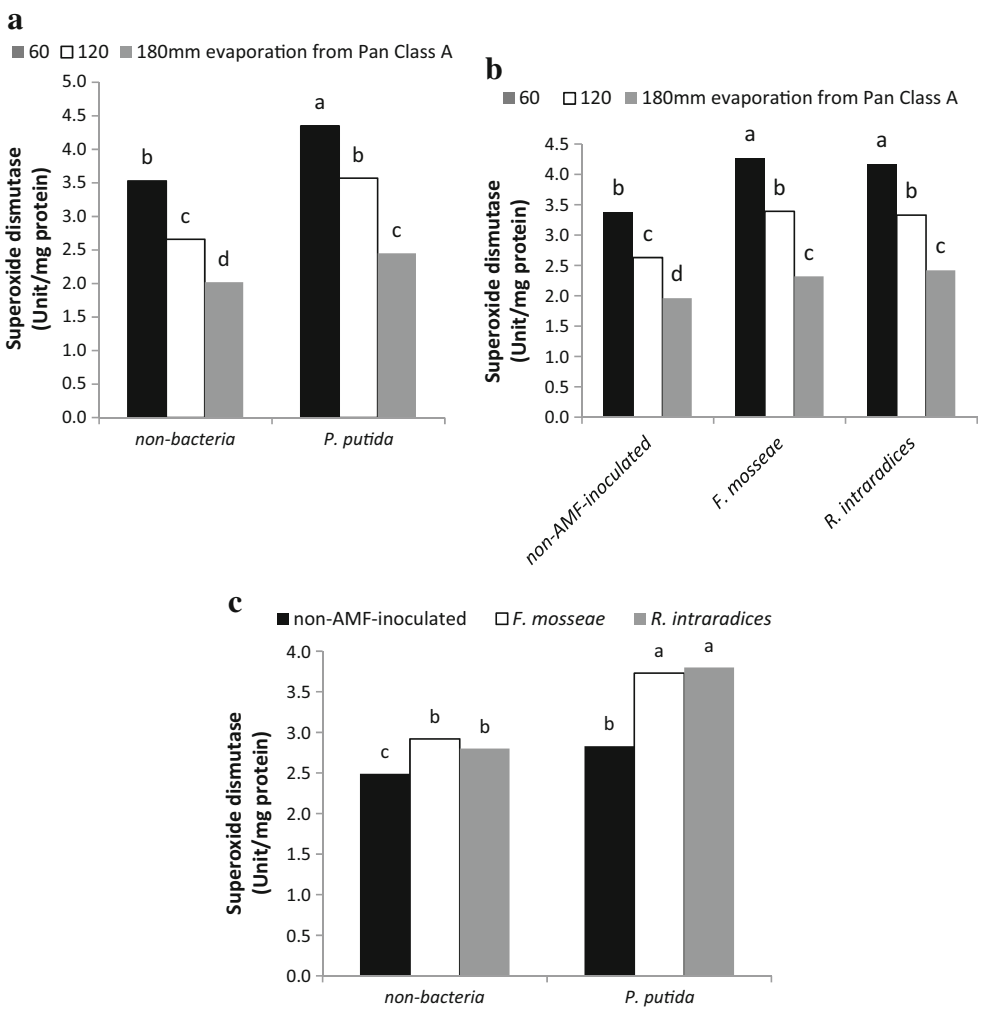

Fig. 1 Interaction effects of irrigation regimes × bacteria (**a**), mycorrhizal fungi ×irrigation regimes (**b**), mycorrhizal fungi × bacteria (**c**) on superoxide dismutase (SOD) activity in the leaves. (Means followed by the same letter are not significantly different)

plants as revealed by electrolyte leakage. In AM-colonized plants, EL significantly dropped to the same extent with both species of inoculant fungi, thus indicating compensation of cell membrane damage (Fig. 2a). We also observed a 22% reduction of EL in plants colonized with the PSB compared to the controls (Fig. 2b). The highest electrolyte leakage was observed in 2015 (Table 4).

Hydrogen peroxide

Leaf H_2O_2, a non-free-radical which is involved in a number of signaling cascades in plants was increased along with increasing water deficit stress from I_1 to I_3. Both AMF species reduced the leaf H_2O_2 in each irrigation regime, although *R. intraradices* was more effective than *F. mosseae*. This reduction was greater in plants inoculated with *P. putida* than for mycorrhizal plants. Dual-inoculated plants, however, exhibited the greatest reduction (45% for I_2 and 24% for I_3) of H_2O_2 compared to non-mycorrhizal control plants (Table 2). Nevertheless, the leaf hydrogen peroxide concentration was in greater in 2015 than in 2014 (Table 4).

Protein concentration

The highest concentration of protein was achieved with well-watered dual inoculation as well as bacterial inoculation alone. Protein concentration significantly decreased with increasing irrigation interval. The single- and dual-inoculated plants showed similar concentrations of leaf protein in I_2 and I_3. In spite of small alterations of leaf protein concentrations under stressed conditions, AM-colonized plants (*R. intraradices* in I_1 and *F. mosseae* in I_3) produced higher protein concentrations than non-inoculated control plants (Table 2). The highest concentration of leaf protein over all treatments was observed in 2015 (Table 4).

Discussion

Despite there being native AMF in the field soil, mycorrhizal colonization was enhanced by AMF inoculation both without and with PSB and likely contributed to all the results. The yield (biomass) improvement in AM and PSB treatments for well-watered plants and in the dual-inoculated plants with *R. intraradices* for I_2 were consequences of plant-microorganism interactions in response to drought.

Because of high solubility of phosphorus in well-irrigated soil, leaf phosphorus concentration was reduced by greater irrigation intervals with which there likely was diminished solubility, mobility (mass flow or diffusivity), transport between roots and shoots, and thus P uptake under drought conditions (Sawers et al., 2008; He and Dijkstra, 2014). The greater P uptake by plants co-inoculated with PSB and AM fungus can be attributed to transport of P by the AM fungus after solubilization by PSB (Minaxi et al., 2013) reflecting access to a large soil volume which facilitates absorption and dissolution of relatively-insoluble P (Neetu et al. 2011).

The reported results of 2-year increase in leaf proline concentration in consequence of increasing water deficit stress suggest that the production of proline probably is a common response of flax under drought conditions as an osmotic adjustment. A lower concentration of proline may be attributed to either greater drought resistance or less injury of colonized plants under drought stress conditions (Wu and Xia 2006), and proline concentration was lower in dual-inoculation treatments than in single-inoculant treatments.

We also observed a reduction in the total soluble sugars concentration when flax plants were subjected to drought. Although previous studies (Masoudi-Sadaghiani et al. 2011; Vijayalakshmi et al. 2012) have demonstrated that water deficit can increase concentrations of TSS in leaves, other studies indicated no change or even diminished concentrations in stressed plants such as we found. Jamil

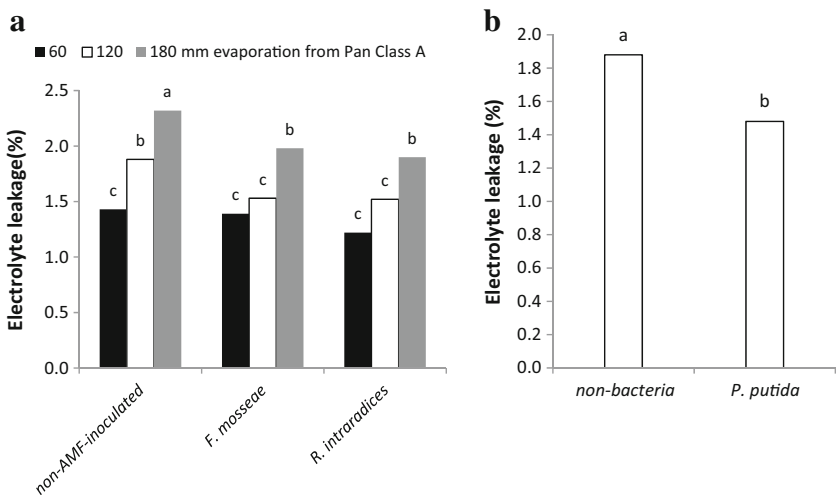

Fig. 2 Interaction effect of irrigation regimes × mycorrhizal fungi on electrolyte leakage (**a**). Effect of bacteria on electrolyte leakage (**b**). (Means followed by the same letter are not significantly different)

Marur et al. (1996) reported a 55% decrease of TSS in stressed plants versus controls. The decrease in TSS in response to drought might be related to limited carbohydrate availability, as a result of reduced photosynthesis (Goicoechea et al. 2005; Gogorcena et al. 1997). 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 total soluble sugars. Nevertheless, we found synergistic effects of dual inoculation (mycorrhizal fungi plus *P. putida*) on TSS concentrations likely because of enhanced photosynthesis.

Increased glycine betaine with water stress conditions (I_2 and I_3) was reduced by the application of AM fungi and PSB. This indicates that AM fungus and PSB inoculation alleviated the water stress. Accumulation of GB was reported in cotton (Lv et al. 2007) as an effective compatible solute enhanced by drought stress. The major role of GB might be protection of the integrity of the cell membrane from drought stress damage and involvement in osmotic adjustment (Lv et al. 2007).

In our study, all enzyme activities diminished with increasing irrigation interval. Increased enzyme activities in mycorrhizal plants, PSB-inoculated plants, and dual-inoculated plants indicated that the microorganisms alleviated the oxidative damage from water shortage. Similar to our results, Sandhya et al. (2010) found that inoculation with *Pseudomonas* augmented antioxidants under severe drought conditions, suggesting that they can alleviate the drought-induced oxidative damage. Additionally, symbiosis with mycorrhizal fungi 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 (Ruiz-lozano et al. 1996). Detoxification of cellular H_2O_2 through the activity of the Asada-Halliwell scavenging cycle, which involves the oxidation and re-reduction of ascorbate and glutathione through the action of APX and GR, among other enzymes, is an important element of plant defense against ROS (Donahue et al. 1997). The reduction in CAT activity is regarded as a general response to water stress (Pan et al. 2006; Liu et al. 2008) as a result of the inhibition of enzyme synthesis or change in the assembly of enzyme subunits under stress conditions. It also may be associated with degradation caused by induced peroxisomal proteases or may be a consequence of the photo-inactivation of the enzyme (Liu et al. 2008). Under stress conditions, the modest increase of leaf CAT activity in *P. putida*-treated and mycorrhizal plants that we observed suggests that inoculated plants might have a potential to activate this enzyme to counteract oxidative, water deficit-induced damage (Ghorbanpour et al. 2013; Wu and Zou 2009). Thus, the inoculants were able to regulate oxidative reactions and antioxidant defense (Ortiz et al. 2015). Effects of each of the mycorrhizal and bacterial inoculants on the water content of plants were obvious, whereas a maximized positive effect on water content was observed in dual-inoculated plants (Armada et al. 2014; Marulanda et al. 2009). Notwithstanding, plants in drought conditions may show different trends (increase, decrease or no change) in antioxidant enzyme activities (Masoudi-Sadaghiani et al. 2011; Armada et al. 2014).

In our experiment, drought stress tended to diminish leaf chlorophyll concentrations (Kpyoarissis et al. 1995). Under well-watered conditions, however, the increased chlorophyll concentrations that we observed with dual inoculation of AMF and PSB might have contributed to an increased rate of photosynthesis (Vafadar et al. 2014) possibly related to a large number of chloroplasts in bundle sheaths in the leaves (Krishna and Bagyaraj 1984).

Overexpression of glutathione reductase in chloroplasts doubled the concentrations of ascorbate and glutathione in leaves and conferred increased resistance to oxidative stress. The decrease in glutathione concentration under drought stress (Foyer et al. 1997) resulted in enhanced lipid peroxidation. Inoculation with AMF increased ascorbate and glutathione as protective compounds to cope with the harmful effects of water shortage. Flax plants benefited not only from the AM symbiosis but also from *P. putida* irrespective of the watering level, similar to the results of Ruiz-Sanchez et al. (2011). Both AMF inoculation and inoculation with PSB alone significantly increased ascorbate concentration versus the control treatment, and they had an additive effect after co-inoculation. Under severe drought, higher MDA concentrations in leaves may be associated with higher accumulation of H_2O_2 in stressed plants which could reveal the degree of membrane lipids peroxidation (Gill and Tuteja 2010). Our inoculated plants, however, showed less MDA than their respective controls suggesting the involvement of both types of microorganisms in ROS metabolism (Mo et al. 2016).

Water deficit-induced electrolyte leakage was reduced by mycorrhizal inoculation as well as by *P. putida* inoculation. The low value of EL in addition to the low MDA concentration with AM symbiosis provides evidence of reduced cell membrane damage (Fouad et al. 2014). Such increased membrane stability has been attributed to mycorrhiza-mediated enhanced P uptake and increased antioxidant production (Feng et al. 2002).

The protection of host plants against oxidative stress by increasing antioxidant enzyme activities is responsible for the elimination of ROS, as evidenced by the lower accumulation of H_2O_2 (Fouad et al. 2014). AM formation contributes to the production of scavenging peroxyl radicals, buffering cellular free-radicals and producing a powerful ROS-scavenging system (Ashraf and Foolad 2007).

Elevated leaf protein concentrations occurred in plants inoculated with AMF and PSB alone as well as in dual-inoculated plants depending upon drought stress. High concentrations of protein might be attributable to improved

efficiency of the osmotic regulation mechanism, which in turn prevents protein catabolism under stress (Kumar et al. 2010) and induces the synthesis of osmotically active proteins. The protein increase can lead to membrane stabilization and, related to MDA and EL, helps plants to grow under stress conditions (Goudarzi and Pakniyat 2009). Protein synthesis can be dramatically reduced or even stopped in stressed plant leaves and roots because of decreased photosynthesis (Mohammadkhani and Heidari 2008).

Conclusions

Based on these results, increased osmolytes (proline and glycine betaine) under drought conditions support their potential to protect flax plant cells via osmoregulation. Drought-induced oxidative damage (H_2O_2, MDA and EL) were reduced in AMF-inoculated plants as well as PSB-inoculated plants, and was lowest after dual inoculation. Dual inoculation increased enzymatic and non-enzymatic antioxidants more than in plants inoculated only with AMF or PSB. The microorganisms maintained an appropriate plant water status under drought through improved osmotic adjustment owing to the enhancement of compatible solutes and the regulation of antioxidant systems. Therefore, we have demonstrated that the use of the biofertilizers (AM fungi and PSB) in an open field trial can mitigate water stress damage by alleviating ROS and can improve water stress tolerance.

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