

The Physiological Properties of Lentil (*Lens culinaris* Medik.) in Response to Mycorrhizal Fungi and Phosphate-solubilizing Bacterium under Irrigation Condition

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ABSTRACT

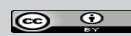
To investigate the effect of mycorrhizal fungi and phosphate-solubilizing bacterium on physiological properties in lentils under irrigation conditions, a factory trial based on a Randomized Complete Block Design was conducted in two years (2018 and 2019) with three replications at Urmia University. The treatments included the combined and individual application of biofertilizers {*Funneliformis mosseae* (mycorrhizal fungi), *Pseudomonas putida* P13 (phosphate-solubilizing bacterium), *F. mosseae* + *P. putida*, control in which no biofertilizer was applied} under rainfed condition and once supplementary irrigation. The contents of hydrogen peroxide, malondialdehyde, and glycine betaine increased by an average of 9.80, 29.36 and 15.31% under rainfed conditions. In dual colonized plants with *F. mosseae* + *P. putida*, the contents of hydrogen peroxide, malondialdehyde, and glycine betaine were substantially reduced. The enzyme activity of catalase, glutathione reductase and the content of glutathione were reduced under rainfed plants. Plants inoculated with *F. mosseae* + *P. putida* had higher enzymatic and non-enzymatic defence than plants inoculated with either the fungal or bacterial inoculum. As compared to control, biofertilizer application improved leaf phosphorus, soluble sugars, and forage yield by an average of 24.13, 19.38, and 25.20 % under irrigated conditions and 9.09, 11.08, and 7.68 % under rainfed conditions. Under supplemental irrigation, plants treated with *F. mosseae* + *P. putida* demonstrated the greatest increases in leaf phosphorus (by 31.03 %), soluble sugars (by 30.25 %), and forage yield (by 32.09 %). The integrated application of biofertilizers in rainfed conditions can enhance lentil growth and forage yield via enhancing antioxidant activities function, which can be desirable for sustainable agriculture.

Keywords: Mycorrhiza, Soluble sugars, Glycine betaine, Catalase, Sustainable agriculture

Lentil belongs to the family Fabaceae, is a plant capable of growing in adverse environments and poor soils. It is highly effective in rotations with crops, especially grains, in rainfed farms. Also, it is a rainfed crop and is moderately drought tolerant,

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but its yield is radically reduced with increasing drought conditions (Sinha *et al.* 2018). In addition to the lentil grains used for human food, the shoots of the plants are harvested before grain maturity (shorter growth period) as high-quality forage. The straw, pod shell, and the residue of lentil plants are of high nutritional value (Ghanem *et al.* 2015).

A significant portion of Iran's land is listed as arid or semi-arid zone where water scarcity is the main limiting factor of crop yield. Increasing water productivity with respect to crop yield per unit area is the best approach for rainfed farming systems (Abbasi *et al.* 2014; Raza *et al.* 2021). In rainfed farming, most phenomena and factors are uncontrollable and non-amendable despite the influences they exert on farming. Rainfall variations across different years, the variations of rainfall amount and distribution, temperature variations, and the lack of rainfall in a part of the growing season, which is a characteristic of rainfed farming, increase the risk of rainfed farming and impair their reliability and stability (Vora *et al.* 2016). Supplemental irrigation is a highly efficient operation that enhances crop production and improves livelihood in highly potent arid regions (Mahmood *et al.* 2015). So that only one or two supplemental irrigations of rainfed legumes can ensure and increase crop production remarkably (Amiri *et al.* 2016; Saadat *et al.* 2019). Indeed, integrated supplemental irrigation ensures the maximum use of rainfall and very limited water reserves of a region to supply the moisture requirements of the plants at suitable times (Saadat *et al.* 2019).

Several studies have reported the increased accumulation of ROS (Reactive Oxygen Species) under water deficit. Plants reduce the produced ROS via enzymatic and non-enzymatic antioxidant mechanisms (Waszczak *et al.* 2018). ROS accumulation in cells impairs membrane lipids, nucleic acids, and proteins. During photosynthesis under water stress conditions, a high rate of electron leakage towards oxygen radicals occurs resulting in the generation of various kinds of ROS, such as superoxide, hydrogen peroxide, hydroxyl radicals, and oxygen radicals (Seleiman *et al.* 2021; Ngugi *et al.* 2021). To cope with ROS-induced oxidative damage, plants use both enzymatic and non-enzymatic antioxidant mechanisms (Choudhury *et al.* 2017).

A mechanism of response to environmental stress is the osmotic adjustment. Osmotic adjustment is a type of adaptation with water deficit stress that can maintain cell turgor and the relevant processes under low water potential via the accumulation of dissolved materials inside the cells (Saadat *et al.* 2019). In plants exposed to water deficit stresses, organic molecules with low molecular weight, e.g. dissolved sugars, proline, protein, and betaine of plant roots and shoots, act as osmotic adjusters (Suprasanna *et al.* 2016). Dissolved sugars work as osmotic adjusters, cell membrane stabilizers, and cell turgor guards. Osmotic adjustment is better in plants in which dissolved sugars are accumulated in reaction to water deficit stress (Thalmann *et al.* 2016).

Mycorrhiza fungi symbiosis with plants has several impacts on plant growth and development so that these fungi can make changes in plant water relations and improve water deficit tolerance in host plants (Mathimaran *et al.* 2017). Mycorrhizas impacts on micro-macronutrients uptake as well as water uptake under water deficit conditions, help the production of physiological processes, mitigate the negative effects of environmental changes, enhance plant tolerance to pathogens, alleviate root injuries, contribute to soil granulation, make improvement nitrogen fixation, and enhance some quantitative traits (Latef *et al.* 2016). Among the main elements that are widely and actively absorbed by mycorrhiza is phosphorous (Williams *et al.* 2017). Mycorrhiza can alleviate water deficit damages via reducing the peroxidation of the lipids and increasing osmotic modification compound accumulation, and antioxidant enzyme activity (Pedranzani *et al.* 2016). Phosphate deficiency in soil can severely limit plant growth productivity of legumes, where both the plants and their symbiotic bacteria are affected and this may have a deleterious effect on nodule formation, development and function (Alikhani *et al.* 2007). Phosphate solubilizing bacterium that can increase cell division in lentil plant, alter root structure, enhance root hairs number, and enhance nutrient absorption (Singh *et al.* 2018).

The present study aims to assess the effect of supplemental irrigation and biological fertilizers on the agrobiological and chemical properties of lentils in rainfed farming systems.

MATERIALS AND METHODS

Study area and experimental design

The research was carried out in the Faculty of Agriculture of Urmia University in two years (2018 and 2019) with eight treatments and three replications, as a factorial experiment based on a Randomized Complete Block Design in a lentil plant. The treatments included the combined and individual application of biofertilizers {*Funneliformis mosseae* (mycorrhizal fungi), *Pseudomonas putida* P13 (phosphate-solubilizing bacterium), *F. mosseae* + *P. putida*, control in which no biofertilizer was applied} under rainfed condition and once supplementary irrigation. The mycorrhizal inoculum was composed of a combination of sterile sand, mycorrhizal hyphae and spores (10 spores g⁻¹ inoculum, mostly yellow-brown with different size), that 35-g of inoculum was added to plots at sowing time, just in the rows below the seeds (Rahimzadeh and Pirzad, 2017). Wet seeds were poured into the suspension of bacteria (108 CFU ml⁻¹) until they were evenly covered. The seed has been cultivated without inoculation for non-microbial and non-bacterial control crops (Deshmukh *et al.* 20007). For the two growing seasons, average monthly rainfall and air temperature are shown in Fig. 1.

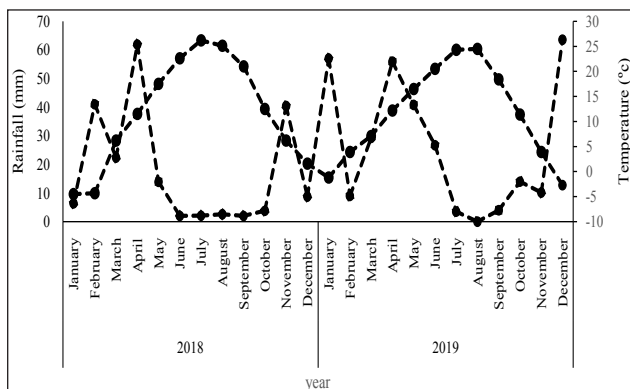


Fig. 1: The average monthly temperature and rainfall in the growing seasons of 2018 and 2019

Seeds were sown on 10th March 2018 and 12th March 2019, by hand at a depth of 5 cm in plots. The distance between the rows was 20 cm and the distance between the plants on the row was 10 cm. The study used lentil seeds with 98% vigour and 99% purity. Farm soil was of loam-clay texture with pH of 8, N content of 0.092%, and P and K contents of 8.1 and 411 mg kg⁻¹, respectively. Supplemental

irrigation was applied at flower initiation by method (Benami and Ofen, 1983). At the full flower stage (82 days after sowing), the treatments were sampled. Then, they were separately placed in nitrogen tanks and were placed in a freezer at -80°C. To determine the final yield, a square meter per plot after eliminating marginal effects was harvested and the forage yield was specified in kg ha⁻¹. Lentil plants in plots were harvested on the last week of June 2019 and 2020.

Determination of agrobiological and chemical parameters

Root colonization

After the flowering stage, the fresh roots of 10 plants per plot were cleared in 10% KOH and stained in 0.05% lactic acid-glycerol-trypan blue based on Phillips and Hayman (1970). Root colonization was assessed using the guiridine intersect method of Liu and Luo (1994).

Leaf nutrients

At the end of the flowering stage, Leaves were sampled for measuring P and K. Leaf nutrients were measured as per AOAC standard by dry digestion method using nitric, hydrochloric, and perchloric acids (AOAC, 2007). The samples were then filtered, and the phosphorus (P) content was determined calorimetrically by the vanadomolybdate method. The colour intensity was determined as an absorbance maximum at 470 nm using a Spectronic 20 colourimeter, and potassium content was determined with a flame photometer (Chapman and Pratt, 1961).

Dissolved sugars

Total dissolved sugars of leaves were estimated by the phenol-sulfuric acid method. This method is based on acidic hydrolysis of dissolved sugars that produces furfural compound. This compound reacts with phenol to produce a colourful complex. 0.5 g of leaves were homogenized with 70 % ethanol. The extract was infiltrated and was well-mixed with 5 phenol. Then, it was added with 5 mL of 98 % sulfuric acid and one hour later, the absorption was read at 485 nm with a spectrophotometer (Dubois *et al.* 1956).

Glycine betaine

Glycine betaine content was determined by Grieve and Grattan (1983) method. To measure glycine betaine content, 0.5 g of plant tissue was mixed with 20 mL of distilled water and it was kept at 25°C for 48 hours. After infiltration, the extract (1:1) was diluted with 1 mol L⁻¹ sulfuric acid. The samples were kept in iced water in a centrifuge tube for 1 hour. Then, it was added with 0.2 mL of cold iodide-potassium iodine reagent and was slowly mixed with a vortex. The samples were kept at 0-4°C for 16 hours. Then, they were centrifuged at 10,000 rpm for 15 minutes, 1 mL of supernatant was isolated with a micropipette, and it was dissolved in 9 mL of 1,2-dichloroethene (as the reagent). Two hours later, its absorption was read at 365 nm with a spectrophotometer.

Determination of enzymatic activity

Fresh sample of lentil leaves (0.25 g) were ground in 4 mL of 0.05 M potassium phosphate buffer (pH = 7.5) containing 1% polyvinylpyrrolidone (PVP) and 0.2 mM EDTA. All extraction stages were performed on ice. Then the extracts were centrifuged at 15,000 rpm at 4°C for 20 minutes, and the clear supernatant was used to estimate the activity of the enzymes.

Catalase

Catalase activity was assessed based on the variation of hydrogen peroxide (H₂O₂) concentration at 240 nm. In this procedure, catalase of the sample decomposes hydrogen peroxide, thereby preventing its absorption at 240 nm. The reaction mixture contains 2.5 mL of 50 mM phosphate buffer (pH = 7.5), 0.1 mL of 1% hydrogen peroxide, and 50 mL of enzyme extract. Enzymatic activity was read based on absorption variations in 60 seconds as per mg of protein (Maehly and Chance, 1959).

Glutathione reductase

The activity of glutathione reductase was measured in terms of the reduction of oxidized glutathione (GSSG) by glutathione reductase using NADPH. The reaction mixture contained 100 mM potassium phosphate buffer (pH = 7), 0.5 mM oxidized glutathione (GSSG), 50 μM NADPH, 1.5 mM MgCl₂, 0.2 mM Na₂EDTA, and enzymatic extract. The absorption was read at 340 nm with a spectrometer (Sgherri *et al.* 1994).

Determination of non-enzymatic defence (activity)

Glutathione

Glutathione content was determined by Smith (1985) method. 0.5 g of the leaf was homogenized with 5 mL of 5% sulfosalicylic acid in a cold mortar and the extract was infiltrated and centrifuged at 1000 rpm for 10 minutes. Then, 1 mL of supernatant was taken and its pH was adjusted to 7.5 using 1.5 mL of 0.5 mM potassium phosphate buffer. The reaction solution was composed of 0.5 mL of 0.1 M phosphate sodium buffer, 5 mM EDTA, 0.2 mL of 6 mM 5,5-dithiobis (2-nitrobenzoic acid), 0.1 mL of 2 mM NADPH, and 0.1 mL of glutathione reductase. The reaction commenced as soon as 0.1 mL of the extract was added. The variation of the absorption was read at 412 nm with a spectrophotometer.

Malondialdehyde

To measure malondialdehyde with respect to thiobarbituric acid (TBA) reaction, 0.5 g of fresh leaf tissue was ground in 1 mL of 5% trichloroacetic acid (TCA). The extract was centrifuged at 4000 rpm at room temperature for 10 minutes. 2 mL of the extract was added to 2 mL of 0.6 % TBA and was placed in a bain-marie for 10 minutes. The absorption of the supernatant was read at 532, 600, and 450 nm, and the malondialdehyde content was estimated by the following equation (Zhang and Qu, 2004):

$$MDA = 645 \times (A_{532} - A_{600}) - 0.56 \times A_{450}$$

Hydrogen peroxidase

0.5 g of leaf sample was homogenized in 5 mL of 0.1% TCA. Then, the samples were centrifuged at 15,000 rpm at 4°C for 15 minutes. Then, it was added with 0.5 mL of supernatant, 0.5 mL of 10 mM potassium phosphate buffer (pH=7), and 1 mL of 1 M potassium iodide. Hydrogen peroxide concentration was estimated by the comparison of its absorption read at 390 nm with a spectrophotometer (Velikova *et al.* 2000).

Statistical analysis

All data were tested to ensure their normality and



then analyzed with SAS 9.1 software package. Means were compared using Duncan's Multiple Range Test at $P \leq 0.05$.

RESULTS AND DISCUSSION

Combined analysis of two-year data showed that the interaction of year and irrigation, the interaction of year and biofertilizer and the interaction of irrigation conditions and biofertilizer had a significant effect on glutathione reductase activity. The concentrations of hydrogen peroxide, malondialdehyde and potassium were affected by the simple effects of irrigation conditions and biofertilizer. The simple effects of year and interaction of irrigation conditions and biofertilizer on phosphorus concentrations, catalase activity and lentil forage yield were significant. Whereas the concentrations of soluble sugars, glycine betaine, glutathione and root colonization were affected by the interaction of irrigation conditions and biofertilizer (Table 1).

Root colonization

The highest root colonization, (70.87 %) was found to be related to supplemental irrigation and the application of *F. mosseae* + *P. putida*. In the rainfed condition, The highest root colonization (54.07%) belonged to *F. mosseae* + *P. putida* which was with the same as *F. mosseae*. Treatment with *F. mosseae* under supplemental irrigation increased root colonization

by 69.64% compared to control rainfed plants (Fig. 2).

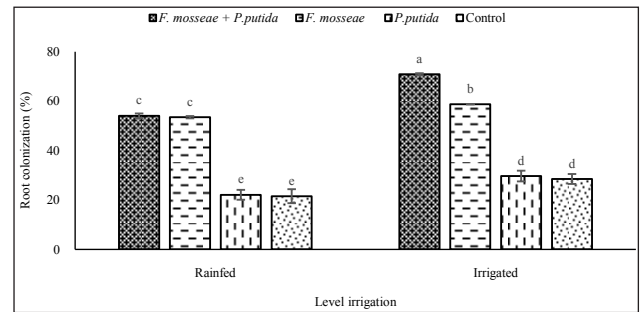


Fig. 2: The interaction of irrigation condition and bio fertilization on root colonization; (Means with same letters are not significantly different based on Duncan's multiple range test $P \leq 0.05$)

The suppression of growth and root colonization under water stress conditions may be caused by modifications in the hyphae's morphological characteristics or by reduced spore development (Saadat *et al.* 2019; Chen *et al.* 2020). The effect of microorganisms in increasing water stress tolerance procedures appears more related to the percentage of root colonized as previously mentioned (Saadat *et al.* 2019; Begum *et al.* 2019). It is mentioned that the positive impact of microorganism inoculation on AMF colonization was important to stimulate the active structures in AMF colonization influencing the metabolic and physiological fungal function which can be regarded as the most significant

Table 1: Variance analysis (ANOVA) of physiological traits of lentil (*Lens culinaris* Medik.) as influenced by the interaction of irrigation conditions and biofertilizer

| S.O.V. | df | Mean Squares | | | | | | | | | | |
|--------------------|----|-----------------------|--------------------------|-----------------------|------------------------|----------------------|-----------------------|----------------------|-------------------------------|----------------------|-----------------------|-------------------------------|
| | | Coloni- zation | Forage yield | Leaf P (%) | Leaf K (%) | Dissolved sugars | Glycine betaine | Cata- lase | Gluta- thione reductase | Gluta- thione | Malondi- aldehyde | H ₂ O ₂ |
| Year | 1 | 14.37 ^{ns} | 452864.74 ^{**} | 0.0004 [*] | 0.006 ^{ns} | 0.05 ^{ns} | 1.97 ^{ns} | 2.41 ^{**} | 23.11 ^{**} | 0.06 ^{ns} | 0.007 ^{ns} | 0.06 ^{**} |
| Replication (Y) | 4 | 7.99 | 1243.46 | 0.0001 | 0.002 | 0.48 | 0.80 | 0.008 | 0.003 | 0.31 | 8.15 | 0.002 |
| Irrigation (I) | 1 | 1005.12 ^{**} | 1559173.33 ^{**} | 0.03 ^{**} | 0.44 ^{**} | 226.72 ^{**} | 129.46 ^{**} | 10.00 ^{**} | 3.61 ^{**} | 295.86 ^{**} | 4300.13 ^{**} | 0.12 ^{**} |
| Fertilization (F) | 3 | 466.80 ^{**} | 350135.70 ^{**} | 0.004 ^{**} | 0.06 ^{**} | 33.27 ^{**} | 20.12 ^{**} | 1.46 ^{**} | 0.47 ^{**} | 25.95 ^{**} | 141.93 ^{**} | 0.05 ^{**} |
| Y × I | 1 | 0.03 ^{ns} | 1018.90 ^{ns} | 0.00003 ^{ns} | 0.00003 ^{ns} | 0.0002 ^{ns} | 0.01 ^{ns} | 0.005 ^{ns} | 1.54 ^{**} | 0.26 ^{ns} | 0.0002 ^{ns} | 0.000008 ^{ns} |
| Y × F | 3 | 0.56 ^{ns} | 790.83 ^{ns} | 0.00002 ^{ns} | 0.000002 ^{ns} | 0.0003 ^{ns} | 0.001 ^{ns} | 0.0002 ^{ns} | 0.19 ^{**} | 0.28 ^{ns} | 0.001 ^{ns} | 0.00003 ^{ns} |
| I × F | 3 | 81.03 ^{**} | 196407.82 ^{**} | 0.001 ^{**} | 0.001 ^{ns} | 6.05 ^{**} | 0.29 ^{ns} | 0.48 ^{**} | 0.16 ^{**} | 6.90 [*] | 6.52 ^{ns} | 0.001 ^{ns} |
| Y × I × F | 3 | 1.44 ^{ns} | 525.09 ^{ns} | 0.00004 ^{ns} | 0.000006 ^{ns} | 0.0005 ^{ns} | 0.00001 ^{ns} | 0.0009 ^{ns} | 0.15 ^{**} | 0.49 ^{ns} | 0.001 ^{ns} | 0.0002 ^{ns} |
| Error | 28 | 4.77 | 1165.55 | 0.00008 | 0.001 | 0.72 | 0.53 | 0.01 | 0.005 | 0.60 | 8.18 | 0.0009 |
| C.V. | | 5.15 | 2.03 | 3.71 | 3.58 | 3.77 | 3.57 | 3.39 | 3.88 | 3.46 | 5.20 | 3.11 |

^{*}, ^{**} and ^{ns} show statistical significance at the $P < 0.05$ and $P < 0.01$ levels and non-significance, respectively.

indexes of beneficial AMF interaction (Saadat *et al.* 2019; Begum *et al.* 2019).

Forage yield

Forage yield was higher in plants treated with supplemental irrigation than those exposed to rainfed conditions. Also, the highest forage yield of 2124.78 kg ha⁻¹ was obtained from supplemental irrigated plants treated with *F. mosseae* + *P. putida* and the lowest one (1442.91 kg ha⁻¹) was obtained from rainfed plants no-inoculated (Fig.3(a)). Forage yield was significantly higher in 2019 (1776.91 kg ha⁻¹) than in 2018 (1582.65 kg ha⁻¹) (Fig.3(b)). It has been reported that supplemental irrigation at the flowering and pod filling period of peas enhanced the biological yield by positively influencing the development of auxiliary branches and plant height (Rao *et al.* 2016). Mycorrhiza fungi can moderate drought stress impacts on plants by increasing leaf water potential, accelerating CO₂ consumption rate, enhancing photosynthetic efficiency, and improving water absorption rate from host plant roots (Qiu-Dan *et al.* 2013; Nasar *et al.* 2021). It was reported that mycorrhiza improves the biochemical activities and growth and development of sorghum plants by increasing the uptake of nutrients, thereby enhancing forage yield (Symanczik *et al.* 2018). It can be stated that the combined application of mycorrhiza and bacteria may improve the nutrient uptake rate of the plants, thereby enhancing growth and development and leaf chlorophyll content followed by the increased level of photosynthesis and assimilation. The final result is the improvement of plant forage yield.

Agrobiological and chemical properties

Potassium percentage

Means comparison revealed that leaf K percentage was significantly higher in rainfed conditions (1.29%) than supplemental irrigated plants (1.10%) (Fig.4(a)).

Means comparison for biofertilizer application clearly showed that K percentage significantly more than the control (Fig.4(b)). The highest K percentage (1.27%) was observed in plants treated with *F. mosseae* + *P.putida* and the lowest (1.10%) was seen in control plants (Fig.4(b)). The increased potassium uptake during water deficit conditions is associated with the active K uptake mechanism. Indeed, to increase drought resistance, plants act against diffusion and spend energy to increase the K content of their roots and shoots (Shabala and Pottosin, 2014). Plants that are exposed to water deficit stress showed an increased concentration of cell sap in root and shoot cells against diffusion, thereby enhancing their water drought tolerance. Higher K uptake improves photosynthesis, photosynthate translocation rate, plant growth, and development, helps to reduce transpiration, regulates the opening and closing of stomatal, and enhances water uptake efficiency by plants (Tränkner *et al.* 2018). According to the results, it can be claimed that the symbiosis of mycorrhiza fungi and bacteria provides the plants with more potential for K uptake and accumulation than their application. This is likely to be related to the optimal expansion and penetration of external hyphae of the fungi to the tiny pores of the soil. In this respect, it has been observed that the

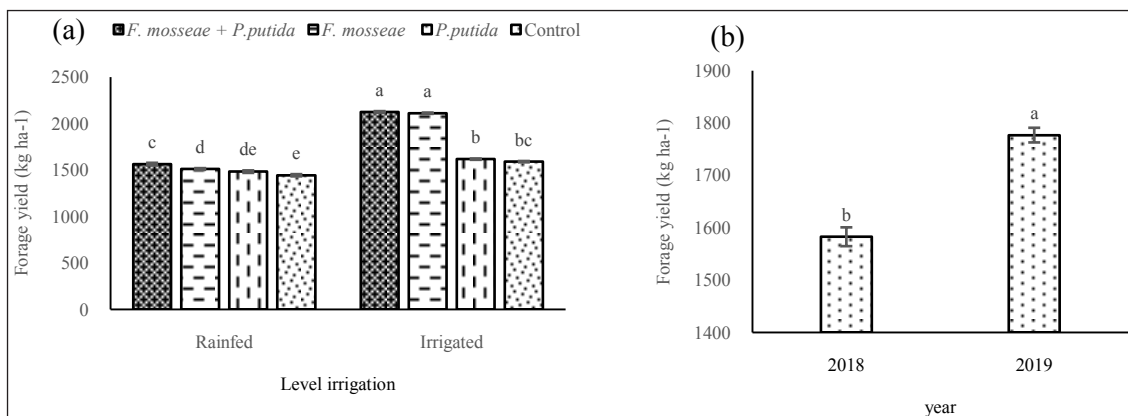


Fig. 3: The interaction of irrigation condition and bio fertilization (a) and year (b) on forage yield; (Means with same letters are not significantly different based on Duncan's multiple range test $P \leq 0.05$)

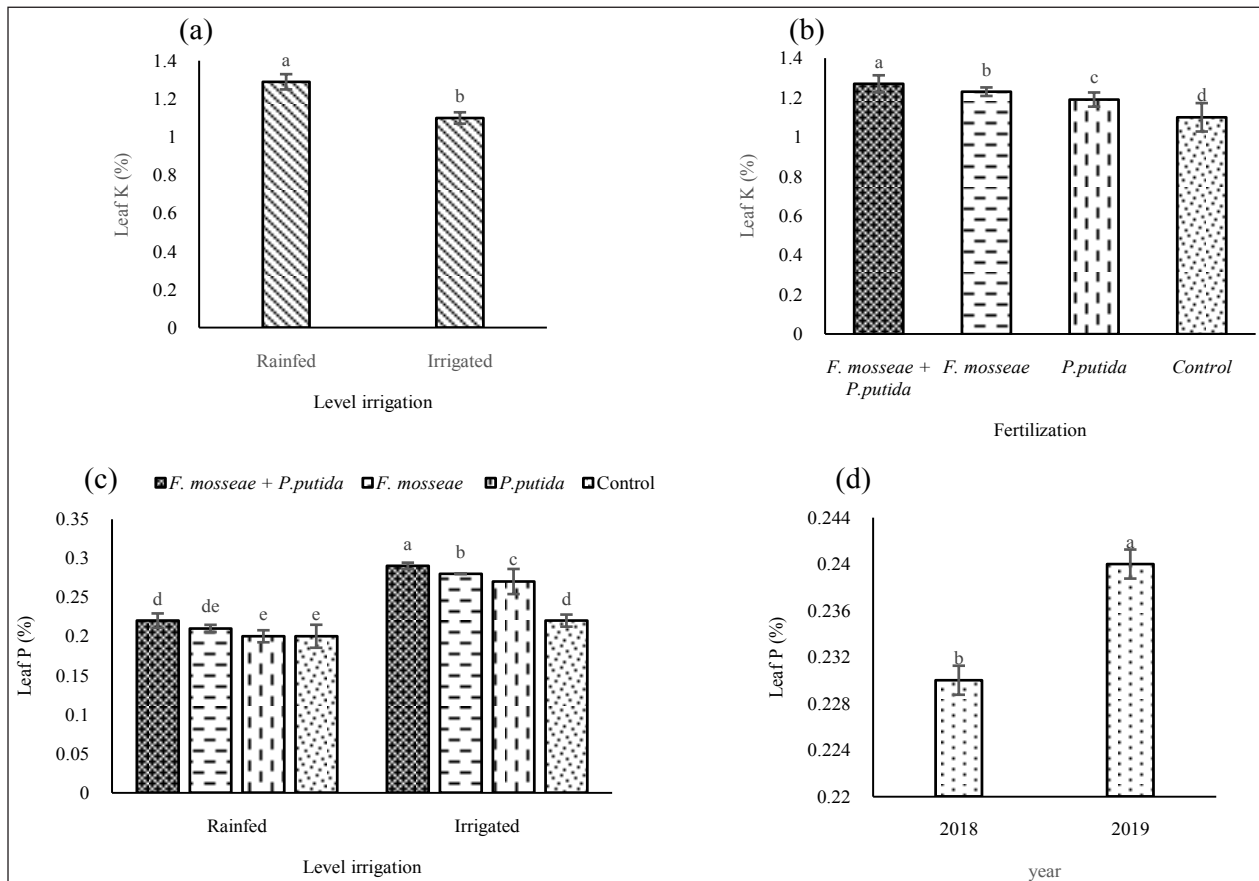


Fig. 4: Effect of irrigation condition (a) and bio fertilization (b) on Leaf K and the interaction of irrigation condition and bio fertilization (c) and year (d) on leaf P; (Means with same letters are not significantly different based on Duncan's multiple range test $P \leq 0.05$)

symbiosis of mycorrhiza with Eucalyptus increased K content as compared with the control remarkably (Arriagada *et al.* 2007).

Phosphorus percentage

The highest leaf P percentage of 0.29% was observed under supplemental irrigation inoculated with *F. mosseae* + *P. putida*. Non-irrigated plants that were not treated with biofertilizer exhibited the lowest leaf P percentage of 0.20%. In rainfed conditions, the concurrent application of biofertilizers did impact leaf P percentage significantly when compared to their application (Fig.4(c)). Nevertheless, leaf P percentage was greater in 2019 than in 2018 (Fig.4(d)). When water availability of soil is decreased, nutrients uptake is limited. In addition, nutrients solubility is reduced as soil moisture is decreased (Suriyagoda *et al.* 2014). Furthermore, water absorption decline results in the decline of photosynthesis and transpiration from a physiological standpoint. Active mobilization

systems are also disrupted in these conditions to save biological energy consumption. These all cause the significant loss of root absorbability and so, the decline of nutrient uptake (Macho-Rivero *et al.* 2018). These results clearly show that the integrated use of mycorrhiza fungi and bacteria versus their individual use improved reserved P percentage and content of grains significantly. This can be attributed to their synergy with one another. In these conditions, mycorrhiza extends the root network and improves P nutrition; it also provides better conditions for water uptake by plants and so, better living conditions for the crop. Besides, *P. putida* improves plant vegetative growth by enriching P nutrition. When these two treatments (*F. mosseae* + *P. putida*) are applied concurrently, they enhance plant growth, dry matter accumulation in the plant, biological yield and the percent of P stored in leaves. It has been documented that the concurrent application of biofertilization improves plant growth and development, photosynthetic

efficiency, and nutrient absorption significantly due to the increased acid phosphatase activity and alkaline phosphatase (Iyer *et al.* 2017; Faridvand *et al.* 2021). It is reported that P affects root growth and development, lateral root development, the formation of root hair and root exudation amount of lentil plant (Singh *et al.* 2005; Gitari *et al.* 2020).

Total dissolved sugars

The highest concentrations of dissolved sugars, ($27.60 \mu\text{mol g}^{-1} \text{FW}$) was found to be related to supplemental irrigation and the application of *F. mosseae* + *P. putida* whereas the lowest one ($19.25 \mu\text{mol g}^{-1} \text{FW}$) was obtained from rainfed control plants (Fig. 5). The loss of total dissolved sugars in response to drought may be chiefly associated with the loss of carbohydrate availability due to the loss of photosynthesis (Goicoechea *et al.* 2005; Benhiba *et al.* 2015). Although the damage to the cell membrane that is exhibited after water deficit stress may limit osmotic adjustment, higher leaf water content under this stress is likely to hinder the accumulation of osmolytes like total dissolved sugars. Nonetheless, the synergic effect of dual inoculation on total dissolved sugars is likely to associate with the increased level of photosynthesis (Wu and Xia, 2006).

Glycine betaine

Under rainfed conditions, lentil plants had higher concentrations of glycine betaine than in

supplemental irrigated plants (Fig. 6(a)). Means comparison for biofertilizer application indicated that the highest amount of glycine betaine ($21.82 \mu\text{mol g}^{-1} \text{FW}$) was obtained in control, whilst the lowest ($18.78 \mu\text{mol g}^{-1} \text{FW}$) was shown by plants treated with *F. mosseae* + *P. putida* (Fig.6(b)). Glycine betaine is a compatible effective factor in protecting the photosynthesis system and increasing photosynthesis capacity in corn plants exposed to drought stress (Ali and Ashraf, 2011). This compound may contribute to reducing water loss from the cytoplasm and maintaining the turgor of the plants (Gupta and Thind, 2015). Increased glycine betaine with water-deficit stress in the plants of legumes was reduced by the application of biofertilizer (Pandey *et al.* 2016). This points to the fact that mycorrhiza-inoculated plants are more effective than non-inoculated plants in stress alleviation. The accumulation of this osmolyte in leaves via the reduction of osmotic potential and cell water potential may allow the cells to keep water uptake.

Enzymatic activity

Catalase activity

The highest activity of this enzyme was $5.09 \mu\text{mol g}^{-1} \text{FW}$ exhibited by plants treated with supplemental irrigation and *F. mosseae* + *P. putida*. But, the lowest was $3.37 \mu\text{mol g}^{-1} \text{FW}$ produced by plants in rainfed conditions not treated with biofertilizer (control)

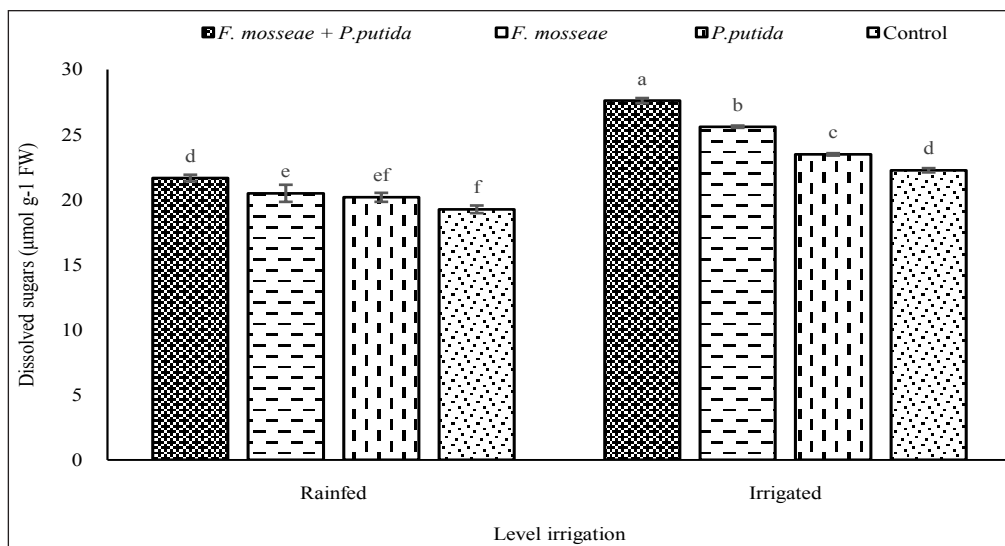


Fig. 5: The interaction of irrigation level and bio fertilization on total dissolved sugars; (Means with same letters are not significantly different based on Duncan's multiple range test $P \leq 0.05$)

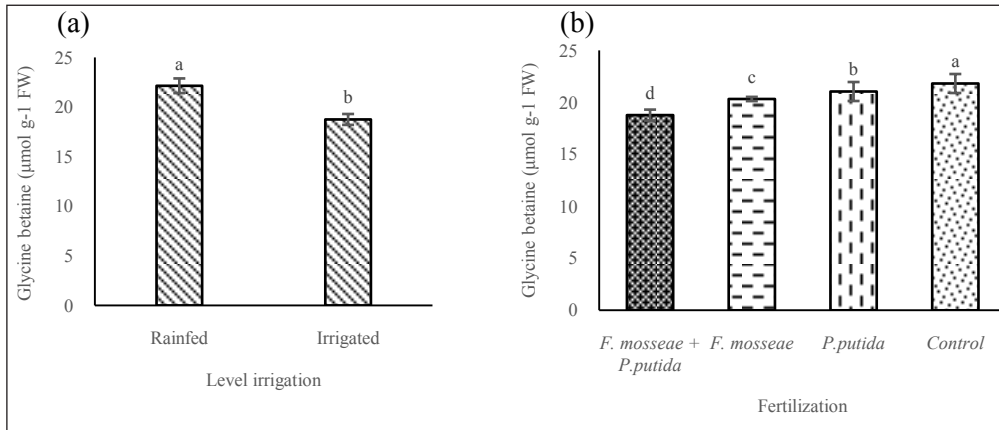


Fig. 6: Effect of irrigation level (a) and biofertilization (b) on glycine betaine; (Means with same letters are not significantly different based on Duncan's multiple range test $P \leq 0.05$)

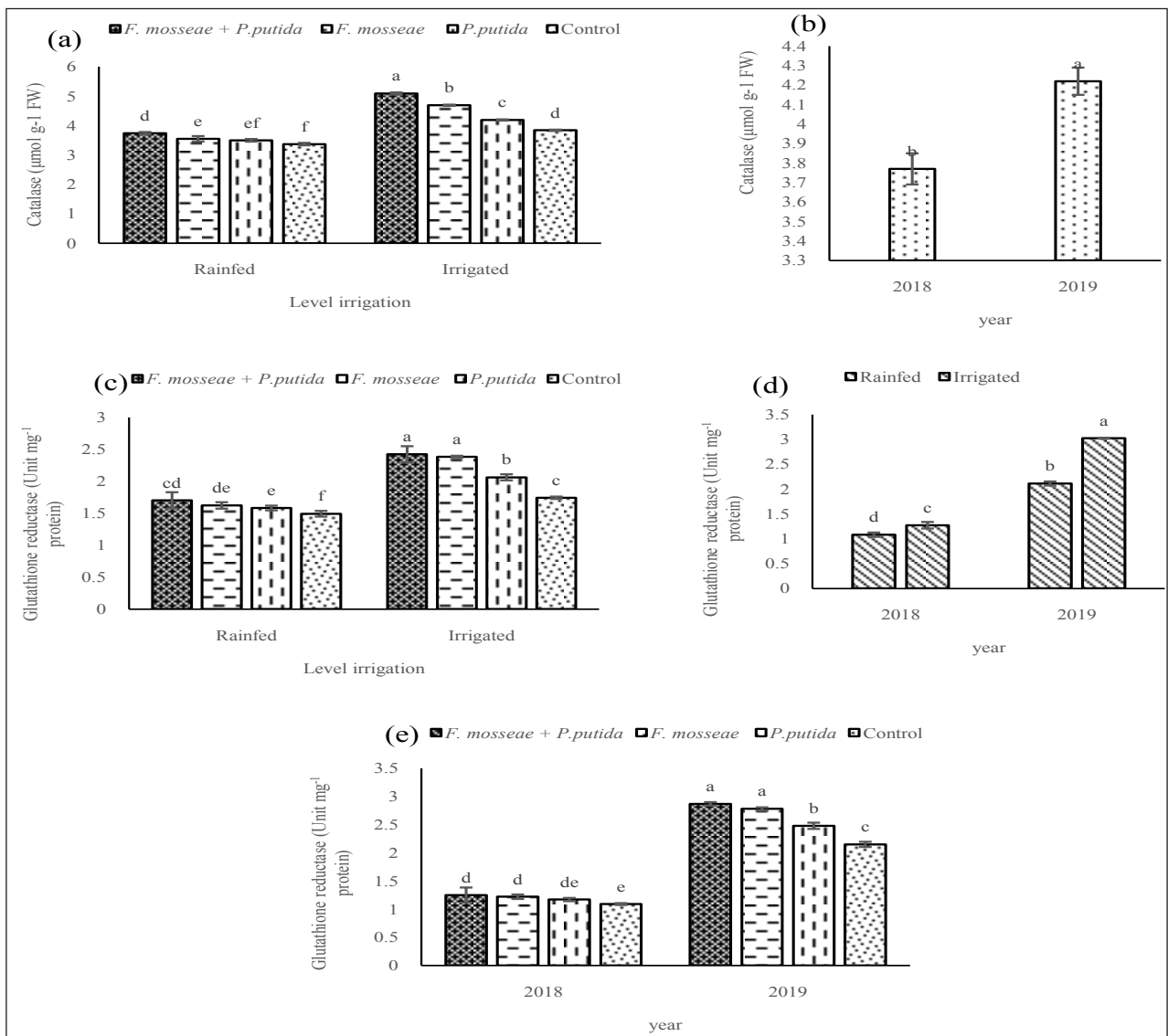


Fig. 7: The interaction of irrigation level and biofertilization (a) and year (b) on catalase activity, the interaction of irrigation level and biofertilization (c) the interaction of irrigation level and year (d) and the interaction of biofertilization and year (e) on glutathione reductase activity; (Means with same letters are not significantly different based on Duncan's multiple range test $P \leq 0.05$)

(Fig.7(a)). The maximum activity of this enzyme was observed in 2019 (Fig.7(b)). The decreased activity of catalase in reaction to water deficit stress is may be caused by the suppression of enzyme synthesis, enzyme photo-inactivation or the modification in the collection of subunit enzymes under drought stress (Liu and Lenardo, 2007; Guo *et al.* 2018). The increased level of leaf catalase activity under water deficit stress conditions in plants inoculated with bacteria and those inoculated with mycorrhiza fungi indicates that inoculation can activate this enzyme to deal with the oxidative damage caused by water deficit stress. Thus, inoculants can regulate antioxidant defence as well as oxidative reactions (Ortiz *et al.* 2015).

Glutathione reductase activity

The activity of this enzyme was maximized to 2.42 unit mg⁻¹ protein under supplemental irrigation treated with *F. mosseae* + *P. putida*. However, concurrent application of mycorrhiza fungi and bacteria was not significantly different from the application of mycorrhiza alone under supplemental irrigation. The lowest activity of this enzyme was 1.49 unit mg⁻¹ protein obtained from rainfed conditions not treated with biofertilizer (control) (Fig. 7(c)). Significant interaction ($P \leq 0.01$) between year and supplemental irrigation on glutathione reductase activity (Table 1) showed that the highest amount of glutathione reductase (3.02 unit mg⁻¹ protein) was observed in supplemental irrigation plants. However, the activity of this enzyme in the 2019 year was higher than in 2018 (Fig. 7(d)). The activity of glutathione reductase was enhanced in plants infected with mycorrhizal fungi and bacteria in both the first and second years. However, the activity of this enzyme was increased in the second year compared to the first year. That is, the highest activity of this enzyme (2.87 unit mg⁻¹ protein) was obtained from plants inoculated with *F. mosseae* + *P. putida* in the second year (Fig.7(e)). We figured out that glutathione reductase activity was declined under water deficit stress. This enzyme's higher activity in plants inoculated with mycorrhiza fungi, bacteria, or both showed that microorganisms alleviate oxidative damage caused by water deficit stress. Symbiosis with mycorrhiza assists plants deals with water deficit, many likely by sustaining photosynthesis mechanisms as influenced by increased antioxidant activity (Mathur *et al.* 2018).

Non-enzymatic defence

Glutathione

The result indicated that the highest glutathione concentrations of 27.62 mg g⁻¹ FW were observed in the plants treated with *F. mosseae* + *P. putida* and the lowest concentrations of 18.49 mg g⁻¹ FW was obtained from control. Nevertheless, Glutathione concentrations were higher in plants treated with supplemental irrigation than those exposed to rainfed conditions (Fig.8(a)). Over-expression of glutathione reductase in chloroplasts increases glutathione concentration in plants and enhances the tolerance to oxidative damage caused by water stress. The loss of glutathione concentration under drought stress increased lipid peroxidation (Rani *et al.* 2018). Non-enzymatic antioxidants, such as glycine betaine and ascorbic acid, were reported to play a key role in preventing oxidative stress in plants under drought conditions (Maziah and The, 2016). There is a report that the inoculation of linseed with mycorrhiza contributes to the increased level of glutathione as a protective compound against the impacts of water deficit (Ruiz-Sanchez *et al.* 2011). Inoculation with mycorrhiza fungi or bacteria, both, increased glutathione concentrations as compared to control. This trait was improved by their concurrent inoculation, too.

Malondialdehyde

According to means comparison, the amount of Malondialdehyde in the rainfed condition was greater than in the supplemental irrigation treatment (Fig.8(b)). Means comparison revealed that the highest malondialdehyde concentrations of lentil (59.36 $\mu\text{mol g}^{-1}$ FW) was obtained in control. The lowest was 49.35 $\mu\text{mol g}^{-1}$ FW obtained in plants inoculated with *F. mosseae* + *P. putida* (Fig.8(c)). Under water deficit stress, a high concentration of malondialdehyde in rainfed plants may be accompanied by an increase in H₂O₂ accumulation in plants, which can reflect the rate of membrane lipids peroxidative damage (Hossain *et al.* 2015; Seleiman *et al.* 2021). However, inoculated plants showed lower concentrations of malondialdehyde than non-inoculated control, reflecting that microorganisms were involved in ROS metabolism.

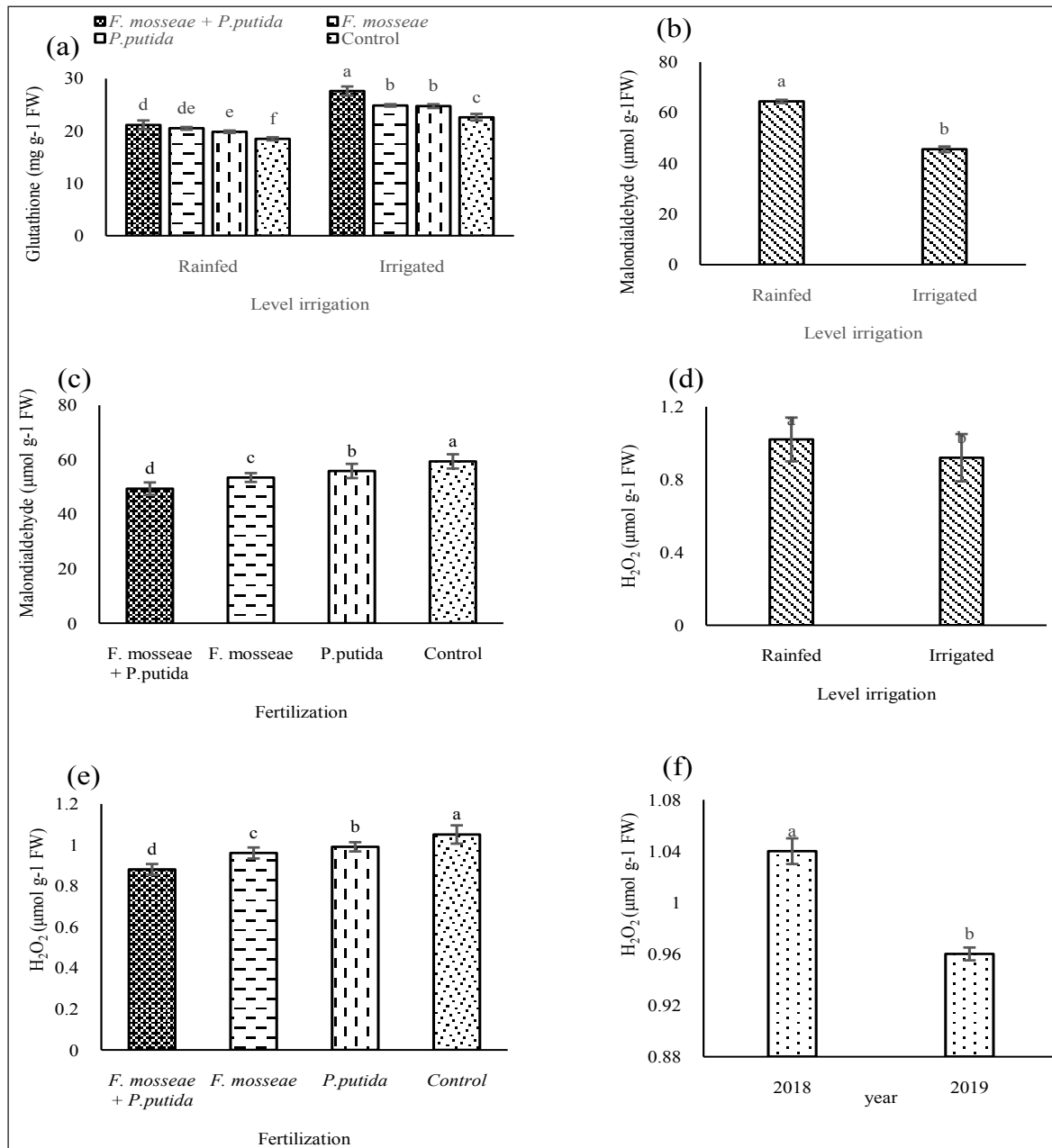


Fig. 8: The interaction of irrigation level and biofertilization (a) on glutathione, Effect of irrigation level (b) and biofertilization (c) on malondialdehyde and Effect of irrigation level (d), biofertilization (e) and year (f) on hydrogen peroxide; (Means with same letters are not significantly different based on Duncan's multiple range test $P \leq 0.05$)

Hydrogen peroxide

According to means comparison, the amount of hydrogen peroxide in the rainfed condition was greater than in the supplemental irrigation treatment (Fig.8(d)). Means comparison indicated the highest rate of hydrogen peroxide ($1.05 \mu\text{mol g}^{-1} \text{FW}$) in control. The lowest was $0.88 \mu\text{mol g}^{-1} \text{FW}$ obtained in plants inoculated with *F. mosseae* + *P. putida* (Fig. 8(e)). The first year had greater

hydrogen peroxide concentrations than the second (Fig. 8(f)). The protection of the host plants versus oxidative damage stress via the increased activity of the antioxidant enzyme is responsible for ROS removal, implying a low concentration of hydrogen peroxide accumulation (Fouad *et al.* 2014). Mycorrhizae contribute to the production of peroxy radical scavengers, cell stability against free radicals, and the provision of a strong suppression system for ROS.

CONCLUSION

The results showed that maximum leaf potassium concentrations, glycinebetaine, malondialdehyde and hydrogen peroxide of lentil was attained in rainfed conditions. Also, the highest percentage of leaf potassium, glutathione, enzyme activity (catalase activity and glutathione reductase activity) and forage yield was achieved in combination treatment of *F. mosseae* + *P. putida*, whereas most glycine betaine, malondialdehyde and hydrogen peroxide were obtained in control (without biofertilizer application). The enzyme activity of catalase, glutathione reductase was increased in irrigated plants. Plants inoculated with *F. mosseae* + *P. putida* had higher enzymatic and non-enzymatic defence than plants inoculated with either the fungal or bacterial inoculum. Hence, the combined application of biological fertilizers (*F. mosseae* + *P. putida*) through supplemental irrigation, in rainfed conditions could improve antioxidant activity and lentil forage yield to move towards sustainable agriculture.

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