

Alleviation of water limited stress in the grass pea (*Lathyrus sativus* L.) through arbuscular mycorrhizal fungi and rhizobium bacteria symbiosis

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14 **Declaration of interests**

15 ☑ The authors declare that they have no known competing financial interests or personal relationships
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Title

Abstract

Grass pea (*Lathyrus sativus* L.) is a considerable leguminous crop, but its growth and development are astricted by drought, which is intimidatory to grass pea production especially in arid or semiarid regions. Farmers are currently adopting good agricultural practices in the crop production. When producing forage, bio organic nutrients are being considered as a better alternative to chemical fertilizers. Here, two consecutive-year of experiments were conduct to investigate the efficiency of bacteria and arbuscular mycorrhizal fungi in changing the growth and yield related traits of grass pea under different irrigation regimes (well watered, mild and severe limited water conditions). The results demonstrated that the unfertilized and stressed plants had the lowest performance of grass pea, while plants inoculated with biofertilizers performed the best. The limited water caused a decline in total chlorophyll content (20.3%), leaf area index (20.32%), relative water index (5.5%), seeds/pod (2.9%), pods/plant (20.23%), 1000-seed weight (2.92%), pod harvest index (0.72%), seed yield (24.5%) and biological yield (17.69%) relative to well-watered treatment, over two years. Rhizobium bacteria inoculation enhanced contents of relative water, seeds/pod, 100-seed weight, biological yield and pod harvest index by average of 9.25%, 4.82%, 4.81%, 16.78% and 1.36%. Moreover, arbuscular mycorrhizal fungi inoculation increased the relative water content (7.7%), seeds/pod (3.8%), 1000-seed weight (3.9%), pod harvest index (1.04%) and pod partitioning index (13.9%). It can be concluded that application of a mixture of biofertilizers belonging to different groups could be a beneficial method and show a positive effect on the productivity of grass pea under limited water conditions.

Keyword: field inoculation, Mycorrhiza fungi, Soil microorganisms, fertilizers, biofertilizer, assimilate remobilization, seed metabolism, plant partitioning, partitioning pattern, leaf, pod

Abbreviations:

Highlights

- The increase in seed yield was further amplified with micro organisms inoculation
- Plants receiving well water supply showed higher pod harvest index
- Pod harvest index was more affected by arbuscular mycorrhizal fungi rather than bacteria
- Combination of two kind of micro organisms did not change the biomass partitioning

Introduction

Nowadays, climate change, high temperature and water limited stress are a significant challenge for agriculture worldwide which predicted to bring about decreased precipitation and increased temperature in the range of 15% and 1.7 °C, respectively by 2040 (Xu et al. 2021). Limited water conditions can have an impact on the metabolism of plant, affecting processes such as photosynthesis which can increase production of hydrogen peroxide (H₂O₂) and superoxide anion (O⁻²) which known as reactive oxygen species (ROS), cause photooxidative injury to membrane lipids, proteins, nucleic acids and photosynthetic pigments, ultimately affecting the relative water content (RWC), leaf area index (LAI), plant height, yield and its related traits (Keshavarz and Khodabin 2019). In response to drought, plant may undergo metabolic adjustments to prioritize the allocation of resources towards essential functions like root growth and water uptake, while temporarily reducing investment in non-essential processes like shoot growth or reproductive development. This phenomenon causing up to 75% of yield losses worldwide and crops have evolved certain mechanisms to tolerate water scarcity.

Grass pea (*Lathyrus sativus* L.) is a high quality and quantity legume that needs lower nitrogen (N) fertilizer rather than other forage crops due to its ability to fix atmospheric nitrogen. Moreover, considering the rising prices of maize due to its higher irrigation consumption and its use in biofuel industry, grass pea hay might be an interesting alternative for source of forage and haymaking production (Rah Khosravani et al. 2017; Haghighi-Hasanalideh and Hojati 2012; Akbari and Hojati 2013). Grass pea (*Lathyrus sativus* L.) in the family of Fabaceae, is a cool season leguminous plant and most important forage crop (multipurpose legume) in Iran after alfalfa and maize. Traditionally grown as an animal feed and cover crop for soil conservation, recently it has gained attention for its seed yield. Grass pea cultivated primarily in regions with dry and marginal

soils, it has been a staple in many parts of Asia, Africa, and Europe. Although, its productivity is limited by water shortage but presents a unique intersection of food security and mental health compared to other forage plant which and could play an important role in supporting the well-being of vulnerable populations worldwide. Moreover, growing cost of chemical fertilizers has become interest in legumes for crop rotation systems. Legumes such as grass pea fix atmospheric nitrogen (N_2) biologically, decreasing the need for chemical N fertilizer and positively benefits subsequent non legume plants (Biswas et al. 2024). It has been reported that grass pea adding 70 kg.ha⁻¹ of N to soil in one growing season and has protein and yield benefit on next non-legume plants (Barat et al. 2024; Gholamhoseini et al. 2012).

Biofertilizers offer a more eco-friendly alternative by providing similar benefits while minimizing negative effects of the excessive use of chemical fertilizers. Biofertilizers play a crucial role in improving nitrogen (N), phosphorous (P) and potassium (K) availability and uptake by plants. Despite its biological nitrogen fixation, biofertilizers activities production of growth hormones, hydrolytic enzymes, siderophore, and hydrogen cyanide (HCN), ultimately, directly or indirectly increase plant growth and productivity. In drought-stressed soils, where nutrient mobilization is often impaired, biofertilizers can help by fixing nitrogen (N_2) from the atmosphere and making it accessible to the plant (Xu et al. 2023). Therefore, biofertilizers contribute to sustainable agricultural practices by reducing the need for synthetic fertilizers and promoting soil health.

Overall, grass pea has evolved a range of adaptive mechanisms to cope with limited water stress and maintain its growth and productivity in challenging environmental conditions. Indeed, studying the effect of biofertilizers on grass pea under limited water stress is a multifaceted research endeavor with implications for food security, sustainable agriculture, environmental

conservation, and economic development. Taken together, the influence of double inoculation with rhizobia (*Rhizobium*) and AM fungi of *Glomus mosseae* on grass pea under water limiting conditions has not yet been studied and the benefit of these biofertilizers for crop management under water limiting conditions are still debated. Therefore, the aim of current study was to compare LAI, total chlorophyll content and biomass partitioning index of grass pea.

Materials and Methods

This two-years experiment (2020-2021) was conducted at the local farm of Ghale Sefid village, Najaf Abad region, Isfahan, Iran (1622 m.a.s.l, 51°39' latitude and 32°38' longitude) on silty loam soil. Plots were performed as a factorial split-split plot assigned in randomized complete block with three replications. Irrigation in three levels: irrigation at 75% (well watered), 60% (mild limited water stress), and 45% (severe limited water stress) of depletion of the available resource of soil water (Keshavarz Mirzamohammadi et al. 2021a) registered as main plot and two treatments of plant growth promoting regulator (PGPR) bacteria of *Rhizobium leguminosarum* (with inoculation and without inoculation) as sub plot and arbuscular mycorrhizal (AM) fungi consisted of *Glomus intraradices* (with inoculation and without inoculation) as sub-sub plot. Therefore, twelve treatment combinations consisting of three irrigation levels, two treatments of PGPR bacteria strains of *R. leguminosarum* and AM fungi strains of *G. intraradices* were the whole 36 plots within each block. The split-split plot configuration was used to minimize errors.

Seeds of grass pea (*Lathyrus sativus* L.) cultivar Mashhad commonly planted in Iran were obtained from National Seed and Plant Improvement Institute, Iran, Karaj. The seeds used had yellow cotyledon types and dark brown seed coats. At the first, the seeds were surface sterilized by immersing in 5% sodium hypochlorite solution (for three minutes) and 96% ethanol (for thirty seconds) followed by 2-3 times successive rinses in distilled water (Keshavarz and Sadegh Ghol

Moghadam 2017). According to the manufacturer's recommendation, inoculation was performed by soaking the grass pea seeds in mixture adjusted to 10^9 CFU ml⁻¹ with sterile normal saline (0.9% NaCl) for 4 hours at 28 °C before sowing (Keshavarz, 2020). Seed inoculation with *PGPR bacteria strains of R. leguminosarum* and AM fungi strains of *G. intraradices* was done by coating a slurry of peat, clay and 10% sugar solution. An uninoculated seeds (control plant) were soaked with sterilized water and served as controls.

During the growth season the amount of water for each level of irrigation level estimated according to the soil moisture using the following equation and accordingly the precise amount of irrigation water was applied by the contour for each treatment (Keshavarz Mirzamohammadi et al. 2021a):

$$V_w = \frac{(FC-PWP)}{100} \times pb \times V_b \times MAD$$

Equation 1

where, V_w : necessary irrigation water volume (cm³), FC: field capacity (%), PWP: wilting point (%), pb: bulk density (g.cm⁻³), V_p : pot volume (cm⁻³), MAD: management allowed depletion. FC and PWP were determined using a pressure plate.

Soil analyses showed that the levels of N, P and K were 0.02%, 11.7 mg.kg⁻¹ and 334 mg.kg⁻¹ respectively, with soil pH between 7.8 and 7.9. Monthly temperature and precipitation were recorded in each year (Figure 1). The seeds of grass pea were planted in line given spacing of 15 × 30 cm in a 1.8 × 3 m plot where the number of rows were maintained 6 plot⁻¹ with considering the 1 and 1.5 m spacing between plots and blocks, respectively. After a deep summer plowing and two tillages, the seeds (40 kg ha⁻¹) of grass pea were drill and lightly harrowed into the soil (15 cm row spacing) in first week of March 2020 which was the year of establishment. The urea was applied in two steps (flowering and podding stage) but triple superphosphate and potassium chloride were broadcasted and incorporated into the soil before planting. Fertilizers urea (CH₄N₂O,

46% N), triple superphosphate ($\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$; 45% P, 15% Ca) and potassium chloride (KCL or muriate of potash; MOP, 60% K) were used to apply 80-90-60 kg ha⁻¹ as of N, P and K, respectively, in each pot (Recommended doses).

For measuring the total chlorophyll content, relative water content (RWC) and leaf area index (LAI), three randomly chosen plants from a single row of each plot at flowering stage. The total chlorophyll contents in the leaves was calculated using the methods developed by Lichtenthaler and Welburn (1983) describe as below:

$$\text{Total chlorophyll} = (\text{A}_{663} \times 8.02) + (\text{A}_{645} \times 20.2) \times \text{V/W} \times 1000$$

Equation 2

where A = Absorbance, V = Extract volume (mL) and W = Fresh weight of leaf tissue (g).

Keshavarz (2020) method was used to estimate relative water content (RWC) of leaves as below:

$$\text{Relative water content} = \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \times 100$$

Equation 3

where FW = fresh weight (g), FW, DW = dry weight (g) and TW = total weight of leaf tissue (g). Leaf area index was measured using electronic leaf area meter, LI-3100-LICOR, Lincoln, NE, USA (Keshavarz et al. 2016).

Harvest treatments were laid out in 2020 and 2021 (end of June), between 09:00 am and 11:00 am at full maturity stage. The cuts were initiated 5 cm high from soil surface. Data were recorded as average of ten randomly selected plants from the central two rows in each plot to evaluated for the number of pods plant⁻¹, the number of seed pod⁻¹, 1000-seed weight (g), seed yield (kg ha⁻¹), biological yield (kg ha⁻¹) and pod dry weight (kg ha⁻¹). Estimating of biological yield was done by harvesting of 1 m² of herbage through the center of each plot. For this purpose, the samples were weighted and dried at 60 °C for 72 hours in an oven until constant weight. The biomass partitioning index (harvest index, pod harvest index and pod partitioning index; equation 4-6) were determined

at mid-pod filling stage which is supposed to be the time that shows the maximum vigor of the plants as described by Beebe et al. (2010):

$$\text{Harvest index (HI) \%} = [\text{seed dry weight} / \text{total biological dry weight}] \times 100$$

Equation 4

$$\text{Pod harvest index (PHI) \%} = [\text{seed dry weight} / \text{pod dry weight}] \times 100$$

Equation 5

$$\text{Pod harvest index (PPI) \%} = [\text{Pod dry weight} / \text{total biological dry weight}] \times 100$$

Equation 6

Data were statistically analyzed by implementing the analysis of variance (ANOVA) methods (Hosseini et al. 2023b) for factorial randomized block design. The LSD test was carried out to comparison the differences between mean values at a 5% level of significance. SAS 9.1 was used for statistical analysis (Keshavarz Mirzamohammadi et al. 2021b; Gholamhoseini et al. 2013).

Results

Analysis of variance

Significant differences were achieved in most parameters under irrigation regimes among biofertilizer treatments (Table 1). Analysis of variance over the entire data showed that all trait except harvest index and pod partitioning index significantly affected by main effect of irrigation regimes and bacteria inoculation. Furthermore, the two mycorrhiza levels differed significantly for total chlorophyll content, RWC, LAI, pods/plant, seeds/pod, 1000- seed weight, seed yield, PHI and PPI (Table). Variation in total chlorophyll content, leaf area index, pods/plant, seed yield and biological yield was observed among irrigation regimes from different year (Table 1). Significant ($P < 0.05$) differences were found in interaction of bacteria $\times$ mycorrhiza for total chlorophyll, LAI, pods/plant and seed yield (Table 1). The two-way interaction among year and bacteria, irrigation and bacteria, year and mycorrhiza, irrigation and mycorrhiza, the three-way interaction

among year and irrigation and bacteria, year and irrigation and mycorrhiza, year and bacteria and mycorrhiza, irrigation and mycorrhiza and bacteria and the four-way interaction among year and irrigation and mycorrhiza and bacteria were not significant for any of the studied traits.

Total chlorophyll content

The mean value for total chlorophyll content ($\mu\text{g g}^{-1}$ FW) among irrigation levels ranged from 2.48 to 3.68 and 2.41 to 3.12 in first and second year, respectively (Table 2). The maximum total chlorophyll content was recorded with well-watered irrigation in first year ($3.62 \mu\text{g g}^{-1}$ FW). In second year mild and severe limited water stress caused 11.2% and 22.7% reduction in total chlorophyll content compared to well-watered conditions (Table 2). The bacteria strain of *R. leguminosarum* and AM fungi strains of *G. intraradices* improved total chlorophyll content of grass pea (Table 3). Under *R. leguminosarum* inoculation, plants treated with *G. intraradices* had the highest total chlorophyll content ($3.40 \mu\text{g g}^{-1}$ FW) which was higher than no *G. intraradices* application by 14.7%. However, the difference between inoculation and non inoculation of AM fungi among with out bacteria application was 7.94% (Table 3).

Relative water content

The imposed of limited water conditions down regulated the RWC and RWC reduced gradually from well-watered conditions to severe limited water conditions. RWC was 80.6%, 77.5% and 74.5% under well-watered, mild and severe limited water conditions (Table 4). Inoculated plants with bacteria boost RWC of grass pea over the control (not inoculation) by 9.25% (Table 5). The greater value of RWC were observed in plants inoculated with AM fungi than the control and the mean value of RWC in inoculated plants increased 7.7% compared with non inoculation (Table 5).

Leaf area index

Leaf expansion was still processed during limited water conditions but at reduced value compared with well-watered conditions. Average over the years, the LAI in the well-watered conditions was 3.95 (some data not shown). This is more than 13.7% and 27.3% compared to mild and severe water limited conditions. However, in first year, plants produced from mild and severe water limited conditions had 15.8% and 31.3% less LIA than well-watered plants (Table 2). In second year, LAI was 11.4% and 22.6% higher in mild and severe water limited stress than well-watered conditions. The inoculation of bacteria and AM fungi significantly increased the LAI (Table 3). The improvement in LAI with AM fungi was 14.8% and 7.3% with and without bacteria inoculation, respectively. Among the treatments, plants inoculated with *R. leguminosarum* and *G. intraradices* attained the maximum LAI (3.98) followed by plants with bacteria and without AM fungi (3.93) and plants without bacteria and with AM fungi (3.25) which were in a same statistical group (Table 3).

Pods/plant

Based on the pooled across the years, plants grown under well-watered conditions recorded significantly the highest pods per plant. Despite of irrigation levels, the number of the pods in the first year was higher than second year by 9.22% (data not shown). However, the interaction treatments, plants grown in the first year under well-watered conditions, registered maximum pods/plant (46.53) which was higher than mild and severe limited water conditions by 15.64% and 31.36% (Table 2). In second year mild and severe limited water stress caused 11.32% and 22.67% reduction in pods per plant compared to well-watered conditions. There was considerable difference among biofertilizer treatments for pods per plant and inoculation of each *R. leguminosarum* or *G. intraradices* increased pods/plant compared to uninoculated plants. The dual inoculation of *R. leguminosarum* with *G. intraradices* proved that both microorganisms were

efficient for plant to reach optimum yield. *R. leguminosarum* colonized plants increased pods per plant by 11.37. However, the effect of AM fungi in increasing the number of pods per plants was 7.44% (Table 3)

Seeds/pod

As evident from the results (Table 4) limited water caused a considerable decrease in seeds per pod. In this regard, the mild and severe limited water conditions caused 0.6% and 3.88% of reduction in number of seeds per pod, respectively. As shown in Table 5, the number of seeds per pod in plants inoculated with *R. leguminosarum* significantly increased by 4.82%. As compared to uninoculated plants, the number of the seeds per pod at *G. intraradices* inoculation was raised to 3.09 seeds per pod which was higher than control by 3.88% (Table 6). The AM fungi also increased the number of seeds per pod compared to non inoculation but the effect of rhizobium bacteria was more evident in seed weigh.

1000-seed weight

Our study showed that limited water conditions caused a considerable reduction in seed weight (Table 4). The 1000-seed weight was decreased up to 1.9% and 3.9% under mild and severe limited water conditions, respectively compared to well-watered conditions. Among the inoculated plants, all of them had higher seed weight than the uninoculated plants (Table 5). In fact, the selected *R. leguminosarum* enhanced 1000 seed weight from 122.6 g to 128.8 g and improved this trait by 4.81% in comparison with uninoculated plants. In terms of the *G. intraradices* treatment, inoculation with *G. intraradices* provided the highest 1000-seed weight (128.2 g) which means that significantly increased the seed weight by 3.9%.

Seed yield

Results in Table 2 depicted that the highest seed yield was observed in first year and well-watered conditions. While further increase in drought severity negatively affected the seed yield. Minimum seed yield was observed in severe limited water conditions with 38.1% and 26.58% reducing over the well-watered in the first and second year, respectively. Data in Table 3 reflected the ability of *R. leguminosarum* and *G. intraradices* to produce higher seed yield rather than uninoculated control plants. Accordingly, the seed yield (on average across AM fungi) was 6152.6 kg ha⁻¹ in inoculated plants and decreased by 16.71% in uninoculated control plants. Although a negligible reduction was achieved in seed yield for plants that not inoculated with *G. intraradices* (on average across *R. leguminosarum*) and plants inoculated with *G. intraradices* had 3.63% higher seed yield. A quick glance on results (Table 3) showed that AM fungi inoculation plants did not response significantly to bacteria inoculation.

Biological yield

On average across irrigation regimes, a slight increment (by 5.8%) was achieved for biological yield at second year compared with the first year (Table 2). However, in both years, as limited water increased the biological yield decreased. Plants grown under well-watered conditions had the highest biological yield (6432.4 and 6335.7 kg ha⁻¹ in first and second year, respectively). Accordingly, plants grown under mild and severe limited water conditions showed a 14.98% and 29.97% reduction in the first year and 8.6% and 17.2% reduction in second year, respectively (Table 2). In comparison with uninoculated plants, inoculation with *G. intraradices* positively affected biological yield and increased biological yield by 16.7% (Table 5).

Pod harvest index

Data in Table 4 showed that limited water stress had negative effect on PHI and this trait decreased substantially with increasing drought severity. Maximum PHI of 86.9% was observed

in well-watered condition with 0.4% and 0.9% increase over mild and severe limited water stress. When compared with uninoculated plants, the improvement of 1.36% in PHI was also achieved in rhizobium bacteria inoculation plants (Table 5). Data regarding the PHI also, revealed a reduction of 1.04% in uninoculated plants compared to *G. intraradices* inoculated plants (Table 6).

PPI

Same trend was achieved in case of PPI, inoculation of plants with *G. intraradices* had positive effect on PPI (Table 6). In comparison with uninoculated plants higher had 13.99 higher PPI.

Discussion

It has been demonstrated that water scarcity and drought stress is one of the limiting factors for plant growth and development due to suppresses effect of limited water and low turgor pressure inhibition on photosynthetic system, cell elongation and expansion growth (Ajoudani et al. 2024; Hosseini et al. 2023a). In limited water conditions, the total chlorophyll content was far below rather than well-watered plants. The reduction of photosynthesis machinery under limited water conditions caused by stomatal closure or metabolic impairment (non stomatal limitation) which lead to generation of ROS in thylakoids membrane, oxidative damage and ultimately reduction in total chlorophyll content. Under stress conditions, the efficiency of Calvin cycle (dark reaction of photosynthesis) is decreased, therefore the amount of ROS increased due to photo oxidative reaction. In one hand, a decrease in total chlorophyll content under water limiting may be due to disorders in synthesis of plant pigments, reduction in activity of 5-aminolevulinic acid and protochlorophyllide reductase (Akram and Ashraf 2013). In other hand, the oxidation of chlorophyll is the solution to reduce the production of ROS to decreases the level of light intercepted by plant and reduces the generation of ROS and further damage to photosynthetic apparatus. Thereby, part of decrease in total chlorophyll content caused by plants' defenses system

to inhibit the production of ROS. We observed that regardless of limited water conditions, biological fertilizers positively affected total chlorophyll content, increased the content of pigment machinery revealing a direct effect on growth and biomass canopy. Similar to these results, Zhu et al. (2017) revealed that *Rhizophagus irregularis* and *Glomus versiforme* inoculation increase the induction of chlorophyll synthesis and decreases the activity of chlorophyllase genes in black locust (*Robinia pseudoacacia* L.), which leads to producing higher chlorophyll content, more photosynthesis and carbon fixation values.

Foliar RW has long been considered as the realistic criteria for measuring plant water status and reduction in RWC value due to loss of cellular turgor that lead in limited cell elongation and consequentially, reduced in expansion plant growth. Although, we did not estimate the rate of Indole-3-acetic acid (IAA), the modification in RWC and several phytohormones have been associated with together to increase the content of inhibit growth substances to allowing the cell to keep the water budget (Nguyen et al. 2016). It has been reported for 80% of bacteria which improves the production of phytohormones like IAA, cytokinin and gibberellic acid (Bi and Zhou 2021).

Stomatal closure and reduction of leaf growth are the first responses to limited water conditions to protect the plant cell from oxidative damages and extensive water loss. A decrease in transpiration rate, gas exchange, photosynthesis, membrane stability index and leaf water potential are the consequence of limited water conditions (Arnold et al. 2019). Moreover, Disturbances in carbohydrate metabolism may lead to a carbon unbalance and reduction of growth. In our study, there were strong and positive correlation between RWC and LAI indicated the reduction in RWC was associated with a decline in cell expansion and more leaf senescence (some data not shown). Therefore, the lowering of RWC in current study might be responsible for the lower value of LAI

under stress conditions. The leaf area index was also higher in inoculated plants than in control plants. Inoculation with root zone microorganisms improved the chlorophyll concentrations, provided the carbohydrate as the prerequisite for leaf area and other yield related traits. Overall, inoculation with *R. leguminosarum* provided a more functional LAI than *G. intraradices* so as to arranged a better facility for photosynthesis. The use of sunlight and the rate of photosynthesis apparatus determine the pod fullness in further growth periods. Biofertilizers increased the chlorophyll content and also the leaf area for photosynthesis and carbohydrate production which finally increased biological yield. In this regard, the LAI in inoculated plants became more developed compared with control to receive more solar energy and improved the photosynthetic capacity to meet plant needs.

In the none inoculated plants, due to lake of adequate nutrients, the leaf senescence began earlier which significantly reduced the total dry weight and pods were small or even non filled. It may be that biofertilizers first improve the root structure and function due to higher mycelia which provided uptake and transporting more elements to leaves and stem, increase robustness of above ground part and optimized plant biomass. Errickson and Huang (2024) has been showed that microbial fertilizers increase the carbon fixation rate which would increase both the growth of the host plant and microorganisms due to the reciprocal P–C relationship between them (Hosseini et al. 2023a). This process leads to increasing the concentration of CO₂ in the soil, turned to carbonic acid and could enhancing P and other nutrients availability. Although, we did not estimate the content of mineral nutrients but an increase in the value of magnesium and calcium by AM fungi and bacterial inoculation was observed (Keshavarz et al. 2021). Hu et al. (2022) reported that, inoculated plants have more transportation of carbohydrates from shoot to root due to meet plants needs because biofertilizers accelerate tricarbones transport and *Pi* cycle, thereby promote the

tricarboxylic acid pathway which leads to higher photosynthetic capacity. Bi and Zhou (2021) documented that utilization of glucose and sucrose increased by inoculated plants to accelerate the transportation of carbohydrate from source to sink.

Muhammad Aslam et al. (2022) stated that abscisic acid produced in the leaves of stressed plants is mobilized to the reproductive parts which could be associated in decreasing 1000-seed, number of pods or seeds per plant. Therefore, higher number of pods or seeds could have an advantage for compensation if pod or seed senescence occurs during limited water conditions. Moreover, limited water availability had adverse effect on the translocation of photosynthetic products like sucrose and other sugars due to disruption of photosynthetic pathway and their related enzymes (Martignago et al. 2020). Inoculated plants had higher seeds number and pods number per plant, which yielded more not because of their lower seed weight (Table 3) but because of their superior photosynthetic efficiency and sink strength to be able to producing more assimilates, with better photosynthate allocation to seed filling. The current data showed that the combination of *R. leguminosarum* and *G. intraradices* has a more beneficial influence than the single symbiotic. A comparable interaction with bio organic fertilizers in legume crops were also documented with an improvement in pod fertility, pods per plant and seeds per pod (Jwar and Neghamish 2021). It's suggested that higher mycelia network and hypha structure of bacteria or AM fungi accelerate root absorption (Qin et al. 2022) explaining the higher pods per plant and other yield related traits.

The cumulative responses of grass pea to limited water conditions microorganisms colonization are reflected in the seed yield. Based on our findings, the seed yield decreased in limited water conditions might be due to the disruption of carbon fixation and remobilization of carbohydrates during flowering, pollination, seed setting and seed filling stages, resulting in pod abortion,

reduced seed number, and less seed weight (Lopes et al. 2023). Under a well watered conditions, improves in the seed yield was cooperated with higher 1000-seed weight, seeds number per pod and pods numbers per plant. By closing initiation of water availability, the stomata closure which followed by proteins and chlorophyll degradation resulted in a decreased photosynthesis capacity (Sabourifard et al. 2023). In line with the present results, Rah Khosravani et al. (2017) reported that limited water conditions decreased the seed yield through the reducing the production and transportation of assimilates.

Our results showed that *R. leguminosarum* increased grass pea seed yield especially through the improving total chlorophyll content, leaf area index and pods per plants. The enhancement in LAI and total chlorophyll content has been associated with the increased carbohydrate production which improve economical yield. AM fungi increased seed yield by diffusion of mycorrhizal mycelium which can link plant root to microorganism's system as well as external hyphae that grow from an infection spot in to the soil (Keshavarz et al. 2018). Our data showed that bacteria inoculation was more efficiency for increasing the seed yield of grass pea compare to AM fungi (Table 3). According to previews studies, different microorganisms had different colonization levels (De Oliveira et al. 2020), indicated that they have various effect on host crops. The superiority of *R. leguminosarum* inoculated grass pea to uptake more nutrients get higher chlorophyll content and LAI (Table 3) most likely clarify our results. Also, higher diffusion of *R. leguminosarum* hyphae rather than *G. intraradices* could explained the observation in enhancement of grass pea seed yield. In addition, due to different penetration abilities to host plants and various spore dormancy schedule, various microorganism species have many potentials to inoculation, subsequently, they differently affect on plants. Overall, the main reason for increasing seed yield may be attributed to increased yield related traits which potentially resulted from strict

and positive influences of AM fungi on solubility and access to nutrients especially P (De Oliveira et al. 2020), longer leaf duration and greater photosynthesis capacity due to a higher photosynthesis apparatus.

The results of our study revealed that inoculation of grass pea with *R. leguminosarum* and *G. intraradices* or the combined of them leads to an improvement in the biological yield of plant due to increase in photosynthetic capacity, yield and yield related traits of grass pea plants. Importantly, AM fungi or rhizobia bacteria inoculation did not affect the value of harvest index (Table 1). It seems that AM fungi and rhizobacteria inoculation induced growth of seed yield as same as biological yield. The results reported by Hamidi (2023), Keshavarz (2020) and Yousefzadeh et al. (2023) showed that inoculation with *Azospirillum lipoferum* or other microorganisms improved plant leaf area and biological yield which indicated that inoculation with the selected biofertilizers could increase economical and biological yield at same time

Pod harvest index (PHI) and pod partitioning index (PPI) are known as the important biomass partitioning indexes that showed the share of dislocation of assimilation from vegetative parts of plant to pod wall and then to seed. PHI shows the content of photosynthates remobilization photosynthates from pod wall to seeds formation. The PPI shows the share of assimilates partitioned to pods related to the total biological yield. The negative impacts of limited water on pods per plant, seeds per pod or seed weight can ultimately lead to relatively lower seed yield, PHI and PPI rates might be due to impaired of assimilates production which caused by the lower rate of photosynthetic efficiency. However, these changes are not necessarily stimulated by the disruption of photosynthesis processes but rather by the change in translocation of photosynthetic assimilates, carbohydrate partitioning, and associated enzymatic activity (Nguyen et al. 2016).

Like other yield related traits, the direct associations have been documented between seed yield and PHI, confirmed that factors contributing to assimilate remobilization were primary determines of seed yield. Moradi-Ghahderijani et al. (2017) emphasized the importance of dislocation of N and carbon for seed filling and assumed that greater rate of PHI and PPI may be attributed to superior remobilization of carbohydrate from leaves and stem to pod wall and ultimately to seeds. It could be concluded that plants gained their higher PPI and PHI due to remobilizing of a higher rate of photosynthetic products from vegetative structures to reproductive parts. This means that selection of PPI and PHI could be useful to recognizing the resistant cultivars.

The PHI showed that the most significant improvement after rhizobium bacteria and AM fungi inoculation rather than non inoculation plants. The enhancement of pods per plant, PHI or PPI mainly could be explained by increasing in number of branches in plant which probably due to better plant development in earlier growth periods that led to higher yield and also due to increasing in phosphorus availability (Keshavarz et al. 2024). The results showed that uninoculated or stressed plants had lower PPI or PHI which could confirmed that weak seed filling and slow development of pods eventually caused a less seed weight and seeds number per pod. This recommends that the plants with better sink strength had better ability to dislocate assimilate to pods and emphasized that PPI and PHI could be an importance of photosynthates partitioning for seed filling. Some pre-podding assimilated was remobilized from the roots, leaves and stems to the seed, but the rate was small and could not reimburse for the lack of present assimilates that resulted from the reduction in photosynthetic efficiency which occur after limited water conditions (Karami et al. 2016). Therefore, process of flowering, podding and seed filling in indeterminate legume plants are strongly dependent on current carbon assimilates from green part of plants including pod walls (Karami et al. 2016). It's suggested that most of the photosynthetic products

and carbohydrate were allocated to the leaves and stems, but after flowering, pods formation and seed setting stage, assimilate was mainly dislocated to the young leaves and reproductive organs because indeterminate legumes (as well as grass pea) have high growth rate even after flowering which made them continued leaf production (Jie et al. 2022). However, these results need more investigation to understand the mechanisms.

Conclusion

The current results reveal that the mixture of *Rhizobium leguminosarum* and *Glomus intraradices* have a positive effect on grass pea and the improved seed yield is showed by enhanced LAI, number of seeds/pod and pod/plant. Although, significant interaction did not exist between biofertilizers inoculation and irrigation regimes but, the biofertilizers application led to a higher seed yield by improving seeds/pod and pod/plant and inhibiting pod senescence, resulted in an improvement in the PHI and PPI. We can conclude that the limited water conditions decreased total chlorophyll content and LAI due to impaired physiological and biochemical processes. Bio organic application promoted chlorophyll and leaf growth. However, this application had no significant effect on HI. Therefore, segregation of yield into its pieces could provide a better understanding on the most associated trait for yield improvement under limited water conditions. The combined application of two kind of micro organisms is a promising approach to improve the quality of important substances while sustaining the long-term use of synthetic fertilizers. The novelty that came out in present experiment consist of higher PPI and PHI achieved in inoculated plants. Although, little information is available on the effects of bio fertilizers on the PPI and PHI of grass pea. To promote production of grass pea, future studies should focus on adjusting the formula of inorganic fertilizers with bio organic fertilizers to improve the quality of important substances while sustaining the long-term use of synthetic fertilizers.

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Author contributions:

Conceptualization and writing—original draft preparation: **H-R.S.**; methodology and formal analysis: **M.O.**; investigation: **M.N.**; data curation: **H-R.L.**; writing—review and editing: **H-R.T-M.** All authors have read and agreed to the published version of the manuscript.

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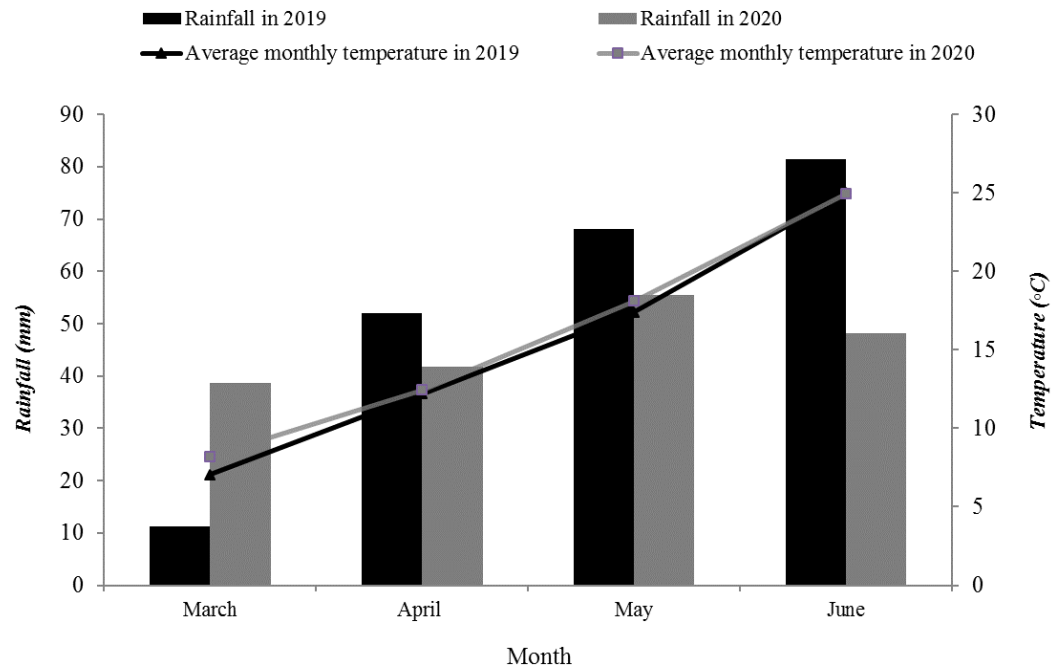


Figure 1. Average temperature and monthly rainfall during the growing seasons in 2019 and 2020

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Table 1- Combined analysis of variance on some agronomic traits, yield and yield components of grass pea (*Lathyrus sativus* L.) as affected by irrigation regimes and biofertilizer treatments

Source of variation	df	TChl	RWC	LAI	Pods/Plant	Seeds/pod	1000-SW	SY	BY	HI	PHI	PPI
Year	1	1.42ns	57.56ns	1.95ns	235.6 ns	0.021ns	38.06ns	1095542 ^{ns}	2077778 ^{ns}	1079ns	1.32ns	1512ns
Year × Replication (Rep)	4	0.34	160.3	0.47	57.5	0.06	106.1	599358	1042733	525.6	3.67	707
Irrigation	2	5.08**	222.6*	6.99**	841.1**	0.088*	150.2*	9425098**	13701831**	229ns	5.48**	243ns
Year × Irrigation	2	0.26*	21.7ns	0.36*	44.6*	0.008ns	14.8ns	587131*	1040623*	0.68ns	0.55ns	0.11ns
Year × Irrigation × Rep	8	0.031	27.39	0.04	5.2	0.01	17.7	78594	159603	71.4	0.58	92
Bacteria	1	4.061**	1020.1**	5.58**	672.7**	0.40**	690.8**	12565142*	19038039**	250ns	25.3**	199ns
Year × Bacteria	1	0.01ns	1.57ns	0.02ns	3.29ns	0.0008ns	1.25ns	68109ns	52054ns	7.7ns	0.06ns	12.8ns
Irrigation × Bacteria	2	0.02ns	13.3ns	0.03ns	4.86ns	0.005ns	10.7ns	35597ns	8259ns	2.56ns	0.57ns	5.5ns
Year × Irrigation × Bacteria	2	0.02ns	9.62ns	0.03ns	3.98ns	0.003ns	5.76ns	115517ns	21922ns	63.3ns	0.14ns	80ns
Year × Irrigation × Bacteria × Rep	12	0.03	18.5	0.05	6.17	0.007	12.1	68881	347076	90.1	0.41	119
Mycorrhiza	1	2.24**	695.2*	3.08**	370.1**	0.26**	450.2**	7334341*	785165ns	1527ns	14.8**	1766*
Year × Mycorrhiza	1	0.002ns	0.49ns	0.003ns	0.42ns	0.0001ns	0.17ns	8515ns	412182ns	17ns	0.000005 ^{ns}	25ns
Irrigation × Mycorrhiza	2	0.019ns	1.06ns	0.025ns	3.13ns	0.0007ns	1.15ns	59103ns	25701ns	4.21ns	0.09ns	5.2ns
Year × Irrigation × Mycorrhiza	2	0.041ns	3.54ns	0.05ns	6.7ns	0.001ns	2.68ns	89190ns	23774ns	14ns	0.12ns	16ns
Bacteria × Mycorrhiza	1	0.38*	0.80ns	0.52*	63.1*	0.0008ns	1.12ns	681642*	1214110ns	360ns	0.12ns	475ns
Year × Bacteria × Mycorrhiza	1	0.022ns	0.22ns	0.03ns	3.72ns	0.000005 ^{ns}	0.08ns	17920ns	3006ns	8.24ns	0.00008ns	10.7ns
Irrigation × Mycorrhiza × Bacteria	2	0.12ns	24.7ns	0.16ns	19.9ns	0.009ns	17.01ns	38796ns	328162ns	4.19ns	0.66ns	3.82ns
Year×Irrigation×Mycorrhiza× Bacteria	2	0.049ns	0.02ns	0.06ns	8.22ns	0.000001 ^{ns}	0.006ns	42784ns	61570ns	1.04ns	0.0004ns	1.71ns
Error	24	0.05	35.2	0.07	9.4	0.013	22.88	125742	607713	123.8	0.76	165.5
CV (%)		8.19	7.65	8.19	8.18	3.77	3.80	11.1	13.82	19.5	1.008	19.6

TChl: total chlorophyll content; RWC: relative water content; LAI: leaf area index; 1000-SW: 1000 seed weight; SY: seed yield; BY: biological yield; HI: harvest index; PHI: pod harvest index; PPI: pod partitioning index. ns, ** and * indicate no significance and significance at *P* levels of 0.01 and 0.05, respectively.

Table 2- Two way interaction of year and irrigation regimes on studied traits of grass pea (*Lathyrus sativus* L.).

Year	Irrigation regimes	Total chlorophyll ($\mu\text{g g}^{-1}$ FW)	Leaf area index	Pods/plant	Seed yield (kg ha^{-1} FW)	Biological yield (kg ha^{-1} FW)
2019	Well-watered	3.62 a	4.24 a	46.53 a	4103.8 a	6432.4 a
	Mild limited water	3.05 b	3.57 b	39.25 b	3283.7 bc	5468.6 bc
	Severe limited water	2.48 d	2.91 d	31.96 d	2538.3 d	4504.8 d
2020	Well-watered	3.12 b	3.66 b	40.18 b	3536.8 b	6355.7 a
	Mild limited water	2.77 c	3.24 c	35.63 c	3052.5 c	5808.3 b
	Severe limited water	2.41 d	2.83 d	31.07 d	2596.4 d	5261.0 c

Means within each column followed by the same letter are not significantly different by LSD test at $P \leq 0.05$.

Table 3- Two way interaction of rhizobium bacteria and arbuscular mycorrhizal fungi symbiosis on studied traits of grass pea (*Lathyrus sativus* L.).

Rhizobium bacteria	Mycorrhizal fungi	Total chlorophyll ($\mu\text{g g}^{-1}$ FW)	Leaf area index	Pods/plant	Seed yield (kg ha^{-1} FW)
With bacteria	With fungi	3.40 a	3.98 a	43.70 a	6127.2 a
	Without fungi	2.90 b	3.39 b	37.29 b	6178.1 a
With out bacteria	With fungi	2.77 b	3.25 b	35.71 b	5358.5 b
	Without fungi	2.57 c	3.01 c	33.05 c	4889.9 b

Means within each column followed by the same letter are not significantly different by LSD test at $P \leq 0.05$.

Table 4- Mean comparison of main effect of irrigation regimes on some studied traits of grass pea (*Lathyrus sativus* L.).

Irrigation regimes	RWC (%)	Seeds/pod	1000-seed weight (g)	PHI (%)
Well-watered	80.6 a	3.09 a	128.2 a	86.9 a
Mild limited water	77.5 ab	3.03 ab	125.7 ab	86.5 ab
Severe limited water	74.5 b	2.97 b	123.2 b	86.04 b

RWC: relative water content; PHI: pod harvest index; Mean comparison of main effect of arbuscular mycorrhizal fungi on some studied traits of grass pea (*Lathyrus sativus* L.).

Table 5- Mean comparison of main effect of rhizobium bacteria symbiosis on some studied traits of grass pea (*Lathyrus sativus* L.).

Rhizobium bacteria	RWC (%)	Seeds/pod	1000-seed weight (g)	Biological yield (kg ha ⁻¹)	PHI (%)
With bacteria	81.35 a	3.11 a	128.8 a	6152.7 a	87.12 a
With out bacteria	73.82 b	2.96 b	122.6 b	5124.2 b	85.93 b

RWC: relative water content; PHI: pod harvest index; Means followed by the same letter are not significantly different by LSD test at $P \leq 0.05$.

Table 6- Mean comparison of main effect of arbuscular mycorrhizal fungi symbiosis on some studied traits of grass pea (*Lathyrus sativus* L.).

Mycorrhizal fungi	RWC (%)	Seeds/pod	1000-seed weight (g)	PHI (%)	PPI (%)
With fungi	80.69 a	3.09 a	128.2 a	86.98 a	70.73 a
Without fungi	74.47 b	2.97 b	123.2 b	86.07 b	60.83 b

RWC: relative water content; PHI: pod harvest index; PPI: pod partitioning index; Means followed by the same letter are not significantly different by LSD test at $P \leq 0.05$.