

Plant growth-promoting bacteria inoculation mitigates drought stress effects in *Trifolium pratense* L. seedlings

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Research Article

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Abstract

Multiple ecological factors have an effect on the capacity of plants to survive, grow, and produce. In the meantime, a significant limitation on plant growth in dry regions is drought. Accordingly, the study's objective was to determine the impacts of plant growth-promoting rhizobacteria (PGPR) on indices of germination, growth, and nutrient absorption in the red clover (*Trifolium pratense* L.) under drought regimes. Treatments included different levels of drought: 100% (FC = field capacity), 70% (0.7FC), and 40% (0.4FC) as the first factor and inoculation conditions of PGPRs, including non-inoculation (control), *Azotobacter vinelandii* (AV), *Pantoea agglomerans* and *Pseudomonas putida* (PA + PP), and combined application of *Azotobacter*+ *Pseudomonas* (AV + PA + PP), as the second factor. Results revealed that the maximum and minimum germination percentages were obtained for interactions of AV + 0.7FC and control + 0.7FC, respectively. The combined treatment of AV + PA + PP had less impact on reducing drought stress on the germination of seeds than the single application of bacteria. Maximum values for length of roots, shoots, and plant's dry weight were obtained for AV + 0.4FC interaction, while the minimum values were recorded for PA + PP + 0.4FC interaction. Additionally, results showed that the treatment of PA + PP had no significant influence on drought stress reduction ($p > 0.05$). The highest uptake of K was observed for the treatment of AV + FC, and the highest amounts of Fe and Zn were measured for the interaction of PA + PP + 0.7FC. The lowest uptake was recorded for Fe, Zn, and Mn under the combined treatment of AV + PA + PP + 0.4FC. In general, the use of *Azotobacter* compared to *Pseudomonas* had a better effect on reducing drought impacts on plant characteristics and increased plant tolerance to drought stress.

Introduction

Biologists believe that plant stress refers to a condition in which increasing demand in plants leads to instability in their activities; however, plant activities return to a normal situation following the elimination of stressful conditions (Heidarpour Monfared et al. 2021). Abiotic stressors' impacts on plant development and biological mechanisms such as photosynthesis, respiration, and mineral absorption are often evaluated in terms of total plant growth (He et al. 2017; Laxa et al. 2019).

In general, drought in plants refers to the exposure of plant cells and tissues to unfavorable moisture conditions, which results in plant wilting and decreasing turgidity in cells and tissues. Furthermore, changes related to the enzyme activities and the decrease of cell division, chlorophyll contents, photosynthesis, nutrient uptake by roots, and transport them upward to aerial parts are other adverse impacts of drought on plants (Brodersen et al. 2019; Seleiman et al. 2021; El-Sawah et al. 2021). According to recent studies, enough water supply is required for plant cultivation to attain high yield. Plants, on the other hand, can survive water stress only up to a certain point (threshold level), after which there is a significant decrease in production (Sheteiwy et al. 2020; Sheteiwy et al. 2021a).

For more than half of the dry regions, drought stress is expected to cause major issues by 2050 on plants, according to the studies (Vinocur and Altman 2005). Hence, plants must have different mechanisms to

manage stress and maintain metabolic processes to survive stressful and dry conditions (Abid et al. 2018). As Osmolovskaya et al (2018) have said, the primary strategies of plant defense against drought are drought escape, prevention, and resistance. Accordingly, understanding techniques related to drought resistance in plants should be one of the most important research objects of researchers. In this regard, since plant water relationships affect all physiological processes of plants (Delshadi et al. 2017a), providing sufficient water for plant growth and development before causing adverse impacts of drought stress on plant physiological processes is of prime importance for plants (Diatta et al. 2020). Drought resistance in plants is also influenced by osmoregulation, a system that affects growth of plant by collecting osmolites and supporting cellular activities (Devincentis 2020). According to other researches, a variety of substances, including phytohormones, antioxidants, proline, polyamines, and soluble sugars, have been demonstrated to be critical in controlling plant osmoregulation under drought conditions (Du et al. 2004; Jogawat et al. 2021).

As a living and dynamic ecosystem, soil health may be maintained most naturally and effectively by using favorable soil microorganisms called biological fertilizers or bio-fertilizers (Bunemann et al. 2018). The most significant advantages of using bio-fertilizers are their participation in the supplying of plant-specific macro- and micronutrients, as well as their increase in biodiversity, promotion of key plant activities, and enhancement of plant yield under difficult conditions (Saharan and Nehra 2011; Khan et al. 2020). Plant productivity is reliant on microorganisms' ability to synthesize and release bioactive chemicals that are necessary for soil fertility and plant production. As a result, inoculating plants with a variety of soil organisms, such as bacteria and fungi, has been recognized as an effective strategy for combating abiotic stresses, notably drought stress, in recent decades (Niu et al. 2018; Arun et al. 2020; Efthimiadou et al. 2020). These microorganisms have an effect on plant function directly or indirectly through one or more specific strategies (Ahemad and Kibret 2014). Due to their stimulant effects on plant, they have been given the name plant growth promoting rhizobacteria (PGPR) (Cakmakci et al. 2007; Martínez-Viveros et al. 2010). The application of PGPR has the ability to provide eco - friendly and sustainable solutions to agricultural production and environmental adaptation challenges. PGPR can improve plant nutrient uptake as well as soil-borne illnesses are suppressed, and biological control agents are used (Bashan and De-Bashan 2010; Sheteiwy et al. 2021a).

The methods involved in enhancing plant yield under the application of PGPRs are not fully understood; however, their effects have been attributed to producing phytohormones (especially cytokinin, gibberellin, and auxin), participating in biological nitrogen fixation, increasing phosphorus solubility, enhancing organic phosphate mineralization, inducing germination-related traits, accelerating root elongation, increasing root weight, and improving plant establishment (Fahad et al. 2015). In this field, different genera of *Azotobacter* and *Pseudomonas* were reported as the most influential PGPRs involved in increasing the bioavailability of minerals and affecting plant production via producing plant growth regulators (Tiwari et al. 2016).

Among PGPRs, *Azotobacter vinelandii* is one of the few PGPRs that can fix atmospheric nitrogen (N). Commonly, this form of N is taken by plants in the rhizosphere as ammonium and utilized as a necessary

mineral nutrient (Bianco and Defez 2011). This bacterium can dissolve phosphate at 15 to 40°C and replicate and develop in NaCl concentrations over 7% and different pH ranges of 5–9. Also, the above bacterium has been introduced as one of the bio-fertilizers enhancing plant growth in conditions of phosphorus and nitrogen deficiency (Nosrati et al. 2014). Evidence has shown that bacteria with the potential of a mutualistic relationship with the host plant, e.g., *Azotobacter*, are not only able to convert atmospheric nitrogen to absorbable forms through plant root system but also reduce the negative impacts of osmotic stresses caused by chemical fertilizers in arid soils (Nosrati et al. 2014). In addition, bio-fertilizers containing *Pantoea agglomerans* strain P5 and *Pseudomonas putida* strain P13 are known as the most effective phosphate solubilizing bacteria (Chen et al. 2015), which are resistant to high temperatures (42°C) and high salt concentrations (5%), tolerant to a wide pH range (5–11), and suitable for Iran's climate conditions (Amiri Farsani et al. 2013). Additionally, it was observed that the enzyme phosphatase produced by *P. putida* strain P13 and acidic extracts released by *P. agglomerans* strain P5 were capable of hydrolyzing mineral and organic phosphate in the soil and converting it to accessible and absorbable forms for plant use. (Goljanian-Tabrizi et al. 2016).

Given that drought is one of the most significant environmental variables limiting global productivity of plants, it is critical to investigate the impact of PGPRs on plant resistance to abiotic stresses, such as drought stress, in order to optimize water usage efficiency. Thus, the purpose of this study was to determine the influence of PGPRs on certain physiological parameters and nutrient uptake in red clover under drought stress conditions. It should be emphasized that this study made the assumption that *A. vinelandii* and *P. agglomerans* + *P. putida* reduce the negative effects of drought stress on red clover germination, growth, and nutrient uptake.

Material and methods

Plant characteristics

The red clover (*Trifolium pratense* L.) is a perennial herbaceous flowering plant of the legume family that is native to Europe, Northwest Africa and West Asia. It grows in temperate regions throughout the world (Rosso and Pagano 2005; Morales et al. 2013; Latifian and Arslanoglu 2018; Colgecen et al. 2020). However, it is currently cultivated in most of the world's arable lands. Some studies also introduced the western, northwestern, northeastern, and Iran's central regions as the distribution sites of different ecotypes of the red clover (Azhir and Fayaz 2019). This plant is cultivated as a seven-year-old plant in favorable conditions and a biennial legume in arid and semi-arid climates. Red clover has a limited upright growth (with a maximum height of 15 cm) and oval, elongated, and trefoil leaves (15–30 mm in length and 8–15 mm in width). The flowers are dark pink and 12 to 15 mm long, which appear densely in the inflorescences. In addition, this plant has long been used as a forage plant in animal feeding due to its richness in nutrients (Van Der Kooi et al. 2015).

Preparing culture substrate

The focus of this research was to find out how PGPRs affect the seed germination, some growth traits and nutrient uptake of red clover under drought conditions. To do this, we used a factorial experiment with three replications in a completely random design. Bio-fertilizer application was the first factor (at four levels): the control (without bio-fertilizer), *A. vinelandii* = AV, *P. agglomerans* + *P. putida* = PA + PP, and the combination of AV + PA + PP. Drought (at three levels) was the second factor: 100% (FC = field capacity), 70% (0.7FC), and 40% (0.4FC).

It should be emphasized that physical and chemical parameters of the soil were determined using a combined sample. In this study, all soil analyses began with passing the samples through a 2 mm sieve for laboratory experiments and a 4 mm sieve for potting experiments before starting the research. Soil texture was determined based on the hydrometric method proposed by Day (1982). Also, the pH value of saturated mud was estimated based on the method presented by Thomas (1996), and the electrical conductivity (EC) value, was measured by EC meter, Model Jenway 4520, England (Rhoades 1996). In addition, values of total nitrogen (N), available phosphorus (P), and available potassium (K) in the soil samples were determined based on methods presented by Hesse (1971) using Kjeldahl method, (model Kjeltac Analyzer Unit 2300), Bray and Kurtz (1954); based on the Olsen method using a spectrophotometer, model GES30, Thermo Scientific, USA), and Simard (1993) flame photometry, model XP, BWB Tech, England), respectively. Additionally, soil organic matter was determined by the method of Walkley-Black (Allison 1965), and the field capacity determination was done using weighting method (Zarik et al. 2016).

This study was conducted in the greenhouse of Zabol University in 2021. Experimental conditions included temperatures of 10°C (min) and 32°C (max) and low and high irradiances (60 and 250 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, respectively). Long and short day lengths (16:8 and 8:16 LD cycles) were also applied at the two irradiances. The pots used in the greenhouse research had a capacity of 2 kg and were irrigated with distilled water to reach saturation conditions after filling pots with sieved soil. Also, a thin cloth was placed into the bottom of each pot to prevent bio-fertilizer leaching and further penetration of plant roots. Then, the seeds were impregnated with solutions containing bio-fertilizers, and each pot was planted with 15 seeds at a depth of 1 cm. Also, the water requirement for the cultivated seeds was provided daily and continued until the complete germination of the studied seeds.

The *Azotobacter* was inoculated in powder form contained 1×10^8 live and active pure bacteria per gram. *Pseudomonas* was inoculated in powder form with an approximate population of 1×10^8 live and active bacteria per gram. Bio-fertilizers were obtained from Sabz Biotechnology Company (Iran). For each bacterial strain, a 250 ml Erlenmeyer flask containing 100 ml nutrient broth was prepared. To prepare the inoculum, a pure colony of each bacterium was removed and added to an Erlenmeyer flask containing the culture medium under sterile conditions. Erlen inoculated with each bacterium was placed on a shaker at a speed of 120 rpm and a temperature of 28°C. After 48 hours of inoculation, the strains with an approximate population of 1×10^8 (based on the McFarland standard) were ready for use (Delshadi et al. 2017a).

Soil water content was kept at 100% (field capacity = FC), 70% (0.7FC), 40% (0.4FC). The FC of the soil was first determined using weighting method before applying drought stress (Diallo and Marico 2013). Then, the required water amount to apply drought stresses was determined based on the calculated FC (Zarik et al. 2016). To that end, first, 2 kg of dry soil of each pot were saturated after weighing (W₁) so that the additional water would be extracted by gravity. The pots were covered with a thin plastic layer to prevent evaporation. Then, there were left in the greenhouse under optimum conditions for 48 h, so that the additional water would be extracted (Azizi et al. 2020). Then, the saturated soil of each pot was weighed (W₂). The weight difference between W₁ and W₂ is the amount for saturation, which, here, was considered as the FC value (100%). The water required for each irrigation time per pot was calculated per stress level using the following equations (Azizi et al. 2020).

$$0.7FC = 0.7 \times (W_2 - W_1) \text{ (Eq. 1)}$$

$$0.4FC = 0.4 \times (W_2 - W_1) \text{ (Eq. 2)}$$

Seed germination

Maguire's formula (1962) was used to calculate germination percentage (GP) and rate (GR). In other words, the number of seeds that germinated in each pot was used to compute the total number of seeds that germinated, and the GR was estimated by Eq. 3:

$$GR = \sum NiDi \text{ (Eq. 3)}$$

In which, GR, Ni, and Di indicate the speed of germination, the number of germinated seeds, and the number of days following planting, respectively. It should be noted that the thinning operation was done after 14 days so that eight plants remained in each pot, and drought stress levels were applied based on experimental treatments, namely FC, 0.7FC, and 0.4FC. Plant were harvested 90 days after planting. After separating the roots from the plant, they were cleaned with distilled water. Indices of stem and root length (by the ruler), total dry weight, and several macro elements were measured in this study. A high-precision digital scale (accuracy of 0.001 g). was used to weigh the samples after they had been dried in an oven at 72°C for 48 hours.

The wet digestion method was used to dissolve the extract samples before determining the content of adsorbed elements (Rayan et al. 2001). Then, the amount of Nitrogen (N) absorption by titration method and Kjeldahl device (Model Kjeltex Analyzer Unit 2300), Phosphorus (P) concentration by vanadate-molybdate method (yellow method) and spectrophotometer (Model GES30, Thermo Scientific, USA), and potassium (K) concentration by flame diffusion method and flame photometer (Model XP, BWB Tech, England) were determined (Rayan et al., 2001). In addition, concentrations of zinc (Zn), iron (Fe), and manganese (Mn) micronutrients were measured by ICP/OES (Model AA240, Varian, USA).

Data analysis

Multivariate analysis of variance was used to analyse all of the data using SPSS 20.0. The Kolmogorov–Smirnov test was applied to ensure that the distribution was normal. The Levene's test for variance homogeneity was used to determine whether the treatments were equal in variance. The Post hoc Duncan test (at level of less than 0.05) was used to examine the significant difference between the means.

Results

Germination indices

Characteristics of the soil are listed in Table 1. Results of analysis showed that bio-fertilizers and the interaction of bio-fertilizers × different regimes of drought had significant effects on seeds germination ($p < 0.01$). The main effects of different regimes of drought determined that the lowest and highest GR (germination rate) (14.21 and 21.11 seeds per day) were recorded for 0.4FC and FC treatments, respectively. In addition, similar outcomes were observed for the GP index (germination percentage).

In contrast, the main effects of bio-fertilizers demonstrated that the highest and lowest germination indices were achieved in treatments of AV and AV + PA + PP, respectively (Table 2). According to mean comparison of bio-fertilizers application (Table 2), the highest GR (germination rate) and GP (germination percentage) (25.77 seeds per day and 90.24%) was obtained for AV treatment, while the lowest values (14.71 seeds per day and 62.14%) were observed for the control treatment. Also, results indicated no significant differences between values of germination indices in AV and PA + PP treatments ($p > 0.01$). In addition, it should be noted that the combined application of AV and PA + PP treatments (AV + PA + PP) led to a significant reduction ($p < 0.01$) in GP (germination percentage) in comparison with the single application of bio-fertilizers.

Results of drought stress and bio-fertilizers interaction (Table 3) showed that the maximum seed germination were measured for AV + 0.7FC, and their minimum values were measured for control + 0.7FC. According to the above results, it can be deduced that the application of bio-fertilizers significantly ($p < 0.01$) reduced the negative impacts of drought on germination indices of red clover; however, the efficiency of AV treatment was higher than PA + PP and AV + PA + PP treatments.

Moreover, although combined treatment of AV + PA + PP was less effective than single application of bio-fertilizers in reducing drought stress affecting germination indices, plant germination significantly improved under the interaction of AV + PA + PP in drought stress of 0.4FC compared to the interaction of control + 0.4FC ($p > 0.01$). Also, similar results were observed for AV + PA + PP + 0.7FC interaction. In other words, there was a significant increase for both germination indices under AV + PA + PP + 0.7FC interaction compared to control + 0.7FC interaction ($p < 0.01$).

Table 1
Physical and chemical characteristics of soil used in the experiments

Soil texture	EC (dS/m)	pH	N _{tot} %	P (mg kg ⁻¹)	K (mg kg ⁻¹)	OM %
sandy clay loam	0.10	4.5	0.23	19.50	630	1.90

Table 2
Impacts of bio-fertilizers and drought on seed germination

Treatment	GR (number/day)	GP (%)
FC	21.11 ± 0.4a	89.56 ± 2.45a
0.7FC	17.09 ± 0.44b	70.31 ± 2.45b
0.4FC	14.21 ± 0.44c	60.00 ± 2.45c
AV	25.77 ± 0.40a	90.24 ± 2.82a
PA + PP	24.09 ± 0.40a	82.00 ± 2.82a
AV + PA + PP	18.29 ± 0.40b	71.13 ± 2.82b
Control	14.71 ± 0.40c	62.14 ± 2.82c

Treatments having different letters indicate a significant difference ($p < 0.01$). (mean ± standard error)

AV = *A. vinelandii*, PA + PP = *P. agglomerans* + *P. putida*, AV + PA + PP = *A. vinelandii* + *P. agglomerans* + *P. putida*.

GR = Germination Rate, GP = Germination Percentage

Table 3
Interaction effect of bio-fertilizers × drought stress on seed germination

Treatment	GR (number/day)	GP (%)
AV + FC	26.23 ± 0.88b	92.00 ± 4.90b
AV + 0.7FC	28.93 ± 0.89a	93.00 ± 4.91a
AV + 0.4FC	21.11 ± 0.87c	84.00 ± 4.85c
PA + PP + FC	20.76 ± 0.86d	80.00 ± 4.80d
PA + PP + 0.7FC	19.20 ± 0.85d	74.00 ± 4.72d
PA + PP + 0.4FC	17.07 ± 0.83e	65.00 ± 4.60e
AV + PA + PP + FC	20.66 ± 0.86d	79.04 ± 4.73d
AV + PA + PP + 0.7FC	19.66 ± 0.85d	73.04 ± 4.66d
AV + PA + PP + 0.4C	17.60 ± 0.84e	67.00 ± 4.61e
Control + FC	17.00 ± 0.83e	65.00 ± 4.61e
Control + 0.7FC	14.17 ± 0.71f	60.00 ± 4.85f
Control + 0.4FC	13.06 ± 0.64f	62.00 ± 4.87f

Treatments having different letters indicate a significant difference ($p < 0.01$). (mean ± standard error)

AV = *A. vinelandii*, PA + PP = *P. agglomerans* + *P. putida*, AV + PA + PP = *A. vinelandii* + *P. agglomerans* + *P. putida*.

GR = Germination Rate, GP = Germination Percentage

Plant growth

Results of analysis showed that main effects of factors, and their interaction effects had significant effects on plant growth traits ($p < 0.01$). The main effect of the drought factor (Table 4) revealed that the treatment with 0.4FC resulted in the shortest shoot and root lengths while the highest values of the shoot and root lengths were obtained in FC, indicating negative impacts of drought stress on both traits in red clover. Results related to main effects of drought and bio-fertilizers on the dry weight of plant had a completely similar trend, and increasing drought stress led to a significant decrease in root and shoot dry weight traits ($p < 0.01$). The main effects of bio-fertilizers (Table 4) revealed that the maximum and minimum values for root length and dry weight were recorded for AV and control treatments, respectively. In addition, there were similar outcomes for traits of shoot length and dry weight.

Table 4
Impacts of bio-fertilizers and drought stress on morphological traits

Treatment	Shoot length (cm)	Root length (cm)	Aboveground dry weight (g)	Belowground dry weight (g)
FC	16.33 ± 0.80a	11.14 ± 0.25a	0.73 ± 0.02a	1.63 ± 0.02a
0.7FC	14.80 ± 0.75b	9.20 ± 0.23b	0.53 ± 0.01b	1.43 ± 0.01b
0.4FC	12.40 ± 0.73c	7.11 ± 0.20c	0.32 ± 0.01c	1.22 ± 0.01c
AV	18.29 ± 0.83a	14.04 ± 0.30a	0.93 ± 0.03a	1.83 ± 0.03a
PA + PP	15.77 ± 0.86b	12.01 ± 0.29b	0.64 ± 0.01c	1.54 ± 0.01c
AV + PA + PP	15.94 ± 0.87b	12.78 ± 0.28b	0.66 ± 0.01c	1.56 ± 0.01c
Control	14.04 ± 0.73b	11.47 ± 0.27b	0.50 ± 0.01c	1.40 ± 0.01c

Treatments having different letters indicate a significant difference ($p < 0.01$). (mean ± standard error)

AV = *A. vinelandii*, PA + PP = *P. agglomerans* + *P. putida*, AV + PA + PP = *A. vinelandii* + *P. agglomerans* + *P. putida*.

Table 5
Interaction effect of bio-fertilizers × drought stress on morphological traits

Treatment	Shoot length (cm)	Root length (cm)	Aboveground dry weight (g)	Belowground dry weight (g)
AV + FC	18.66 ± 0.78a	10.73 ± 0.45a	0.96 ± 0.02a	1.86 ± 0.05a
AV + 0.7FC	18.81 ± 0.79a	10.63 ± 0.45a	0.98 ± 0.02a	1.88 ± 0.05a
AV + 0.4FC	18.90 ± 0.80a	10.80 ± 0.46a	0.99 ± 0.02a	1.89 ± 0.05a
PA + PP + FC	16.11 ± 0.64c	9.90 ± 0.42c	0.71 ± 0.01c	1.61 ± 0.04c
PA + PP + 0.7FC	14.51 ± 0.61d	9.77 ± 0.41d	0.51 ± 0.01d	1.41 ± 0.03d
PA + PP + 0.4FC	13.25 ± 0.60e	9.00 ± 0.40e	0.44 ± 0.01e	1.34 ± 0.02e
AV + PA + PP + FC	13.12 ± 0.60e	9.18 ± 0.41e	0.41 ± 0.01e	1.31 ± 0.02e
AV + PA + PP + 0.7FC	11.44 ± 0.59f	8.16 ± 0.39f	0.21 ± 0.01f	1.11 ± 0.02f
AV + PA + PP + 0.4C	10.43 ± 0.56f	8.99 ± 0.39f	0.10 ± 0.01f	1.00 ± 0.02f
Control + FC	14.37 ± 0.60b	10.12 ± 0.41b	0.52 ± 0.01f	1.42 ± 0.03f
Control + 0.7FC	12.43 ± 0.58f	10.00 ± 0.40f	0.30 ± 0.01f	1.20 ± 0.01f
Control + 0.4FC	11.31 ± 0.56f	8.42 ± 0.39f	0.21 ± 0.01f	1.10 ± 0.01f

Treatments having different letters indicate a significant difference ($p < 0.01$). (mean ± standard error)

AV = *A. vinelandii*, PA + PP = *P. agglomerans* + *P. putida*, AV + PA + PP = *A. vinelandii* + *P. agglomerans* + *P. putida*.

The effects of bio-fertilizers × drought (Table 5) indicated that the single application of bio-fertilizers was more effective than their combined application. The highest values for shoot and root lengths, as well as plant dry weight, were attained when AV + 0.4FC treatment was used. However, there was no significant difference between values of shoot and root lengths, as well as plant dry weight under the interaction of AV + 0.4FC, AV + FC, and AV + 0.7FC ($p > 0.01$). Accordingly, it can be indicated that *Azotobacter* is more effective in modifying the negative effects of drought at a level of 04FC and increasing plant tolerance to

existing situations. On the other hand, since the lowest root length, shoot length, and dry weight were obtained under PA + PP + 0.4FC interaction, it showed that the application of PA + PP treatment was not effective in reducing the impacts of drought stress. Combined treatment of bio-fertilizers (AV + PA + PP) also showed that the maximum values for studied traits were measured under the treatment of AV + PA + PP + FC, and there was an inverse relationship between improving the growth traits and drought stress levels at $p < 0.01$. In other words, the lowest values for root and shoot length, and dry weight were obtained under drought stress of 0.4FC (Table 5).

Nutrients uptake

Drought stress, bio-fertilizers, and their interactions exhibited a significant impact on P, Zn, and Fe absorption ($p < 0.01$). The uptake of N, Mn, and Fe was unaffected by the bio-fertilizers. Drought stress had no influence on K, Fe, and Mn uptake in the presence of bio-fertilizer. The absorption of K and Mn was not significantly affected by drought stress ($p > 0.01$).

Drought stress had the main effect of increasing the content of N at the FC, while decreasing it at the 0.7FC (Fig. 1a). The 0.4 FC and FC had the most and least concentration of P, respectively (Fig. 1b). Results showed that the 0.7FC and 0.4FC were associated with the largest and the least levels of K (Fig. 1c), Fe (Fig. 2a), and Zn (Fig. 2b), respectively. In FC and 0.7FC, the maximum amount of Mn was measured and 0.4FC caused to absorb this element (Fig. 2c).

The main impacts bio-fertilizers showed that the maximum concentration of K (Fig. 1f) and Fe (Fig. 2d) was related to the treatment AV. While treatment AV caused to reduce the uptake of P (Fig. 1e) and Zn (Fig. 2e), most concentrations of N (Fig. 1d), P (Fig. 1e) and Zn (Fig. 2e) were measured in the treatment PA + PP. Treatment AV + PA + PP not only didn't cause to uptake none of these elements, but also the least concentrations of Fe (Fig. 2d) and Mn (Fig. 2f) were observed in this treatment. Means comparison showed that the control treatment had the most Mn (Fig. 2f); meanwhile the least concentration of K (Fig. 1f) and N were measured in the control treatment (Fig. 1d).

The interaction effects of factors showed that treatment AV + FC and control + 0.7FC caused to increase and decrease of N concentration, respectively (Fig. 1g). The most and the least concentrations of P were measured in the control + 0.4 FC and treatment of AV + FC, respectively (Fig. 1h). Treatment AV caused to increase concentration of K at FC, while in the control treatment at 0.4FC drought stress, the least amount of K was measured (Fig. 1i). AV + FC and PA + PP + 0.7FC treatments showed the maximum concentrations of Fe (Fig. 2g) and Zn, respectively (Fig. 2h). The least concentration of Fe (Fig. 2g) and Zn related to the treatment AV + PA + PP + 0.4FC (Fig. 2h). It was found that the treatment PA + PP + FC had the highest and lowest concentrations of Mn, respectively (Fig. 2i).

Discussion

Stress caused by droughts is a major element in the decline of plants growth, development, and productivity through affecting physiological, physicochemical, and morphological characteristics of

plants and has been caused significant problems in meeting population food demand in the world (Kapoor et al. 2020; Khodabin et al. 2020; Chandra et al. 2021).

Our findings indicated that increasing the amount of drought stress resulted in a considerable decrease in germination indices and growth of red clover. Drought stress can lead to changes in seed structural composition and consequent reduction of seed germination by reducing plant cell moisture and inducing germination phytohormones, e.g., hormones involved in hydrolyzing seed storage materials such as amylase (Abbassi and Koocheki 2008). Also, since increasing drought stress levels from 0.7FC to 0.4FC is associated with impaired water uptake by the seed, reducing seed germination and root emergence indices is not unexpected. Accordingly, Mena et al. (2015) reported that reduced water potential is associated with poor germination, seedling establishment, and plant yield. In their investigation of the associations of spectral features with yield and nutritional quality of *Phaseolus vulgaris* L. in drought years, Nemeskéri et al (2017) found that under non-irrigated situations, the plant height reduced and the leaf area index was low. In addition, it has been reported that limitation of water uptake by seeds is closely related to the reduced seed storage transport, and reduced organic matter and protein production and distribution in the seed embryo may have an effect on seed germination (Alsaedi et al. 2017; Radnejad et al. 2017; Sabeti et al. 2019).

Overcoming negative effects of drought and improving plants growth require comprehensive strategies. Drought stress increased bacterial counts, according to Sheteiwy et al. (2021a), and this could be due to soil drying and rewetting, which could cause a large shift in the makeup of organic matter and its components, making it more sensitive to microbial activity. Furthermore, during drought stress, microorganisms do not always perish, but may enter latent phases to be swiftly degradable organic compounds by bacteria following rewetting. According to Chandra et al (2021), using PGPR may be a safe way to increase crop yields under drought circumstances through a variety of processes, including the production of phytohormones, free radical-scavenging enzymes, and stress-combating enzymes. In the study, the use of *Azotobacter* moderated the unfavorable effects of drought stress on red clover. One of the main reasons for increasing germination in the presence of bacteria may be due to enhancing the synthesis of some phytohormones, especially gibberellin, which affect germination by activating some enzymes involved in starch metabolism, e.g., amylase (Kaymak et al. 2009; Batool et al. 2020). Seed inoculation with PGPRs could lead to the synthesis of indole acetic acid (IAA) as an affecting factor in stimulating plant cells and cell elongation and increasing germination of seed in response to amino acids secreted in the seed (Barnawal et al. 2017; Lu et al. 2018). Seed priming with bio-fertilizers also reduced seed exposure to drought stress, induced radicle emergence, and decreased adverse effects of stressful factors (Sabeti et al. 2019). Omara and Elbagory (2018) also reported that applying PGPRs under drought stress conditions increased the seeds germination in wheat (*Triticum aestivum* L.). The latter researchers confirmed that seed inoculation with PGPRs accelerated the release of enzymes involved in the seed germination and increased seed germination compared to non-inoculation conditions.

According to the data, combined treatment of bio-fertilizers had fewer effects on reducing drought stress on seed germination than the single application of bio-fertilizers. Sabeti et al (2019) stated that although

inoculation of seeds with only a specific strain of bio-fertilizers could increase plant yield by absorbing nutrients and promoting plant yield due to the secretion of secondary metabolites, sometimes their combined use has antagonistic effects on each other and leads to reduced plant growth and yield. Our findings also showed that increasing drought stress led to a decrease in morphological traits of the red clover. In agreement with the negative impacts of drought stress on plant, some researchers stated that reducing water potential caused by drought stress could change plant morphology and physiology via disturbing ionic balance, intensifying toxic ions, disrupting physiological and metabolic processes, including absorption, transport, reduction, and metabolism of nitrogen and protein, closing stomata, degrading cell membrane, decreasing cell division, reducing photosynthetic efficiency, and increasing metabolite toxicity (Zawoznik et al. 2011; Rojas-Tapias et al. 2012). Drought stress reduces plant growth phytohormones, e.g., gibberellic (GA) and auxin, and increases growth inhibitors, especially abscisic acid (ABA), by affecting plant hormonal balance (Andersen et al. 2015). ABA is crucial in limiting plants growth, especially shoot and root height. Since turgor pressure is one of the primary factors involved in cell division, especially meristematic cells, increasing drought leads to decreasing turgor pressure in the cell wall and plant growth (Awari and Mate 2015; Li et al. 2018).

Reducing indices of fresh and dry weight in plants under drought conditions has been widely confirmed in other studies, which was attributed to osmotic pressure in the root region and reduced some characteristics, such as water uptake, growth, and finally plant weight (Emadi et al. 2009). Drought stress causes rapid the senescence of root nodules and increases the formation of reactive oxygen species (ROS). As a result, nodule, root, and shoot weights are reduced. As the root-to-shoot ratio rises, water stress causes a shift in the proportion of root weight (Nemeskéri and Helyes 2019). Under water-stressed conditions, the small root volume aids to prevent yield loss (Nemeskéri 2001).

Drought stress, apart from decreasing capacity for carbon storage and limiting leaf gas exchange, also affects the transport of photosynthetic materials that can lead to saturating leaves with these compounds, limiting the photosynthesis process, and reducing the plant biomass. Therefore, the reduction of plant photosynthesis under adverse environmental conditions, especially drought stress, can be attributed to changes in chlorophyll contents, damage to photosynthetic apparatus, or both (Nadeem et al. 2014).

Plants have a decreased stomatal conductance in order to preserve water. As a result, CO₂ fixation and photosynthetic rate decline (Mafakheri et al. 2010; Delshadi et al. 2017b), resulting in lower assimilate production for plant yield (Nemeskéri et al. 2018). Under drought, diffuse resistance of the stomata to CO₂ entry is most likely the principal factor restricting photosynthesis (Boyer, 1970). Water deficit also reduces plant photosynthesis by changing chlorophyll concentration, modifying chlorophyll particles, and harming the photosynthetic system (Mafakheri et al. 2010; Nemeskéri et al. 2019). According to Nemeskéri and Helyes (2019), in dry periods, tomatoes cultivated under non-irrigated circumstances exhibited poor photosynthetic activity, but under moderate and optimum water supply situations, photosynthetic operation was higher. In an investigation of the impact of water supply on water use-related physiological characteristics and produce of snap beans in dry periods, Nemeskéri et al. (2018)

found that non-irrigated conditions had the maximum stomatal tolerance of the leaves and the lowest leaf area index and harvest index regarding the pods.

In general, a reduction in the root water content and an increase in the biosynthesis of ABA, which is transported to plant aerial parts via the xylem under drought stress, occurred when plants are exposed to drought stress; eventually, it leads to shrinking the guard cells and closing stomata (Talaat et al. 2015). Sheteiwy et al. (2021b) discovered that different bio-fertilizer treatments caused changes in the cell development of soybean roots at 50 and 90 days after sowing. They predicted that using bio-fertilizers reduced the inhibitory effects of drought on cell development, resulting in a shorter period for cell development and cycle division. Cell production may be reduced in drought-stressed plants owing to a lesser number of dividing cells, such as meristem size reduction. Results also confirmed that plants treated with *Azotobacter* bacteria under drought stress conditions of 0.4FC had a better growth status than other treatments. El-Sawah et al. (2021) reported that *Cyamopsis tetragonoloba* L. treated with bio-fertilizers showed significant increases in shoot length, root length, plant dry weight, chlorophyll content, and nutrient uptake, as compared with control plants.

Horváth et al. 2020 found that multicomponent bio-fertilizers that induce physiological responses in very water-intensive plant species impact plant output under various water supply conditions. In drought years, tomatoes inoculated with bio-fertilizers dramatically enhanced photosynthetic rate as measured by chlorophyll fluorescence and raised the ratio of green output in non-irrigated circumstances (Horváth et al. 2020). The presence of *Azotobacter* may increase the plant's ability to support photosynthesis capacity, synthesize organic acids to produce carbon-based structures, and provide the energy needed for osmotic regulation. In addition, increased uptake of several ions, e.g., NO_3^- , NH_4^+ , and K^+ , under inoculation of seeds with different strains of *Azotobacter* can be another primary cause of increasing dry weight of aerial parts and roots (Anna et al. 2013). The production of siderophores and chelating compounds by PGPRs may help plants withstand a wide variety of environmental challenges and enhance the availability of trace elements when plants are under stress (Hayat et al. 2010). Other methods of plant growth and development under PGPRs include changes in the root system's structure and physiology. These bacteria are critical for increasing root elongation, the quantity of secondary/hairy roots, and the overall volume and weight of the roots, which results in an increase in root surface area, access to water and nutrients, and eventually an improvement in plant water status (Askary et al. 2009; Davoodifard et al. 2012). Seed inoculation enhanced plant height and root length in cotton, wheat (Narula et al. 2005; Kumar et al. 2007), and maize (Zahir et al. 2004), confirming the beneficial effects of PGPRs on plant morphology.

Other studies have shown that PGPRs improve pistachio seedling development and nutrient intake, as well as rice and wheat growth indices, when grown under drought-stressed conditions (Sarcheshmehpour et al. 2013; Rana et al. 2015).

Since plants interact with environmental changes (Brooker, 2006), our findings determined that the interaction of AP + PP + 0.4FC resulted in the shortest shoot and root length, and plant dry weight. *P*

putida was also tested on *Aloe vera* by Khoshbakht et al (2011), who discovered that when compared to other treatments, *P. putida* reduced the dry weight of the plant's aerial parts. Additionally, Cardinale et al (2015) demonstrated that different *Pseudomonas* strains isolated from the rhizospheres of two salt-tolerant plants (*Hordeum secalinum* and *Plantago winteri*) inhibited barley growth and development. Volatile substances produced by direct contact of PGPR with plant roots, such as cyanide, can have antagonistic and synergistic effects on plants growth. Cyanide, which is generated by many *Pseudomonas* strains, may operate as a biological control agent against infections and inhibit plant productivity. Also, these strains reduce plant access to the micro-element of iron and consequently reduce plant growth to overcome pathogens (Alstrom and Burns 1989). It should be noted that ethylene production is a significant component restricting root longitudinal growth and auxin transfer (Vacheron et al. 2013). In contrast, several studies have shown that ethylene concentrations rise under various degrees of drought stress (Valluru et al. 2016; Montila Bascon et al. 2017; Dubois et al. 2018). Thus, *Pseudomonas* (PA + PP treatment) may not be beneficial in lowering ethylene concentration in red clover. However, further research is need to fully explain this behavior.

The results showed that *Pseudomonas* (PA + PP treatment) was more effective on uptake of N, Zn and P by the plant than the control treatment. Increased concentrations of N in wheat (Kizilkaya 2008), P and Zn in *Bromus tomentellus* (Delshadi et al. 2017a), and uptake of macro- and micro-nutrients in wheat (Turan et al. 2012) have also been reported. *Pseudomonas* spp. bacteria can release insoluble P as organic phosphorous acids through lowering the soil pH, increasing EC of the soil and contributing to enzymatic processes, thereby increasing the mobility of this element in the soil (Delshadi et al. 2017a). Additionally, the researchers have discovered an increase in plant uptake of P when P-solubilizing microorganisms coexist in the soil (Rodriguez and Fraga 1999) as well as development of plant roots due to increased uptake of nutrients in bio-fertilizer treatments (Bhatia et al. 2014; Budania and Yadav 2014). Withholding water harmed the growth of soybeans, according to Sheteiwy et al. (2021a), but bio-fertilizer treatments improved soybean yield during growing seasons. This could be due to bio-fertilizer's ability (*Bradyrhizobium*) to fix nitrogen, solubilize phosphate, and produce IAA, all of which are linked to increased plant growth. Furthermore, they reported bio-fertilizers can increase plant growth by increasing the concentration of nutrients, particularly P, in plant parts. Furthermore, the increased biological nitrogen fixation and the relatively lower uptake of nitrate from the soil are acidifying the rhizosphere more strongly, possibly contributing to phosphate mobilisation from the soil. In this study, *Azotobacter* had a significant effect on uptake of Fe and K. Therefore, it can be stated that using bacteria of the genus *Azotobacter* through improving plant biomass and continuous and stable supply of minerals, especially N for the plant, may increase plant germination, increase plant shoots and absorb some nutrients by the plant. Additionally, the secretion of hormones such as cytokinin and auxin enhances plant efficiency by increasing water and nutrient absorption (Karimi et al. 2020). It was found that *Azotobacter*-inoculated wheat had the greatest effect on increasing N uptake, according to Kizilkaya (2008).

The findings indicated that when exposed to 0.4FC stress, the amount of P in the aerial parts increased. While K, N, Mn, Fe and Zn had the lowest values under 0.4FC stress. The mechanisms of uptake and transfer of nutrients in plants such as mass flow, diffusion or uptake and transfer by osmotic phenomena

are all a function of the content of moisture in the soil and roots and change in the case of reduced moisture, intensity and uptake of nutrients (Karimi et al. 2020). When drought stress occurs, the active uptake of nutrients also reduces, but under these conditions, with the closure of the stomata, the rate of production of photosynthetic matter and its storage in plant tissues is more than the reduction in nutrients (Hu and Schmidhalter 2005). Perhaps for this reason under stress conditions at different times the content of some nutrients such as P by the plant under drought stress conditions is higher than the control treatment (Karimi et al. 2020).

The treatment of PA + PP had the greatest effect on uptake of nutrients under 0.7FC stress and under 0.4FC conditions compared to FC and 0.7FC, we had the lowest uptake of nutrients. The inoculation of the two bio-fertilizers demonstrated a synergistic and intensifying interaction that enhanced microbial biomass and activity to increase mineral intake from the soil and subsequently improved some plant traits. It seems as if combining different types of PGPRs might result in an intensifying interaction, so enhancing bacteria's favorable effects, such as improving the plant's uptake of water and nutrients from the soil and thus increasing plant development (Delshadi et al. 2017a).

Conclusion

In conclusion, drought-stressed plants showed a substantial reduction in seed germination, root and shoot length, plant biomass, and nutritional content when compared to well-watered plants. Drought stress significantly reduced stages of growth and development in red clover; however, the application of plant growth-promoting rhizobacteria moderated drought stress-induced adverse effects and led to improving germination, growth, and establishment of the plants (Fig. 3). In the present study, *Azotobacter* increased germination indices, shoot and root dry weight, shoot length, and root length. On the other hand, despite the lower efficacy of *Pseudomonas* aerobic solutions than *Azotobacter*, they mitigated the negative effects of drought on plant. Additionally, since drought stress occurs during seed germination and is related with a decrease in the water potential of the plant growth medium and water availability, a decrease in germination indices, plant growth, and development is not unexpected. In general, inoculation of red clover seeds with *Azotobacter* is recommended to improve its growth and development characteristics under drought conditions because of the positive effects of plant growth-promoting rhizobacteria on the growth characteristics of plants under drought stress and the reduction of negative impacts of drought stress. The findings might help in decreasing on the usage of chemical fertilizers. However, further research is necessary to confirm how much the rhizobacteria can help the host plant avoid drought impacts. Overall, additional study should be conducted to determine which strains of plant growth-promoting rhizobacteria are beneficial to plant development and drought stress reduction.

Declarations

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Conflict of interest

The author states that no conflict of interest exists in connection with the publication of this work.

Author Contributions

ME proposed the concept for the research and designed the research plan. ME carried out the experiments, evaluated the findings, and wrote the first version of this paper. Also ME worked on developing methodologies and overseeing the project on a local level. The author did all the editing of the work, reviewed and approved the version submitted.

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Figures

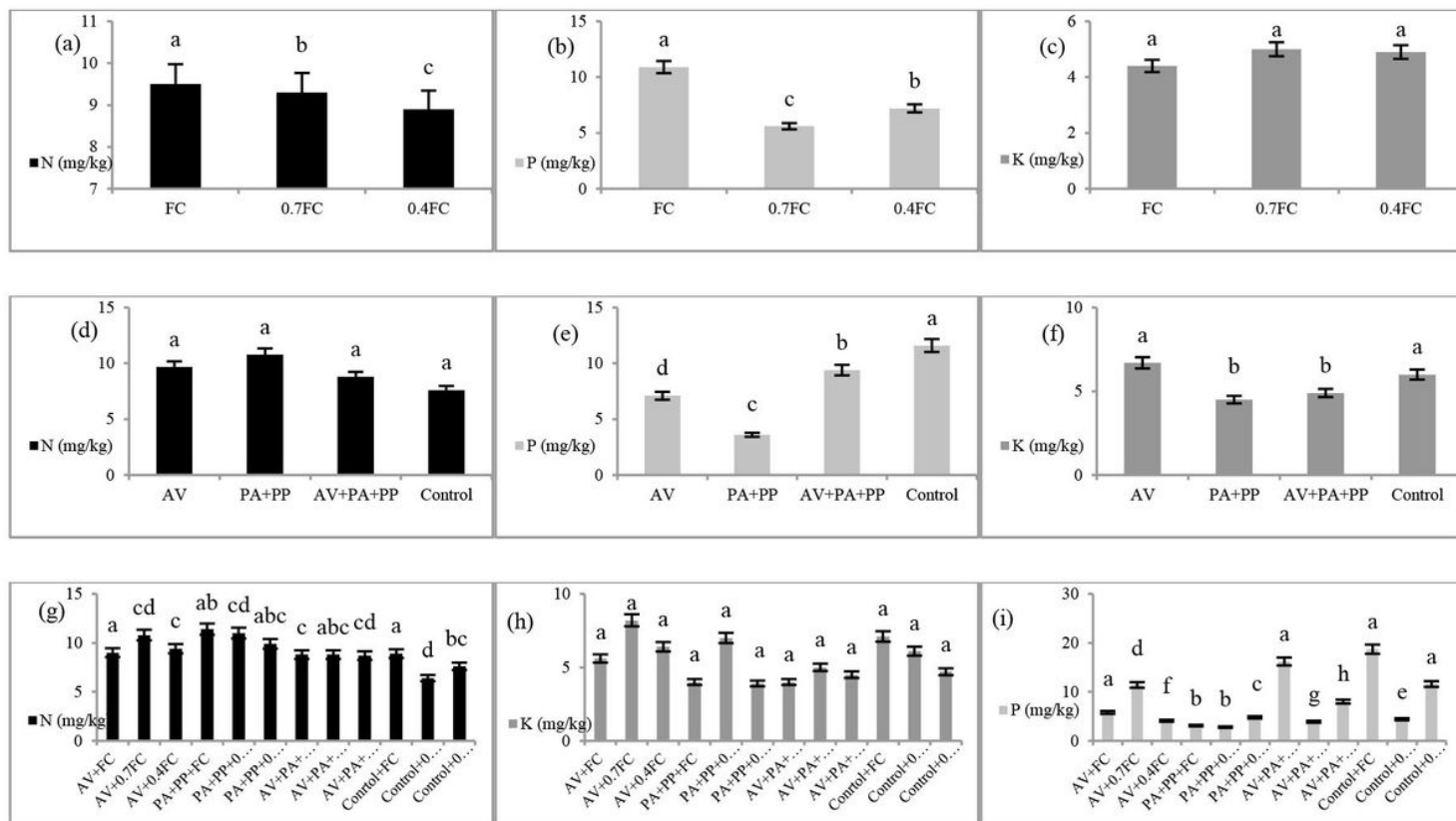


Figure 1

Impact of drought stress (a, b, c), bio-fertilizers (d, e, f), interaction effect of bio-fertilizers × drought stress (g, h, i) on absorption of N/P/K (Data ± SE).

AV= *A. vinelandii*, PA+PP=*P. agglomerans*+ *P. putida*, AV+PA+PP= *A. vinelandii*+*P. agglomerans*+ *P. putida*.

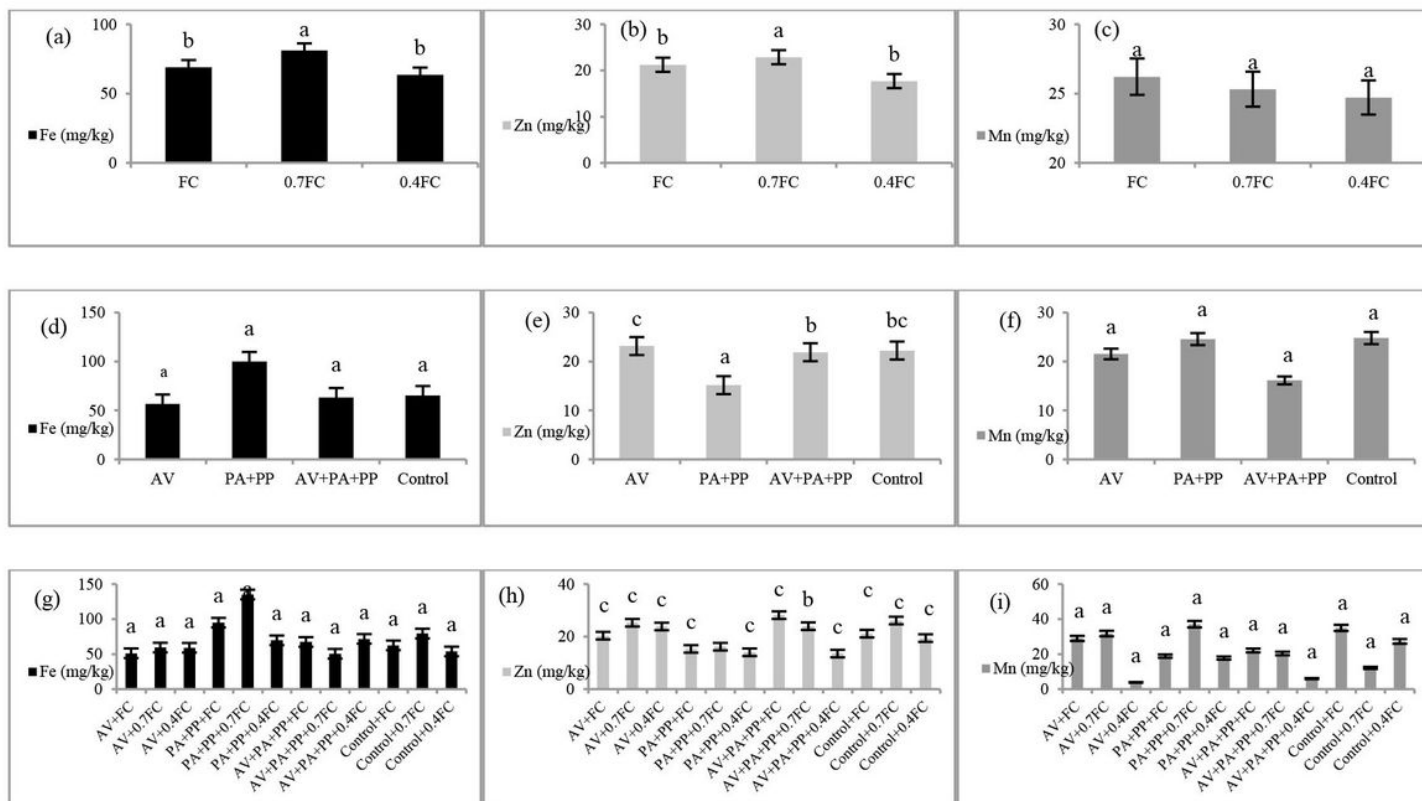


Figure 2

Impact of drought stress (a, b, c), bio-fertilizers (d, e, f), interaction effect of bio-fertilizers × drought stress (g, h, i) on absorption of Fe/Zn/Mn (Data ± SE).

AV= *A. vinelandii*, PA+PP=*P. agglomerans*+ *P. putida*, AV+PA+PP= *A. vinelandii*+*P. agglomerans*+ *P. putida*.

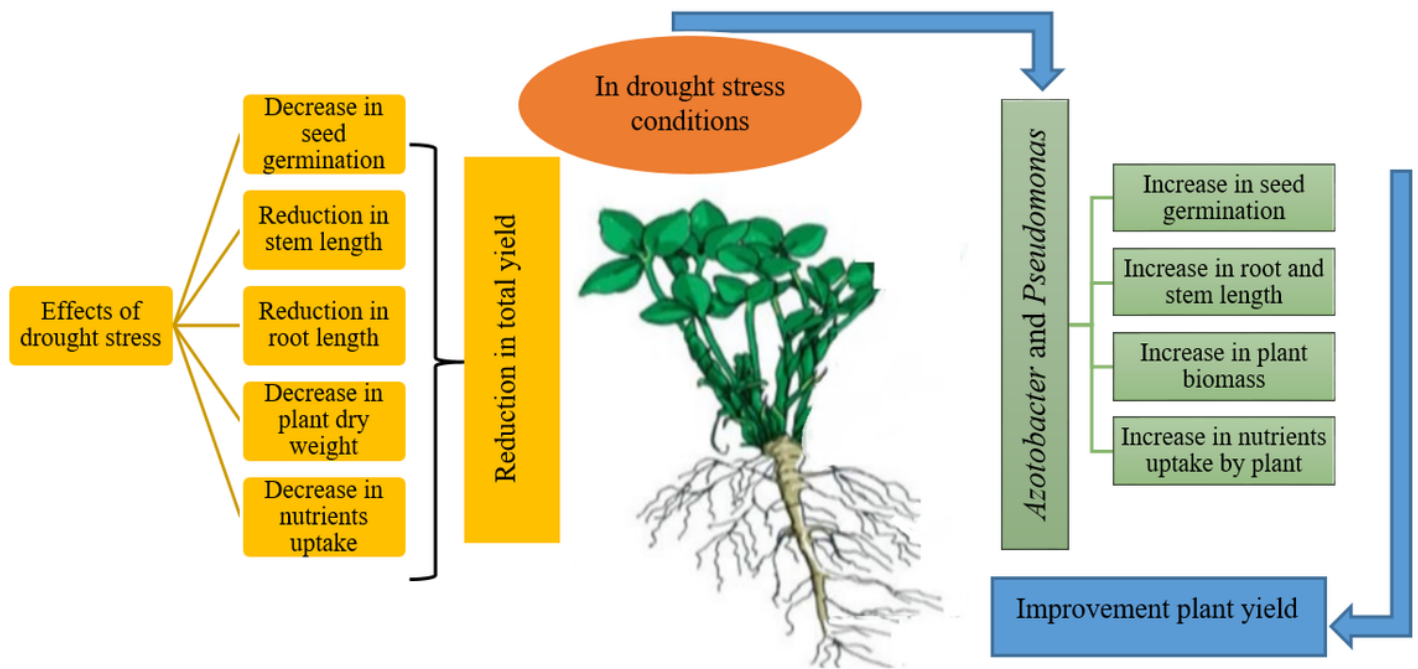


Figure 3

A diagram depicting the interaction between plants and bio-fertilizers to help enhance red clover growth under drought stress.