# The Role of Eco-Friendly Soil Amendments Co-Application on the Performance of Lingrain (*Linum Usitatissimum* L.) Under Late-Season Irrigation Limitation

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**Research Article**

**Keywords:** antioxidant enzymes, combined use of bio/organic fertilizers, grain yield, limited irrigation, Rhizophagus irregularis, Trifolium pretense

**Posted Date:** December 7th, 2020

**DOI:** https://doi.org/10.21203/rs.3.rs-112924/v1

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# Abstract

This study's main purpose was to investigate the probable amelioration of limited irrigation conditions by soil amendments for lingrain plants. The experiment was accomplished as a completely randomized factorial design along with three replications. The first factor was green manure (without (Gc) and with Trifolium pratense (Gr)), the second factor consisted of Rhizophagus irregularis mycorrhiza (Fm), vermicompost (Fv), both of mycorrhiza and vermicompost (Fm+v) and none of them (Fc), and also the third factor was irrigation regime (full irrigation and late-season water limitation). Green manure, vermicompost and mycorrhiza single-use enhanced the plant performance under water limitation conditions in comparison with the control. However, in the presence of vermicompost, along with green manure or mycorrhiza developed a positive synergistic effect on most of the traits. Combining green manure with the dual fertilizer (Fm+v) resulted in the vermicompost and mycorrhiza synergistic effects, especially under limited irrigation. Consequently, the triple fertilizer (Gr×Fm+v) experienced the highest amount of LRWC, root colonization, leaf nitrogen, chlorophyll a, chlorophyll b, carotenoids, antioxidant enzymes activity, grain yield and oil yield, which would lead to more resistance of plants to limited irrigation conditions.

# Introduction

Climate change and water scarcity have sparked drought as the most critical environmental stress that affect the crops growth, development and productivity in all over the world[91]. Along with the increase in greenhouse gas emissions under the constant global climate change, an increase in the drought intensity and incidence is expected soon. Approximately half of the agricultural lands in all over the world are constantly endangered to water shortages, resulting in a 50% reduction in grain yield[23]. Drought stress can seriously disturb secondary metabolites concentration, also along with the morphological and physiological traits[24,64]. Plants could create different mechanisms in order to defend against water stress, for instance: root development increasing for more water absorption, increasing the free radical scavenging enzymes activity, and osmolytes accumulation for moderating the osmotic pressure[40,95]. Plant resistance to drought can also be increased using other external methods, for instance; by different soil amendments of which the eco-friendly treatments (for example mycorrhizal inoculation and organic fertilizer like vermicompost and green manure) have great importance. Organic fertilizers increase the products yield and quality by soil structure improving and nutrient availability increasing[7]. using nutrients achieved from organic sources helps maintaining the organic matter balance along with improving the chemical, physical and biological properties of the soil[60]. Green manure plays a significant role in nutrients supplying for crops, decreasing dependency on chemical fertilizers, enhancing the crops yield, enriching the cultivated fields ecological environment, and lowering the soil destruction and contamination[86].

Vermicompost, derived from organic wastes of the earthworm, has nutrients that are readily absorbable by a plant. Vermicompost as a nutrient-rich and nature-friendly substance has several potential applications as soil conditioners[20]. It stimulates microbial growth and activity and increases the soil nutrient mineralization. It increases fertility and the soil quality and contains plant growth stimulating agents (like vitamins, hormones, and enzymes), which help the plant grow better[96]. Arbuscular mycorrhizal fungi (AMF) are omnipresent in land-dwelling ecosystems and indicates symbiotic interactions with many plant species roots[51]. The AMF can protect plants against the biotic and abiotic stresses by different mechanisms such as increasing the nutrient absorption, photosynthetic activity, osmolytes accumulation, antioxidant enzymes production, and also improving the rhizosphere environment[93]. The methods used by the AM fungi for increasing the host plants-water relationships are not clear enough, but this may be caused by the water cumulative absorption through exterior hyphae, the adjustment of the stomatal system, the increased antioxidant enzymes activity and nutrients uptake mainly phosphorus[1].

Lingrain is one of the earliest identified cultivated plants, and since ancient times, it has been extensively cultivated for its fine, cellulose-rich, bast fibres and its oil[48]. Lingrain oil is a superb dietary supplement being rich in omega-3 fatty acids and α-linolenic acid. The oil is also benefited in the industrial raw materials production[58]. Although, the worldwide lingrain harvested area and its production went down from 1993 to 2014, the oil yield increased at a rate of 1.72%. Hence it was cultivated on 2.3 million hectares of lands in all over the world with 560000 tons of oil production[22].

There is often a severe shortage of water supply for irrigation of crops in the late growing season in most semi-arid countries, including Iran, which coincides with mid and late-summer. The significant effect of various soil amendments, including organic and biologic fertilizers, has separately been reported on improvement of different plants performances under water deficit stress. Since Iranian and most semi-arid regions soils are poor in organic matter, and few available studies were conducted on the simultaneous integrated use of organic and biologic fertilizers, this research purpose was to investigate the effect of single and combined use of these eco-friendly fertilizers for possible mitigation of lingrain performance under late-season irrigation limitation.

## Results

# Leaf Relative water content (LRWC)

LRWC considerably decreased in water-limited condition. However, Green manure noticeably increased the LRWC in mycorrhizal plants (Fm and Fm + v). In both irrigation conditions, single and combined use of vermicompost and mycorrhiza had the ability of increasing the LRWC significantly, in comparison with the control, with the highest increase (92.56%) belonging to the Fm + v as shown in Table 3.

# Colonization

Sole and dual usage of mycorrhiza and vermicompost (Fv, Fm and Fm + v) would lead to more colonization in comparison with non-fertilized treatment (Fc) (Table 3). Green manure usage increased colonization in all fertilizer treatments except Fc in both irrigation and mycorrhizal plants in water limited condition. The greatest colonization was achieved in the combination of mycorrhiza and vermicompost (Fm + v) that were co-applied with green manure as indicated in Table 3. Moreover, limited- irrigation significantly declined the colonization in all fertilizer treatments (Table 3).

# Leaf N

Water limitation decrease the levels of leaf nitrogen. The single and dual usage of vermicompost and mycorrhiza would result in increased leaf nitrogen in both irrigation conditions in comparison with Fc. Application of green manure and mycorrhiza significantly elevated leaf nitrogen in both irrigation conditions except fv. Green manure combination with mycorrhiza and vermicompost could generate the highest leaf nitrogen percentage as indicated in Table 3.

# Leaf P

Water inadequacy could reduce the leaf P concentrations. Under normal irrigation conditions, single and double fertilizers (Fm, Fv and Fm + v) brought approximately a higher percentage of leaf P in comparison with control (Fc), but there was no significant difference amongst the fertilizer treatments (Table 5).

Although, dual usage of mycorrhiza and vermicompost (Fm + v) accumulated more P in the leaf, but it indicated no significant difference with the single applications of mycorrhiza and vermicompost (Fm and Fv) in water-limited condition (Table 5). In second year leaf P increased significantly than first year (Table 6).

# Leaf K

Water stress strongly decreased the leaf K content in all fertilizer treatments. Leaf K percentage was higher in second year in comparision of first year (Table 5). Under normal irrigation conditions Fm + v) were more effective in leaf K content increasing in comparison with single vermicompost and mycorrhiza. However, they were not significantly different from each other under limited irrigation conditions. On the other hand, they all produced more leaf K in comparison with the control treatment (Fc).

## Photosynthetic pigments

Water limitation significantly reduced chlorophyll a, chlorophyll b, and carotenoids contents. Green manure application soared chlorophyll a content in both irrigation condition. Also, Fm + v had the highest content of chlorophyll a in all conditions, after that Fm and Fv were in the second and third place, respectively. In addition, the lowest chlorophyll a quantity belonged to Gc × Fc (Table 3).

Green manure increased the amount of chlorophyll b, and single use of mcorrhiza and vermicompost could not affect the chlorophyll b under full irrigation conditions, but it mycorrhizal plants resulted in a significant increase of chlorophyll b content under limited-irrigation (Table 3). Using green manure significantly increased the level of carotenoids in Fm + v in both full irrigation and late-season water stress. In both conditions, the combined treatment of vermicompost and mycorrhiza produced the highest carotenoids content as shown in Table 3.

## Proline

At this stage, Water limitation significantly increased. green manure didn’t change the proline concentration except (fm + v) in full irrigation regime. The single and dual application of vermicompost and mycorrhiza increased the proline content in water-limited conditions, also Fm + v indicated the highest proline accumulation under water limitation. Also the single usage of fertilizers had same effect on proline concentration in both irrigation regimes (Table 5).

## Total soluble sugar

Total soluble sugar concentration inhanced in seconde year in comparision of first year in both irrigation regimes (Table 6). Application solo (fm) and (fv) didn’t have significant effec on TSS concentration in full irrigation regime. Also fm + v decreased TSS concentration in this condition. All fertilizer sources increased TSS concentraion in irrigation cut-off conditinon. So that the dual fertilizer treatment (Fm + v) produced more TSS than the others under irrigation cut-off conditinon (Table 5).

## Glycine betaine (GB)

Water limitation considerably enhanced the levels of GB in lingrain leaves. Although, the application of vermicompost and mycorrhiza increased GB content under limited-irrigation circumstance, but the highest GB was obtained from dual fertilizer treatment (Fm + v). Additionally, under full irrigation, only mycorrhizal fertilizers (fm, fm + v) led to an increase in GB content (Table 5).

## Hydrogen peroxide

Drought stress considerably increased the level of leaf hydrogen peroxide. However, using fertilizer treatments caused a significant decline in its amount, in comparison with the control (Fc). Dual fertilizer (Fm + v) brought approximately the highest reduction percentage for hydrogen peroxide. The mycorrhiza-containing treatments more effectively reduced the amount of hydrogen peroxide compared to vermicompost (Table 5). Green manure usage had significant effect on H2o2 concentration in all treatments except (Fv) in both irrigation regimes (Fig. 1).

## Malondialdehyde (MDA)

Water limitation enormously increased the levels of MDA, which was as a result of lipid peroxidation increasing. However, single and dual usage of the fertilizers resulted in a remarkable drop in MDA content compared to the control (Fc). Dual fertilizer (Fm + v) brought approximately the maximum reduction for MDA. The mycorrhizal treatments have the ability of decreasing the level of MDA more effectively, in comparison with vermicompost. Also, the single application of green manure was helpful in the MDA level reducing. Green manure usage decreased MDA level only in mycorrhizal treatmens in irrigation cut-off irrigation (Table 4).

## Catalase (CAT)

Water limitation increased CAT activity. Incorporation of green manure under water limitation increased the activity of CAT enzyme in all treatments (Fig. 2). By applying mycorrhiza, with or without vermicompost, indicated a higher influence on the enzyme activity under water limitation, as well. Furthermore, the highest activities of CAT (8.71, 8.61. mmol min-1/g FW) were observed in mycorrhizal plants (Table 5).

## Ascorbate peroxidase (APX)

Water deficit elevated the APX activity. Green manure increased the activity only under water limitation condition in all treatments excep (fm). Furthermore, vermicompost and mycorrhiza, single or combined, had a more stimulatory effect on the enzyme activity under water limitation. The mycorrhiza and vermicompost combination caused the highest activity of APX (Table 4)

## Superoxide dismutase (SOD)

In both years, SOD activity was significantly increased by water limitation as shown in Table 6. The application of green manure enhanced the activity of SOD in the limited-irrigation situation (Fig. 3). The synergistic effects of the co-application and the separate usages of mycorrhiza were more than that of vermicompost under limited-irrigation (Table 5).

## Guaicole peroxidase (GPOX)

Water deficit elevated the Guaicole peroxidase activity. Green manure increased the anzyme activity in all treatments except vermicompost in full irrigation regime. Furthermore, enzyme activity was equal when using mycorrhiza under both irrigation conditions. The mycorrhiza and vermicompost combination caused the highest activity of GPOX (Table 4)

## Grain yield

Limited irrigation significantly reduced the grain yield compared to full irrigation; however, the application of green manure increased grain yield relative to non-application of green manure in this condition. Although, co-application of green manure with vermicompost and mycorrhiza had no influence on grain yield in full irrigation. Green manure application under complete irrigation situation produced the maximum grain yield (4862 kg ha-1) in Fm + v treatment. In addition, using green manure reduced the adverse effects of water restriction on grain yield, and the presence of mycorrhiza and/or vermicompost will additionally mitigate these adverse effects (Table 4).

## Oil yield

The sole and conjoint usage of mycorrhiza and vermicompost along with co-application of green manure under full and limited irrigation would result in an increase in oil yield during comparing them with their corresponding controls (Table 4).

Using mycorrhiza was more effective oil yield in increasing compared to vermicompost, but the dual fertilizer produced the highest oil yield in both irrigation regimes with or without green manure. However, adding green manure to them produced more oil yield than control (Gc × Fc). Furthermore, the green manure inclusion to Fm + v produced the maximum level of oil yield (1602.66 kg h-1) under full irrigation condition as displayed in Table 6.

## Discussion

Water deficit significantly reduced the leaf relative water contents (LRWC)[59]. By Considering the high water absorption efficiency of the soil by peripheral hyphae[36], using different types of mycorrhizal fungi could cause a substantial rise in the water status of inoculated plants, which is in association with non- mycorrhizal plants, under water- limited conditions[97].

Using organic fertilizers improves the water potential and maintains leaf turgor pressure, which is In agreement with this study result[12]. With respect to the green manure and vermicompost roles in soil organic matter and water content enhancement, dual-fertilized plants (Fm + v) indicated high percentage of LRWC in green manured plots.

This study results demonstrated that water stress inhibited mycorrhizal colonization in lingrain roots. These results are in agreement with earlier findings, proposing that mycorrhization will be decreased at low levels of soil moisture[79]. AMF are obligate biotrophic fungi, consequently very slow germination of spores and expansion of the hyphae or their complete inhibition under drought conditions could elucidate the drought adverse consequences on colonization[32]. Therefore, lower colonization can occur because of two reasons; the carbon obtainability reducing from host plants and also the fungal spore germination reducing in limited water conditions[92]. Up to the best of our knowledge, organic matter can affect the activity and frequency of soil microorganisms like AMF, which can increase the plant nutrients[53]. Vermicompost promotes the mycorrhiza colonization in the roots[39,49,80]. The highest percentage of AMF colonization (66%) in *Ocimum basilicum* roots was observed in using vermicompost + *G. intraradices* combination in comparison with control[89]. Therefore, the combination of vermicompost, mycorrhiza and green manure exhibit high root colonization under full irrigation condition in this research.

Nutrients absorption directly relates to the soil water status, thus the nutrient flow from soil to root decreases in moisture shortage conditions[5]. With respect to better solubility of phosphorus in irrigated soil, the phosphorus concentration of the leaves indicates a reduction along with water restriction, which is probably caused by reduced solubility, mobility (mass flow or propagation), transfer between the roots and branches, and also the absorption of P under drought conditions[43,77]. Mycorrhizal inoculation can increase the infection percentage and absorption range in the extramatrical mycelium, and after that increase the nitrogen absorption rate and phosphorus and improve the nutrition in plants[63]. However, inoculated plants under limited irrigation contained less nitrogen and phosphorus content, which may be due to drought constraints in mycelium development and reduced absorption range, in comparison with infected plants in well-watered condition[52].

Green manure will increase the soil holding-capacity for water and nutrients content and mobility in the upper layer of soil, by providing organic matter. Plants can use these elements, and therefore increase their growth and productivity[82]. Moreover, there is an extensive record on the green manure advantages for nitrogen nutrition of succeeding crops[21,45,68]. Mineralized nitrogen from leguminous plant residues can provide considerable quantities of N to a subsequent crop[73]. Green manure effect on phosphorous availability could be caused from its P uptake and unavailable inorganic P changing to more available organic forms to the subsequent crop[42]. Furthermore, biodegradation of green manure remainders can produce bicarbonate (H2CO3) that can provide higher bio-available P for the plants by dissolving mineral P of the soil[78].

Vermicompost provided high nutrition by balancing the nutrients, due to the fact that it contains all macro and some micro-nutrients. In regard with our results, vermicompost treatments increased the K uptake of *Setaria* grass[75] and *Lilium* plant[62]. Moreover, vermicompost increased N percentage of spinach[69] and leaf P content of corn (Gutiérrez-Miceli *et al.* 2008). Also, the available form of N (nitrate) in vermicompost is more than traditional composted manure[84].

Water limitation reduced the leaf chlorophyll, and also carotenoid concentrations. One of the common symptoms of oxidative stress under drought conditions is the chlorophyll content reduction, which may be due to photo-oxidation and the pigment degradation[2,66]. This study results indicated that the amount of carotenoids, chlorophyll b and a significantly decreased under water limitation. These result are not consistent with several studies reported that carotenoid increased under water deficit conditions[6,9].

Earlier researches[9,46,47] approved our results that vermicompost application in soil brought about a remarkable growth in carotenoids content under water deficit condition. mycorrhizal inoculation resulted in the maintenance of a very high chlorophyll content relative to non-mycorrhizal plants in those plants subjected to late-season water limitation. This study results are in agreement with those reported by (Metwally *et al.* 2019)[59]. Using green manure can enhance the nitrogen availability, which can be used by lingrain in the chlorophyll production. Chlorophyll plays a crucial role in photosynthesis, such that increased chlorophyll formation will improve photosynthetic reactions and finally raise plant growth and production. Also, Subaedah and Aladin (2016)[82] clarified the enhancement of chlorophyll content by using of green manure in maize, which is in parallel with our findings.

Amassing proline under stress conditions is identified as one of the plant remedies for osmotic regulation improving in the leaves. Proline plays a leading role during stress in the plant. In addition to a great osmolyte, it acts as a metal chelator, a signalling molecule and an antioxidant molecule[81]. Having a higher capacity for osmotic regulation is one of those plants characteristic with higher drought tolerance. In this study, AMF-treated plants indicated an enhanced accumulation of proline in water-limited condition. These results are in agreement with Gheisari Zardak *et al.* (2018)[29] and Tyagi *et al.* (2017)[87] findings. Consequently, AM plants can be physiologically more tolerant against drought and indicate more osmotic regulation during exposing to stress conditions. Chinsamy *et al.* (2013)[17] demonstrated a substantial elevation in proline concentration within tomato grainlings under salinity stress by the use of vermicompost. Also, The same effect was observed with vermicompost in pomegranate[14] and rice grainlings (García *et al.* 2012)[28] under water limitation stress.

The lingrain plants exposure to drought stress could increase the TSS concentration. Results of a study accomplished by Masoudi-Sadaghiani *et al.* (2011)[57] and Vijayalakshmi *et al.* (2012)[90] have indicated that water constraints can elevate the concentration of TSS in the leaves. Increasing the TSS concentrations occurred in those plants that were treated with one of Fm and Fv, along with their combination in both irrigation regimes. However, under limited irrigation condition, TSS increasing was remarkably higher in mycorrhizal treatments and the presence of green manure. AMF enhance TSS and electrolyte concentrations in host plants. Furthermore, carbohydrates increasing in mycorrhizal plants may be attributed to increased photosynthetic carbon and enzymes activation reduction. Other researchers have reported increased carbohydrate in AM plants[3,94]. Furthermore, Tejada *et al.* (2008)[86] reported that soluble carbohydrate contents of maize plants were the highest in *Trifolium pratense* L. amended soils. The increment of leaf TSS of plants treated with vermicompost was also reported by Tejada and Benítez (2011) and Salehi *et al.* (2016)[76,85].

The main role of the GB could be considered as protecting the plasma membrane integrity from drought stress injury and contribution to osmotic regulation. Dual application of mycorrhiza and vermicompost increased the GB content under the water-limited conditions, and this indicating that the fertilizers could alleviate drought stress impacts. In addition, GB accumulation in cotton has been reported[54] as an effective compatible solution under drought stress. Moreover, Hashem *et al.* (2015)[41] reported that enhanced GB accumulation leads to better growth and salt tolerance, and after that improvement in the performance of photosynthetic traits.

Lower accumulation of H2O2 provides proof that increasing the antioxidant enzymes activity bring about the removal of ROS and the protection of host plants against oxidative stress[26]. A higher concentration of MDA in leaves, which can indicate the amount of peroxidation of membrane lipids, may be accompanied by higher H2O2 accumulation in drought stress plants[30]. Additionally, fertilized plants (Fm and Fv) indicated less MDA than control that indicates the fertilizers involvements in ROS metabolism[14,61,72]. In addition, mycorrhiza inoculation reduced the H2O2 and MDA concentration in foxtail millet leaves in drought[33]. In consistent with our results, Bidabadi *et al.* (2017)[14] demonstrated a reduction in H2O2 content in vermicompost application. Moreover, using green manure would resulted in MDA and H2O2 concentrations reduction, and also along with that, enhancement of the catalase and peroxidase activities in water limitation. It appeared that green manure could impede H2O2 accumulation and lipid peroxidation in plants by increasing the water content of the soil. Limited-irrigation causes oxidative stress by increasing the production of ROS in plant cells, and oxidative damage is also eased by antioxidant enzymes like APX, CAT, and SOD[74,83]. Amongst anti-oxidants enzymes, SOD and CAT are recognized as the most frequently used detoxification enzymes, which play a major role in controlling excessive ROS, in collaboration with involved enzymes of the ascorbate–glutathione cycle[10,15,38]. In this study, the triple application of mycorrhiza, vermicompost and green manure increased the CAT activity in both irrigation conditions, consequently, they can facilitate quick sweeping of ROS especially H2O2, so that metabolic reactions are less affected. Earlier results confirmed that the mycorrhizal and vermicompost-applied plants indicated considerably higher enzyme activities in stress condition[13,47,61,67].

Applying the green manure in soils at high doses increased maize grain yield in comparison with the control soil[50]. Similar results were obtained with the green manure application in wheat grain yield by Gruter *et al.* (2017)[37].

Vermicompost along with biofertilizers-treated plots indicated a substantial increase in essential oil content, essential oil yield and biomass of *Ocimum basilicum* in comparison with control[89]. Similarly, Doan *et al.* (2015)[19] and Goswami *et al.* (2017)[34] reported an increase in grain yield by using vermicompost. Mycorrhizal symbiosis enhanced the grain and oil yields in comparison with non-mycorrhizal plants under both irrigation conditions. The results demonstrated that mycorrhizal colonization was a momentous factor for lingrain development, especially under limited water condition with respect to playing a significant role in nutrient and water absorption in water-limited plants[4].

# Conclusions

These results demonstrated that water limitation could reduce the pigments contents, nutrient contents, LRWC, colonization percentage, and (grain and oil) yields. However, it is responsible for a significant increase in H2O2, MDA, osmolytes and antioxidant enzymes activities in this condition. With respect to these results, the inclusion of vermicompost increased the lingrain performance for most of the investigated traits in comparison with the control (Fc). Although, Fv had no advantage compared mycorrhizal fertilizers (Fm and Fm + v), but its presence along with green manure or mycorrhiza could develop a positive synergistic effect on these traits.

Moreover, adding green manure to vermicompost treatments (Gr × Fv) resulted in a significant increase of colonization percentage, oil yield in comparison with Gc × Fv. Also, Gr × Fv significantly increased the APX activity and grain yield under drought stress conditions. However, it caused no significant difference in non-stressed condition, in comparison with single vermicompost treatment (Gc × Fv). In other words, green manure increased the vermicompost efficacy under stress conditions. Also, adding the green manure could result in a remarkable grain yield superiority of Fm + v in comparison with Fm. In the green manure absence, these two fertilizer treatments effect were similar in full irrigation condition. Consequently, combining green manure with the dual fertilizer (Fm + v) led to the synergistic effects of vermicompost and mycorrhiza.

In drought stress conditions, adding the green manure improved mycorrhiza efficiency on elevating RWC, leaf nitrogen, chlorophyll a, chlorophyll b, grain yield, oil yield and antioxidant enzymes activity (catalase, ascorbate peroxidase, superoxide dismutase and Guaicole peroxidase),

All fertilizer treatments (FV, Fm and Fm + v) imposed less hydrogen peroxide and malondialdehyde on lingrain in both irrigation conditions in comparison with control. The lowest amount of hydrogen peroxide and malondialdehyde was achieved from the application of dual fertilizer under normal irrigation conditions followed by mycorrhiza and vermicompost fertilizers, and also the highest amount was achieved from non-fertilized control and under the limited irrigation conditions .

The co-application of eco-friendly soil amendments would result in the profitable lingrain production under water limitation. Therefore, as a result the triple fertilizer (Gr × Fm + v) experienced the highest amount of LRWC, leaf nitrogen, chlorophyll a, chlorophyll b, carotenoids, grain yield and oil yield. In addition, GrFm + v enjoyed the maximum quantity of and also antioxidant enzymes activity under drought stress conditions. Consequently, it can be concluded that the combined usage of organic and biological fertilizers (green manure, vermicompost, and mycorrhiza) can be also applied as a solution in order to maintain the lingrain plant performance under the water-limited condition.

# Materials And Methods

## Experimental design

Field experiments were accomplished on 2017 and 2018 at the Saatlou Station (44"° N, 45°10’95" E), Urmia Agricultural Research Centre in Iran. The average of rainfall was 17.5 mm and 32.07 mm during the lingrain growing season in 2017 and 2018, respectively. This experiment was a □□ factorial with respect to randomized complete block design with three replications. Factors included (1) irrigation regimes (full irrigation (Iw), limited irrigation (Is) (irrigation cut-off from flowering stage), (2) green manure (no green manure (Gc), *Trifolium pratense* as green manure (Gr) and (3) mycorrhiza inoculation ((*Rhizophagus irregularis*) (Fm) 300 g m-2), vermicompost (Fv) (5 t/ha), their combination (mycorrhiza + vermicompost (Fm + v)) and no fertilizer (Fc).

## Soil amendments

The soil texture was salty loam with a pH of 7.8. Other soil physical and chemical properties are indicated in Table 1.

Red clover (*Trifolium pratense*) was grained on August 2016 and 2017. Also, fresh Gr was manually incorporated into 20 cm depth of surface soil in March 2017 and 2018, at the flowering stage.

Mycorrhizal inoculum included a blend of sterile sand, mycorrhizal hyphae and spores (30 spores g-1 inoculum), and colonized fragments of canola root that were isolated from an AMF community of a canola farm by plant protection department of Agricultural and Natural Resources Research Centre, West Azerbaijan province, Urmia, Iran. Vermicompost was prepared with respect to the method suggested by Ayyobi *et al.* (2013)[8]. Chemical properties of the vermicompost that was used in these experiments are tabulated in Table 2.

## Plant culture

By passing a month from green manure integration, the land was manually prepared. At first and second year, the measured dry biomass production by green manure was 4.72 t ha-1 and 4.95 t ha-1, respectively. Also, inoculum (300 g m − 2) was incorporated into the soil of grainbed before the grain sowing. The exact amounts of vermicompost were manually spread onto the soil surface and incorporated into the top 20 cm of the soil Lingrain Grains were achieved from the Agricultural and Natural Resources Research Centre of West Azerbaijan province, Urmia, Iran. The grains were sown in 8 rows on 21 April 2017 and 24 April 2018, respectively (with two cm intra-row and 25 cm inter-row spaces). The full irrigation treatment included five irrigations (after sowing, at the 4-leaf stage, at stem elongation, at flowering and capsule formation stages), and limited irrigation treatment (irrigation cut -off from flowering stage) had three irrigations (after sowing, at 4-leaf and stem elongation stages). The irrigation water amount in each plot was measured by the use of a water meter. Moreover, the average humidity at the surface and depths of 20 and 40 cm of soil was determined using a soil moisture meter (EXTECH MO750) in each plot before each irrigation. After that, with respect to the lack of moisture under the field capacity point, the amount of water that was required for each irrigation was calculated by following equation:

$Vn = (Fc - \theta) X (A X h)$,

Where, *Vn*, the volume of irrigation water (m3), *Fc* moisture content at the field capacity point (volume), θ soil moisture (volume), *A* area of the plot (m2) and *h*, Effective depth of lingrain root (m).

In terms of the above equation, the amounts of water used for irrigation were 2346 and 1420 m3/ha (for the first year), and 1750 and 600 m3/ha (for the second year) for full and limited irrigation, respectively.

All the practices were manually handled in this experiment, in order to prevent interference with all materials.

## Parameters measured

# Leaf relative water content (LRWC):

Leaf relative water content was assayed in leaf samples with respect to García-Mata and Lamattina (2001)[27].

# AMF Colonization

The mycorrhizal root colonization percentage was determined for each plot in five roots. In order to achieve this goal, roots were cleared by 10% KOH at 90 °C for 10 min, and after that were stained in 0.05% lactic acid-glycerol-Trypan Blue[70]. The mycorrhizal colonization was estimated with respect to Giovannetti and Mosse (1980)[31] gridline intersect method.

# Nutrient content

The leaf samples were dried in an oven for 72 h at 70 °C, and after that, were grounded using an electric mill. The Kjeldahl method was used for Nitrogen measurement[65]. Spectrophotometry and flame photometry methods were applied in order to determine P and K contents, respectively.

# Photosynthetic pigment content assay

Approximately 10 ml of acetone 80% was added to extracts of fresh leaf tissue samples gradually (0.2 g). After that, it was centrifuged for 10 min at 400 rpm and the absorbance was recorded using a spectrophotometer at 645, 663, and 470 nm. Chlorophyll and carotenoids were obtained based on the following equations:

chlorophyll a = (19.3 X A633–0.86 X A645) V/100W

chlorophyll b = (19.3 X A645–3.6 X A663) V/100W

carotenoid = (1000 A470–1.82 Chlorophyll a – 85.02 chlorophyll b

# Proline

Leaf fresh tissue (1 g) was used for leaf proline extracting in sulfosalicylic acid 5% (w/v). Leaf proline was determined using spectrophotometric analysis at 520 nm of the ninhydrin reaction, with respect to (Bates *et al.* 1973)[11].

# Total soluble sugars (TSS)

Leaf total soluble sugar was measured by the anthrone method[56]. Also, the TSS concentration was determined using the glucose standard curve.

# Hydrogen peroxide (H2O2)

The fresh leaf tissue (0.5 g) was grounded in a pre-chilled mortar with 5 mL of 0.1% (w/v) TCA. After that, this homogenate was centrifuged at 12000 × g for 15 min at 4 °C. also, 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 and the OD of the suspension was read at 390 nm[88].

# Malondialdehyde (MDA)

Malondialdehyde was measured with respect to the thiobarbituric acid (TBA) reaction, as it was explained by Heath and Packer (1968)[44]. Fresh leaf tissues (0.1 g) were frozen in liquid nitrogen, and homogenized in 5 ml of 0.1% trichloroacetic acid (TCA). The homogenates were transferred into tubes and centrifuged at 3600 rpm for 10 min at room temperature. Two millilitres of this extract was added to 5 ml 0.5% TBA. The mixture was incubated for 30 minutes at 95 ° C, and then was immediately placed on ice for 5 minutes and centrifuged again at 10000 × g for 10 minutes, respectively. The supernatant absorbance was determined at 532 and 600, nm (A532 and A600). The MDA concentration was calculated due to following equation:

$[(A532 - A600)/155]$ X 100

# Glycine betaine (GB)

Glycine betaine analysis was performed with respect to the method of Grieve and Grattan (1983)[35].

# Catalase (CAT), Ascorbate peroxidase (APX) and Superoxide dismutase (SOD)

The fresh leaf tissues (5.0 g) were macerated in 10 mL potassium phosphate buffer [pH 7.8; 50 mM], by the use of an ice-cooled sterilized pestle and mortar. After that, the homogenates were centrifuged for 20 min at 4 °C at12000 g. The supernatant was applied to the antioxidant enzymes activity assay.

Catalase activity was assayed in regard with Maehly and Chance (1954)[55]. 50 ml enzyme extract was added to 2.5 ml of 50 mM phosphate buffer (pH 7.4), and 0.1 ml of 1% hydrogen peroxide in the ice bath, in order to determine CAT activity. The H2O2 content reduction was obtained at 240 nm for 1 min.

Ascorbate peroxidase activity was determined b absorption reducing at 290 nm due to ascorbate oxidation in 3 min, with respect to Chen and Asada (1989)[16]. The reaction mixture involved 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. Superoxide dismutase activity was measured regarding Dhindsa *et al.* (1981)[18] method in terms of its ability to inhibit the nitro blue tetrazolium reduction by superoxide radicals generated by photochemical reactions. The 3 ml reaction mixture contained 50 mM phosphate buffer, pH 7–8, 13 mM methionine, 75 µM NBT, 2 µ*M* riboflavin, 0–1 mM EDTA, and 0–50 µl enzyme extract. NBT solution, riboflavin, EDTA and 1.0 ml DDW were incubated in light under 15 W fluorescent lamps for 15 min. The blank reaction was processed also for 15 min in darkness. The samples and controls absorbance was measured at 560 nm. Guaicole peroxidase was measured regarding[25].

# Grain Oil Extraction

Soxhlet technique was utilized in orderto extract grain oil[71]. Oil yield was calculated by the following formula:

Oil yield = % oil X seed yield

# Grain yield

At plant maturity, above-ground biomass was harvested from 1 m2 of each experimental plot by ignoring marginal effects. Each plot Grains were separated and weighed in order to measure grain yield.

# Statistical analysis

Combined factorial analysis of variance (three factors) including those gathered data for about two years (with one year as a random factor) was performed using SAS software and by applying the general linear model (GLM) procedure to establish these three factors main and interactive influences. After that, this model was approved by checking the residues normality and homogeneity (using the Bartlett test). Also, No data transformation was performed. The mean values were compared using the protected least significant difference (PLSD) at $P \leq 0.05$, due to the reason that the treatment factors were qualitative.

## Declarations

### Conflicts of Interest

The authors declare no conflicts of interest.

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# Tables

**Table 1.** Soil physicochemical properties

| Characteristic | Saturation extract | pH | EC | P | K | N | Organic carbon | Sand | Silt | Clay | Texture |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | (dS/m) | (mg/kg) | (mg/k) | (%) | (%) | (%) | (%) | (%) | |
| 0-30 cm | 42 | 7.8 | 0.58 | 7.1 | 184 | 0.09 | 0.91 | 23 | 42 | 25 | Silty loam |

**Table 2.** Vermicompost properties

| Properties | pH | EC (dS/m) | Organic matter (%) | Nitrogen (%) | P (%) | K (%) | Mg (%) | Ca (mg/kg) | Cu (mg/kg) | Zn (mg/kg) |
|---|---|---|---|---|---|---|---|---|---|---|
| Vermicompost | 7.64 | 5.22 | 12.3 | 1.3 | 0.88 | 0.67 | 1.1 | 8.75 | 3.14 | 46.76 |

**Table 3. Comparison between 2-year mean values of physiological traits of lingrain affected by green manure× mycorrhiza × vermicompost under full and limited irrigation**

Where $I_w$ (full irrigation), $I_s$ (limited irrigation), $G_c$ (no green manure), $G_r$ (green manure), $F_c$ (no fertilizer), $F_m$ (mycorrhiza inoculation), $F_v$ (vermicompost application) and $F_{m+v}$ (combination of mycorrhiza and vermicompost). Means followed by the same letter in each column are not significantly different

| Treatment | Leaf Relative water content (%) | Colonization (%) | Leaf N % on dry matter | Leaf k % on dry matter | Chlorophyll a (mg/g FW) | Chlorophyll b (mg/g FW) | Carotenoids (mg/g FW) |
|---|---|---|---|---|---|---|---|
| $I_{W}\times G_{c}\times F_{c}$ | $66.64^{f}$ | $7.50^{ij}$ | $2.42^{fg}$ | $0.18^{h}$ | $2.19^{h}$ | $1.68^{c}$ | $1.87^{h}$ |
| $I_{W}\times G_{c}\times F_{m}$ | $79.66^{c}$ | $56.25^{c}$ | $3.04^{c}$ | $0.25^{cd}$ | $3.35^{d}$ | $2.08^{b}$ | $2.9^{d}$ |
| $I_{W}\times G_{c}\times F_{v}$ | $75.04^{d}$ | $13.03^{g}$ | $2.77^{de}$ | $0.25^{cd}$ | $2.71^{f}$ | $1.96^{b}$ | $2.67^{e}$ |
| $I_{W}\times G_{c}\times F_{m+v}$ | $83.71^{b}$ | $58.25^{c}$ | $3.22^{b}$ | $0.28^{ab}$ | $4.42^{b}$ | $2.11^{b}$ | $3.83^{b}$ |
| $I_{S}\times G_{c}\times F_{c}$ | $51.35^{h}$ | $4.94^{k}$ | $1.89^{i}$ | $0.9^{i}$ | $1.19^{m}$ | $1.06^{g}$ | $1.02^{l}$ |
| $I_{S}\times G_{c}\times F_{m}$ | $63.16^{g}$ | $42.55^{e}$ | $2.3^{g}$ | $0.21^{ef}$ | $1.66^{j}$ | $1.54^{cd}$ | $1.63^{i}$ |
| $I_{S}\times G_{c}\times F_{v}$ | $62.73^{g}$ | $9.40^{hi}$ | $2.41^{fg}$ | $0.22^{ef}$ | $1.34^{kl}$ | $1.31^{ef}$ | $1.24^{k}$ |
| $I_{S}\times G_{c}\times F_{m+v}$ | $65.15^{fg}$ | $52.91^{c}$ | $2.41^{fg}$ | $0.22^{ef}$ | $1.83^{i}$ | $1.6^{cd}$ | $1.85^{h}$ |
| $I_{W}\times G_{r}\times F_{c}$ | $73.69^{d}$ | $8.29^{ij}$ | $2.49^{f}$ | $0.20^{fg}$ | $2.41^{g}$ | $1.94^{b}$ | $2.16^{g}$ |
| $I_{W}\times G_{r}\times F_{m}$ | $81.98^{bc}$ | $67.40^{b}$ | $3.27^{b}$ | $0.27^{abc}$ | $3.68^{c}$ | $2.1^{b}$ | $3.17^{c}$ |
| $I_{W}\times G_{r}\times F_{v}$ | $75.56^{d}$ | $18.39^{f}$ | $2.88^{b}$ | $0.26^{bc}$ | $2.98^{d}$ | $1.96^{b}$ | $2.7^{e}$ |
| $I_{W}\times G_{r}\times F_{m+v}$ | $92.56^{a}$ | $71.08^{a}$ | $3.38^{a}$ | $0.29^{a}$ | $4.86^{a}$ | $2.44^{a}$ | $4.37^{a}$ |
| $I_{S}\times G_{r}\times F_{c}$ | $53.74^{h}$ | $5.92^{jk}$ | $2.17^{h}$ | $0.18^{h}$ | $1.23^{ml}$ | $1.22^{fg}$ | $1.13^{kl}$ |
| $I_{S}\times G_{r}\times F_{m}$ | $71.08^{e}$ | $44.04^{e}$ | $2.79^{f}$ | $0.23^{de}$ | $2.46^{g}$ | $1.96^{b}$ | $2.29^{g}$ |
| $I_{S}\times G_{r}\times F_{v}$ | $62.96^{g}$ | $11.57^{gh}$ | $2.41^{e}$ | $0.23^{de}$ | $1.43^{k}$ | $1.46^{de}$ | $1.44^{j}$ |
| $I_{S}\times G_{r}\times F_{m+v}$ | $72.95^{de}$ | $56.58^{c}$ | $2.90^{de}$ | $0.23^{de}$ | $2.71^{f}$ | $1.98^{b}$ | $2.46^{f}$ |

**Table.4 Comparison between 2-year mean values of physiological traits of lingrain affected by green manure× mycorrhiza × vermicompost under full and limited irrigation**

Where $I_{w}$ (full irrigation), $I_{s}$ (limited irrigation), $G_{c}$ (no green manure), $G_{r}$ (green manure), $F_{c}$ (no fertilizer), $F_{m}$ (mycorrhiza inoculation), $F_{v}$ (vermicompost application) and $F_{m+v}$ (combination of mycorrhiza and vermicompost). Means followed by the same letter in each column are not significantly different

| Treatment | Glycine betaine (µmol/g FW) | Hydrogen peroxide (mol/g FW) | Malondialdehyde (*mol*/g FW) | Ascorbate Peroxidases activity (mmol min$^{-1}$/g FW) | Guaicole peroxidase activity (mmol min$^{-1}$/g FW) | Grain yield (kg h$^{-1}$) | Oil yield (kg h$^{-1}$) |
|---|---|---|---|---|---|---|---|
| $I_{W}\times G_{c}\times F_{c}$ | $19.20^{gh}$ | $11.02^{e}$ | $31.80^{fg}$ | $1.35^{j}$ | $0.65^{g}$ | $3298^{gh}$ | $870.12^{gh}$ |
| $I_{W}\times G_{c}\times F_{m}$ | $13.05^{j}$ | $7.32^{g}$ | $24.13^{hi}$ | $3.43^{fg}$ | $2.38^{e}$ | $4228^{bc}$ | $1209.92^{d}$ |
| $I_{W}\times G_{c}\times F_{v}$ | $18.27^{h}$ | $7.67^{g}$ | $26.22^{h}$ | $2.78^{i}$ | $2.36^{c}$ | $3995^{cd}$ | $1102.8^{ef}$ |
| $I_{W}\times G_{c}\times F_{m+v}$ | $11.99^{j}$ | $5.65^{h}$ | $16.93^{j}$ | $3.24^{gh}$ | $3.45^{c}$ | $4456^{b}$ | $1287.72^{c}$ |
| $I_{S}\times G_{c}\times F_{c}$ | $21.95^{e}$ | $19.02^{a}$ | $70.05^{a}$ | $3.97^{e}$ | $1.75^{f}$ | $2050^{j}$ | $452.09^{j}$ |
| $I_{S}\times G_{c}\times F_{m}$ | $26.67^{cd}$ | $12.18^{cd}$ | $36.56^{d}$ | $5.51^{b}$ | $2.59^{de}$ | $3233^{gh}$ | $842.91^{h}$ |
| $I_{S}\times G_{c}\times F_{v}$ | $25.47^{d}$ | $13.05^{c}$ | $43.45^{c}$ | $4.8^{d}$ | $2.47^{e}$ | $2350^{i}$ | $604.20^{i}$ |
| $I_{S}\times G_{c}\times F_{m+v}$ | $33.93^{a}$ | $10.80^{e}$ | $34.26^{e}$ | $5.06^{c}$ | $3.69^{bc}$ | $3237^{gh}$ | $868.69^{g}$ |
| $I_{W}\times G_{r}\times F_{c}$ | $20.40^{fg}$ | $9.67^{f}$ | $29.69^{g}$ | $1.71^{j}$ | $3.24^{c}$ | $3463^{fg}$ | $1095.92^{ef}$ |
| $I_{W}\times G_{r}\times F_{m}$ | $19.81^{fg}$ | $5.74^{h}$ | $23.94^{i}$ | $3.81^{ef}$ | $4.08^{b}$ | $4359^{b}$ | $1422.83^{b}$ |
| $I_{W}\times G_{r}\times F_{v}$ | $20.98^{ef}$ | $7.93^{g}$ | $24.10^{hi}$ | $2.93^{h}$ | $3.1^{cd}$ | $3999^{cd}$ | $1290.68^{c}$ |
| $I_{W}\times G_{r}\times F_{m+v}$ | $16.03^{i}$ | $5.52^{h}$ | $16.40^{j}$ | $3.37^{fgh}$ | $4.24^{b}$ | $4862^{a}$ | $1602.66^{a}$ |
| $I_{S}\times G_{r}\times F_{c}$ | $21.93^{e}$ | $17.10^{b}$ | $59.19^{a}$ | $4.48^{d}$ | $3.37^{c}$ | $2312^{i}$ | $637.43^{i}$ |
| $I_{S}\times G_{r}\times F_{m}$ | $28.41^{b}$ | $11.37^{de}$ | $33.21^{ef}$ | $5.92^{b}$ | $4.13^{b}$ | $3587^{ef}$ | $1052.97^{f}$ |
| $I_{S}\times G_{r}\times F_{v}$ | $27.48^{bc}$ | $12.45^{c}$ | $42.04^{c}$ | $5.61^{c}$ | $4.06^{b}$ | $3187^{h}$ | $928.2^{g}$ |
| $I_{S}\times G_{r}\times F_{m+v}$ | $34.84^{a}$ | $9.85^{e}$ | $31.14^{fg}$ | $7.15^{a}$ | $5.27^{a}$ | $3806^{d}$ | $1129.08^{e}$ |

**Table 5. Comparison amongst 2-year mean values of physio-biochemical traits, leaf P, Total soluble sugars, Catalase and Superoxide Dismutase activityaffected by mycorrhiza × vermicompost under full and limited irrigation**

Where $I_{w}$ (full irrigation), $I_{s}$ (limited irrigation), $F_{c}$ (no fertilizer), $F_{m}$ (mycorrhiza inoculation), $F_{v}$ (vermicompost application) and $F_{m+v}$ (combination of mycorrhiza and vermicompost). Means that were followed by the same letter in each column, are not significantly different

| Treatment | Leaf P % on dry matter | Proline (µmol/g FW) | Total soluble sugars (µmol/g FW) | Glycine betaine (µmol/g FW) | Hydrogen peroxide (µmol/g FW) | Catalase activity (mmol $\text{min}^{-1}$/g FW) | Superoxide Dismutase activity (Units/g FW) |
|---|---|---|---|---|---|---|---|
| $I_w \times F_c$ | $0.47^{d}$ | $23.04^{d}$ | $22.78^{e}$ | $19.80^{cd}$ | | $4.76^{f}$ | $19.14^{e}$ |
| $I_w \times F_m$ | $0.65^{ab}$ | $18.45^{e}$ | $15.93^{f}$ | $16.43^{e}$ | | $7.89^{b}$ | $23.21^{c}$ |
| $I_w \times F_v$ | $0.64^{b}$ | $18.63^{e}$ | $15.26^{f}$ | $19.62^{d}$ | | $7.23^{d}$ | $21.71^{d}$ |
| $I_w \times F_{m+v}$ | $0.67^{a}$ | $12.68^{f}$ | $10.89^{g}$ | $15.82^{e}$ | | $7.71^{bc}$ | $23.21^{c}$ |
| $I_s \times F_c$ | $0.25^{f}$ | $25.09^{c}$ | $27.85^{d}$ | $21.94^{c}$ | | $5.33^{e}$ | $23.72^{c}$ |
| $I_s \times F_m$ | $0.44^{de}$ | $27.80^{b}$ | $39.10^{b}$ | $27.54^{b}$ | | $8.61^{a}$ | $32.91^{a}$ |
| $I_s \times F_v$ | $0.42^{e}$ | $27.42^{b}$ | $33.74^{c}$ | $26.47^{b}$ | | $7.42^{cd}$ | $29.08^{b}$ |
| $I_s \times F_{m+v}$ | $0.53^{c}$ | $36.47^{a}$ | $44.12^{a}$ | $34.39^{a}$ | | $8.71^{a}$ | $33.22^{a}$ |

**Table 6. Comparison amongst 2-year mean values of physio-biochemical traits, leaf P, Total soluble sugars , Superoxide Dismutase activity under full and limited irrigation**

Where $Y_1$ (first year), $Y_2$ (second year), $I_w$ (full irrigation), $I_s$ (limited irrigation),. Means that were followed by the same letter in each column, are not significantly different

| Treatment | Leaf P % on dry matter | Total soluble sugars (µmol/g FW) | Superoxide Dismutase activity (Units/g FW) |
|---|---|---|---|
| $Y_1 \times I_w$ | $0.57^{b}$ | $15.72^{d}$ | $21.6^{c}$ |
| $Y_1 \times I_c$ | $0.37^{d}$ | $35.10^{b}$ | $22.03^{c}$ |
| $Y_2 \times I_w$ | $0.66^{a}$ | $16.71^{c}$ | $28.86^{b}$ |
| $Y_2 \times I_c$ | $0.45^{c}$ | $37.31^{a}$ | $30.6^{a}$ |

# Figures

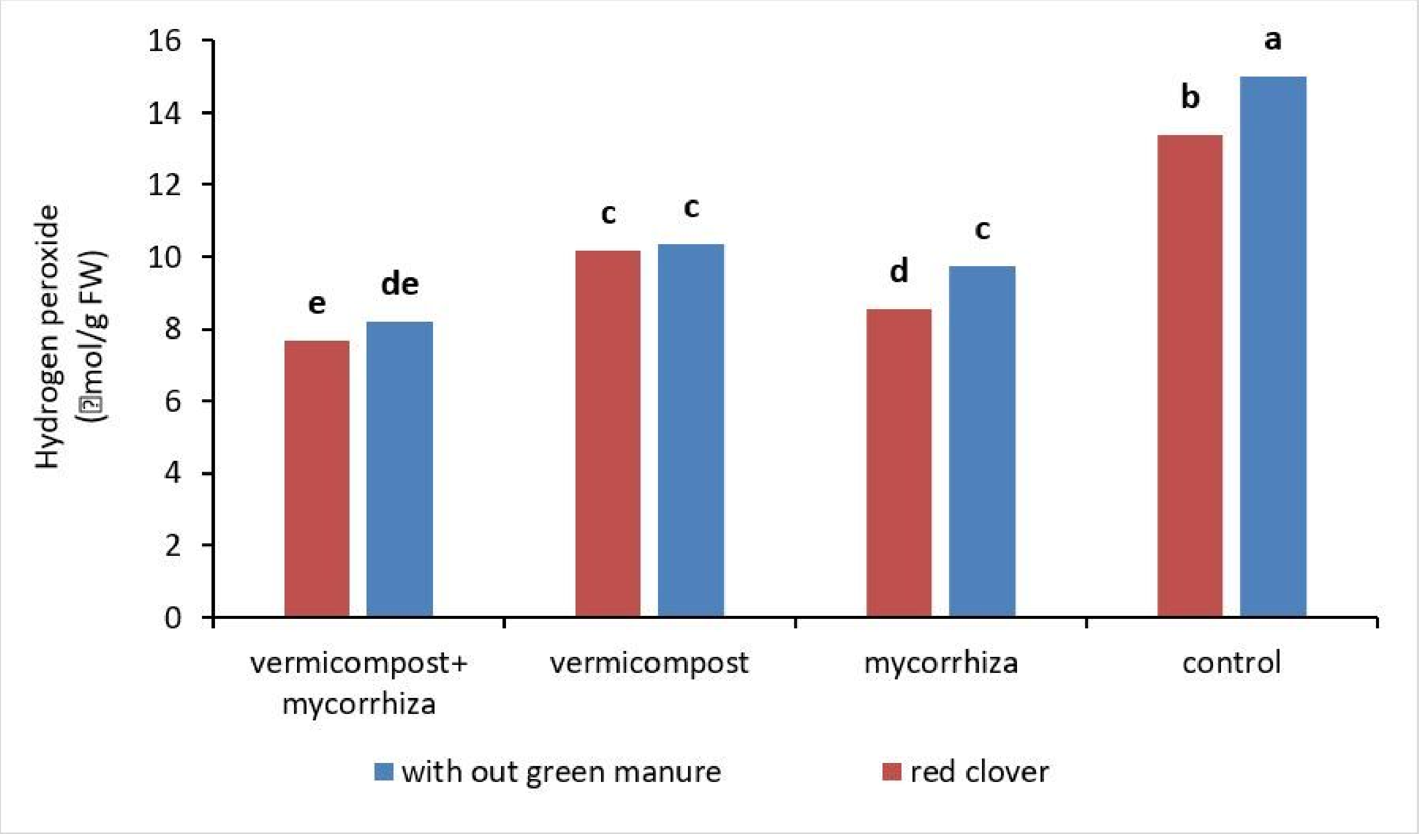

**Figure 1**

Interaction effects of green manure * fertilizers on Hydrogen peroxide concenttation (Means followed by the same letter are not significantly different)

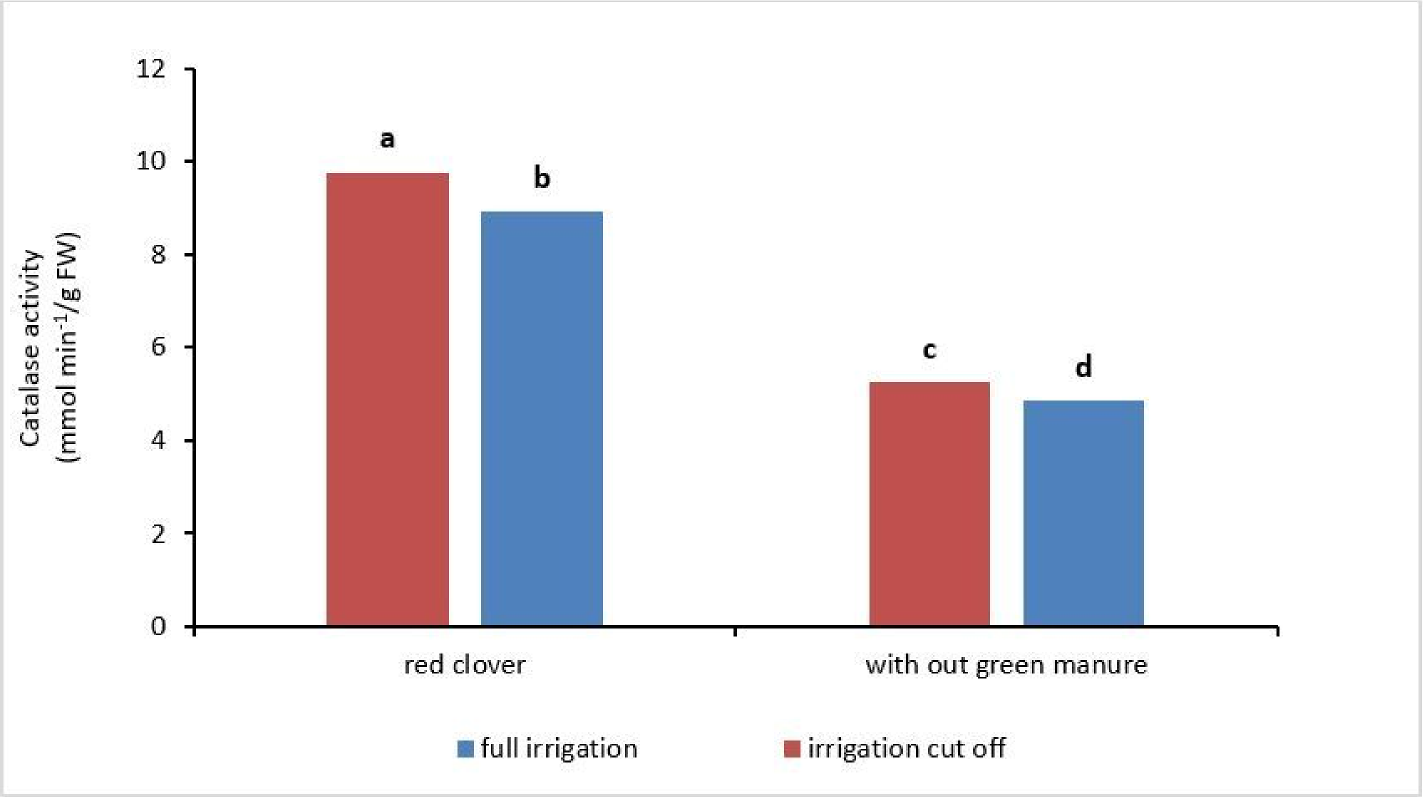

**Figure 2**

Interaction of green manure and irrigation regimes on catalase activity.

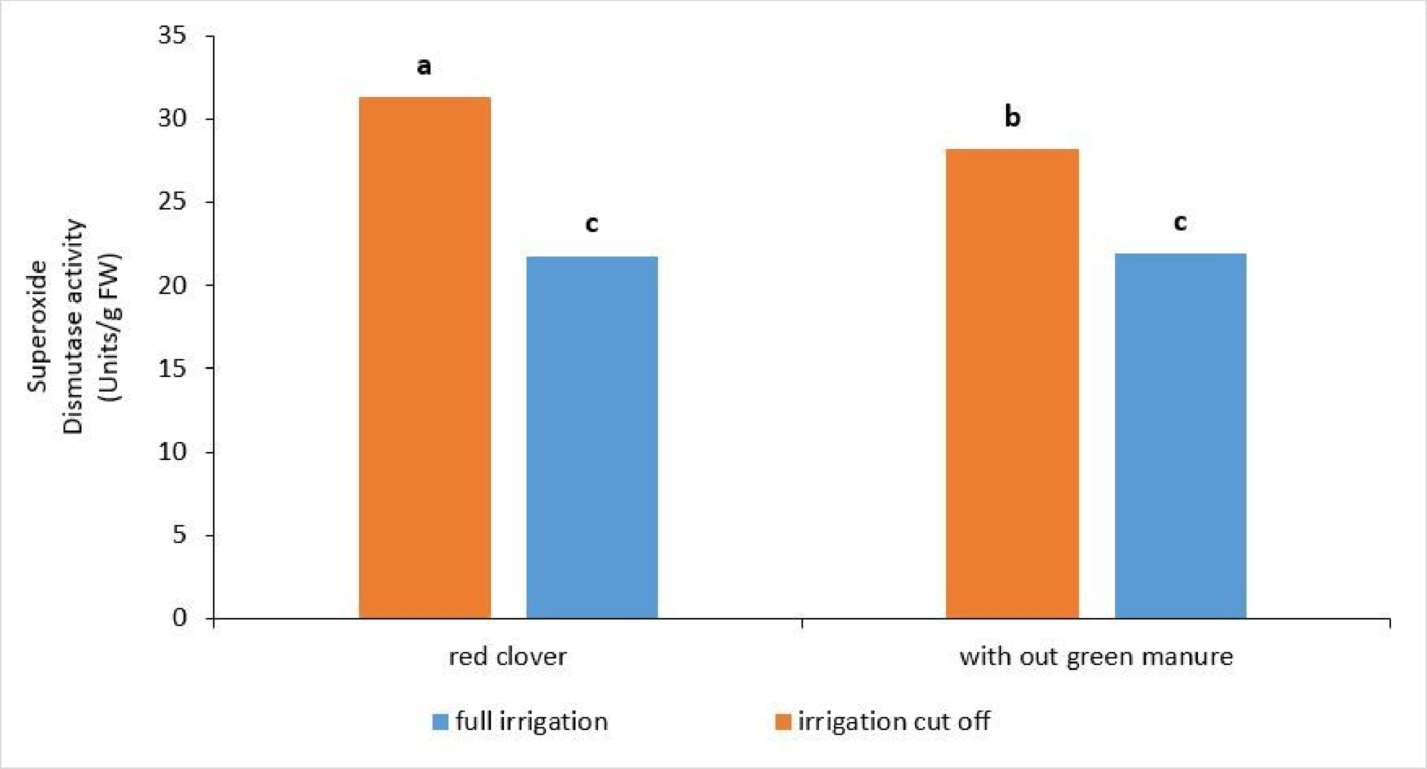

**Figure 3**

Interaction of green manure and irrigation regimes on Superoxide Dismutase activity.