ISSN: 2538-2152; ECOPERSIA 2026;14(3): 217-235.

Enhancing Drought Resistance in Red Clover (*Trifolium pratense* L.) using Growth-Promoting Bacteria

ARTICLEINFO

Article Type
Original Research

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How to cite this article
Ebrahimi M., Erfani M. Enhancing Drought Resistance in Red Clover (*Trifolium pratense* L.) using Growth-Promoting Bacteria. ECOPERSIA 2026;14(3): 217-235.

DOI:
10.48311/ecopersia.2026.119757.82884

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Article History
Received: April 17, 2026
Revised: July 10, 2026
Accepted: July 12, 2026
Published: July 25, 2026

ABSTRACT

Aims: Arid environments present significant constraints on plant development, with water scarcity being a primary impediment. In this study, we investigated whether plant growth-promoting microorganisms (PGPRs) are effective in reducing the negative effects of water deficit on red clover (*Trifolium pratense* L.).

Materials & Methods: A factorial test, using a completely randomized design, investigated the effect of PGPRs on seed germination, growth, and plant nutrient uptake under water deficit conditions. Three soil moisture regimes were established: field capacity 100% (FC100), 70% (FC70), and 40% (FC40). Experimental treatments comprised inoculation with *Azotobacter vinelandii* (AV), a consortium of *Pantoea agglomerans* and *Pseudomonas putida* (PA+PP), and AV+PA+PP.

Findings: Germination rate varied considerably, with AV×FC70 exhibiting the highest percentage (93%) and control+FC70 the lowest (60%). Synergistic applications of AV, PA, and PP were less effective at mitigating drought stress than single treatments. Root length (10.79cm), shoot length (18.89cm), and dry biomass (aboveground: 0.98g.plant^{-1}; belowground: 1.88g.plant^{-1}) reached their maxima under AV×FC40, whereas (PA+PP)×FC40 yielded the minimal measurements. PA+PP demonstrated no statistically significant impact on water deficit reduction ($p<0.01$). AV×FC40 maximized potassium uptake. Iron and manganese were highest in (PA+PP)×FC70, while zinc was maximum in (AV+PA+PP)×FC100. Minimum uptake of iron, zinc, and manganese occurred with (AV+PA+PP)×FC70, (AV+PA+PP)×FC40, and AV×FC100, respectively.

Conclusion: Typically, inoculation of red clover with *A. vinelandii* has demonstrated efficacy in enhancing drought tolerance, offering a biotechnological strategy for forage production in water-limited environments. Nevertheless, a comprehensive understanding of the mechanisms by which rhizobacteria confer drought resilience to host plants necessitates further investigation.

Keywords: *Azotobacter*; Environmental Stress; Nutrient Uptake; PGPRs; Rhizosphere.

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Introduction

Plant physiology posits that stress in flora arises when heightened environmental or internal pressures disrupt normal physiological processes. Typically, upon removal of these perturbing agents, plants revert to their customary functional states [1]. The impact of abiotic parameters on plant ontogeny and metabolic processes, such as Carbon assimilation, Oxygen consumption, and mineral acquisition, is commonly assessed through macroscopic observations of plant morphology and overall growth trajectory [2]. Specifically, water deficit, or drought, is characterized by an inadequate supply of soil moisture, resulting in turgor loss and subsequent wilting. This condition can lead to reduced enzymatic activity, slowed mitotic activity, and a decline in photosynthetic pigment concentration. Furthermore, drought impairs photosynthetic efficiency and impedes the absorption and translocation of essential nutrients from the rhizosphere to aerial vegetative structures [3].

Studies suggest that by the mid-21st century, the majority of arid zones globally will experience substantial drought-induced stress, presenting considerable obstacles to botanical survival. To persist in these arid and demanding ecological niches, plants must employ a diverse array of adaptive mechanisms to mitigate stress and maintain physiological processes. Such acclimatization is paramount to their continued existence amid escalating environmental challenges [4,5]. The primary defensive stratagems employed by vegetation against water scarcity can be broadly classified into three principal modalities: drought evasion, avoidance of its detrimental consequences, and tolerance to its effects. Consequently, scientific inquiry must prioritize developing techniques that enhance plant resilience to drought [3].

Soil functions as a vibrant, biological system that can be preserved through the application of bio-fertilizers. These microbial inoculants provide plants with essential macro- and micronutrients in an assimilable state. They play a crucial role in enhancing biodiversity, supporting critical plant physiological functions, and maintaining agricultural output even under challenging environmental conditions. By fostering a robust microbial population, bio-fertilizers enable crops to thrive, thereby contributing to long-term agricultural viability [6].

The productivity and growth of plants are notably affected by microbes' ability to generate and release biologically active substances. These substances enhance soil richness and encourage development [4, 6]. In current farming methods, incorporating soil microbes has emerged as a successful strategy for lessening environmental pressures, including drought. Because of their beneficial effects on plant growth and yield, these microbes are classified as plant growth-promoting rhizobacteria (PGPRs) [6, 7].

Certain PGPRs, notably those belonging to the *Azotobacter* and *Pseudomonas* families, exhibit pronounced efficacy in enhancing mineral availability and promoting plant development by elaborating growth-regulating metabolites. These microorganisms are instrumental in refining nutrient uptake and elevating crop yields [4,6]. Specifically, *Azotobacter vinelandii* is among a select group of PGPRs capable of atmospheric N fixation. Plants typically assimilate this Nitrogen within the rhizosphere primarily as Ammonium, a critical mineral nutrient

[8]. This bacterial species can solubilize Phosphate over the temperature range of 15 to 40°C, proliferate in saline environments with NaCl concentrations exceeding 7%, and remain viable within a pH range of 5 to 9. Moreover, *A. vinelandii* has been identified as a biofertilizer that promotes plant growth under conditions of Phosphorus (P) and Nitrogen (N) scarcity. Investigations have revealed that rhizobacteria that establish symbiotic associations with host plants, such as *A. vinelandii*, not only transform atmospheric N into plant-assimilable forms via the roots but also ameliorate the detrimental effects of osmotic stress induced by synthetic fertilizers in xeric substrates [9]. In addition, microbial inoculants featuring *Pantoea agglomerans* and *Pseudomonas putida* are recognized as the most effective Phosphate-solubilizing bacterial agents [10]. These microbial populations exhibit robustness to elevated temperatures (up to 42°C) and high salinity (5%). Moreover, they maintain viability across a wide pH range, from 5 to 11, and are demonstrably adapted to the prevailing climatic regimes of Iran [11]. Investigations have further revealed that the Phosphatase enzyme secreted by *P. putida*, coupled with acidic exudates from *P. agglomerans*, possesses the capacity to cleave both forms of Phosphate compounds (organic and inorganic) present in the soil, thereby facilitating their transformation [12].

The increasing global importance of legumes is particularly evident in their contribution to N fertilizer alternatives and feed concentrate production [13]. Within the framework of regenerative agriculture, which prioritizes enhancing soil health and biodiversity, legumes are recognized as instrumental [14]. *Trifolium pratense* L., commonly known as red clover, is a perennial forage (short-lived) noted for its rich protein levels and significant yield potential. However, it is increasingly facing drought-related challenges in various growing regions [15]. The effectiveness of N fixation in legumes is negatively affected by environmental stressors such as drought, which hinder root nodule formation and reduce Nitrogenase activity [5].

This species thrives in temperate climates worldwide [16] and is currently cultivated across a significant portion of the planet's agriculturally viable terrain. Research has further identified the western, northwestern, northeastern, and central territories of Iran as geographical areas harboring a variety of red clover ecotypes [17, 18]. Under optimal environmental conditions, red clover exhibits a seven-year perennial life cycle; however, in dry and semi-dry areas, it functions as a biennial legume [17].

Red clover exhibits a considerable water demand, requiring approximately twice the water volume to produce equivalent dry matter compared to cereal plants. Optimal development necessitates an annual precipitation of 500–600 mm, with 350–460 mm being critical during the vegetative and reproductive phases. Staniak [7] reported that when rainfall deviates from optimal levels, dry matter yield (DMY) can decrease by 3% to 34%. The peak water requirements for red clover coincide with phases of accelerated growth and floral development. Water scarcity during these critical periods results in diminished stem and leaf biomass and impairs entomophilous pollination by reducing nectar secretion. Generally, crops exhibit reduced yield under water stress [16]. Given that aridity is a paramount environmental determinant limiting global plant productivity [1,2], a rigorous investigation

into the capacity of PGPRs to enhance plant resilience to abiotic stresses, specifically drought, is imperative for optimizing water-use efficiency. Consequently, the study was undertaken to elucidate the role of PGPRs in specific growth indicators of red clover subjected to drought conditions.

Drought represents a significant environmental constraint on global plant biomass accumulation [1,2]. Consequently, investigating the influence of PGPRs on plant resilience to water scarcity is paramount for enhancing water-use efficiency. This investigation was therefore undertaken to ascertain the impact of PGPR inoculation on specific physiological characteristics and nutrient assimilation in red clover under drought conditions. Our central hypotheses posited that (1) PGPRs would have a beneficial influence on the germination and subsequent growth of red clover when exposed to drought conditions, and (2) the administration of these rhizobacteria would lead to an enhancement in nutrient acquisition by red clover experiencing water deficit.

Materials & Methods

Plant Characteristics

The red clover, a perennial herbaceous angiosperm (Fabaceae family), originates from Europe, northwestern Africa, and western Asia [17, 18]. The plant's vegetative structure is characterized by modest erect growth, reaching a maximum height of 15 cm. Its leaves are oval and elongated, trifoliate, measuring 15–30 mm long and 8–15 mm wide. The flower clusters feature deep pink blossoms, 12 to 15 mm long, grouped into compact heads. Traditionally, red clover has been esteemed as a nutrient-dense feed crop for livestock [17].

Preparing the Plant Growing Medium

A factorial experimental setup incorporating three replications was structured using a completely randomized design. The investigation comprised two principal factors. The initial factor concerned the application of bio-fertilizer, with four distinct treatment levels: a null application (control), the sole application of *A. vinelandii* (AV), a composite formulation of *P. agglomerans* and *P. putida* (PA+PP), and a synergistic combination of AV, PA, and PP. The microbial inoculants were procured from Sabz Biotechnology Company (Iran). They are sold under the names of Barvar 1 (containing *A. vinelandii* alone) and Barvar 2 (a combination of two bacteria: *P. agglomerans +P. putida*). Barvar 2 is the most well-known fertilizer containing Phosphorus-releasing bacteria in Iran.

The subsequent factor addressed drought stress, evaluated at three intensity levels: full saturation at 100% field capacity (FC100), reduced moisture at 70% field capacity (FC70), and severely limited water availability at 40% field capacity (FC40). For experimental cultivation, a composite soil substrate with a sandy clay loam texture was used. This substrate contained 1.90% organic matter, exhibited an EC of 0.10 $dS.m^{-1}$, and possessed a pH of 4.5. The macronutrient profile of this soil included 0.23% Nitrogen, 19.50 $mg.kg^{-1}$ Phosphorus, and 630 $mg.kg^{-1}$ Potassium. Prior to initiating laboratory analyses and potting trials, a total of 27 mixed soil samples were processed; specifically, they were sieved using a 2 mm mesh for laboratory examinations and a 4 mm mesh for seed planting.

The experimental investigations were conducted within the controlled environment of the greenhouse facilities at Zabol University. Each pot used in this study had

a capacity of 2 kg of prepared soil. After the pots were filled to volume with sieved soil, they were thoroughly saturated with distilled water. A fine-mesh material was strategically placed at the base of each container to prevent the release of bio-fertilizer and to restrict excessive root elongation. Afterward, the seeds were treated with bio-fertilizer solutions and planted about 1 cm deep, with each pot containing 15 seeds.

This experimental design incorporated two photoperiodic regimes: long days (16 h light, 8 h dark) and short days (8 h light, 16 h dark), each at two distinct light intensities. Initial seed watering was maintained daily until complete germination occurred in darkness. Post-germination, seedlings were cultivated under short-day conditions (8 h light and16 h dark) at 32°C during the day and 10°C at night, with photosynthetically active radiation (PAR) at either 60 $\mu M.m^{-2}.s^{-1}$ (low light) or 250 $\mu M.m^{-2}.s^{-1}$ (high light). Once germination was complete, all samples were moved to long-day conditions and maintained under these settings for the remainder of the experimental duration, at 32°C during the day and 10°C at night, with the aforementioned PAR levels.

Cultures of *Pseudomonas* and *A. vinelandii* were prepared in powdered form, with each gram containing 100 million viable microorganisms. For the cultivation of each bacterial species, 100ml of nutrient broth was dispensed into separate 250 ml Erlenmeyer flasks. A sterile aliquot of each bacterial strain was aseptically introduced into its respective flask containing the growth medium. Incubation was carried out on a rotary shaker operating at 120 rpm and 28°C. After the incubation period (48 h), the bacterial populations were standardized to approximately 100 m cells. Ml^{-1} (McFarland standard) was deemed suitable for application [19].

Soil moisture regimes were established at three levels: 100% of field capacity (FC100), 70% of field capacity (FC70), and 40% of field capacity (FC40). Before applying the water deficit factor, the soil's field capacity was measured using the gravimetric method [20]. Subsequently, the required water volumes to achieve the targeted drought conditions were calculated using the determined field capacity [21]. To establish these conditions, 2 kg of oven-dried soil from each experimental unit was initially saturated to a known weight (W_1). Gravitational drainage was then allowed to remove excess water. To mitigate evaporative losses, the containers were sealed with a thin plastic film. The pots were subsequently maintained in a greenhouse for 48 h under controlled conditions to facilitate the removal of excess water [21]. Following saturation, the soil within the pots attained a specific mass (designated as W_2). The quantitative determination of water content at field capacity (FC), expressed as 100%, was calculated as the difference between the initial dry weight (W_1) and the saturated weight (W_2), both measured at the same temperature. The requisite water volume for each subsequent irrigation event, calibrated to individual stress parameters, was determined using Eqs. (1) and (2) [21].

$$FC\ 70=0.7\times(W2-W1) \quad \text{Eq. (1)}$$

$$FC\ 40=0.4\times(W2-W1) \quad \text{Eq. (2)}$$

Seed Germination Record

To quantify seed viability and progression, germination percentage (GP) and

germination rate (GR) were determined employing the method outlined by Maguire [22]. Specifically, the aggregate count of germinated seeds in each experimental unit was used to determine the total number of viable seedlings. Subsequently, GR was computed using Eq. (3).

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

where GR represents the germination rate, Ni denotes the count of germinated seeds, and Di signifies the number of days post-sowing. Following an initial 14-day growth period, a thinning procedure was implemented to ensure that each experimental vessel contained eight individual plants. Subsequently, drought stress treatments were administered and categorized as FC100, FC70, and FC40. Plant harvesting occurred approximately 90 days after the initial sowing. Post-harvest, roots were meticulously detached from the aerial portions of the plants and thoroughly rinsed with distilled water. The study encompassed determining stem and root length indices, measured with a calibrated ruler, as well as measuring total dry biomass and macronutrient content. Following a drying protocol at 72°C for 48 h, sample masses were measured using a digital balance with an accuracy of 0.001 g.

Measurement of Nutrient Uptake

Before elemental quantification, a digestion protocol was implemented to solubilize the extracted samples [23]. Subsequently, Nitrogen (N) assimilation was determined by Kjeldahl titration. Phosphorus (P) uptake was evaluated using the vanadate-molybdate colorimetric method (yellow method), with absorbance measured with a spectrophotometer. Potassium (K) accumulation was quantified through a diffusion technique utilizing a flame photometer [23]. In addition, the uptake of micronutrients (zinc (Zn), iron (Fe), manganese (Mn)) was estimated via inductively coupled plasma-optical emission spectroscopy (ICP/OES).

Data Analyses

The data underwent statistical analysis using Multivariate Analysis of Variance (MANOVA) in SPSS 20.0. A Generalized Linear Model (GLM) served as the analytical framework for data examination. The distribution's normality was assessed using the Kolmogorov–Smirnov test, and Levene's test was used to assess equal variances among the treatment groups. Following this, the Duncan multiple range analysis was used for post hoc analysis to demonstrate significant variance among average values, with a significance level below 0.05.

Findings

Germination Indices

Statistical analysis, detailed in Table 1, revealed that both bio-fertilizers and the synergistic effects between bio-fertilizers and varying drought conditions significantly influenced germination ($p<0.01$). Examination of the primary impact of distinct drought regimes demonstrated that the lowest and highest germination rates (GR), quantified as 14.21 and 21.11 Number.d^{-1}, respectively, were associated with the FC40 and FC100 treatments. Analogous trends were observed for the GP index. Conversely, the principal effects attributable to bio-fertilizer application demonstrated that the most favorable germination indices were achieved with the AV treatment, whereas the AV+PA+PP combination yielded the least desirable outcomes (Table 1).

Table 1) Effects of drought and PGPRs (main and

interaction) on seed growth.

Treatment	GR (Number.d^{-1})	GP (%)
FC100	21.10±2.42^{a}	89.55±3.45^{a}
FC70	17.08±1.42^{b}	70.30±3.45^{b}
FC40	14.20±1.38^{c}	60.01±3.45^{c}
F-Ratio	4.70	6.46
P-Value	0.00	0.00
AV	25.76±1.50^{a}	90.23±3.82^{a}
PA+PP	24.08±1.50^{a}	81.01±3.82^{a}
AV+PA+PP	18.28±1.50^{b}	71.12±3.82^{b}
Control	14.70±1.38^{c}	62.13±3.82^{c}
F-Ratio	5.39	5.66
P-Value	0.00	0.00
AV×FC100	26.22±1.89^{b}	92.01±3.90^{b}
AV×FC70	28.92±1.90^{a}	93.01±3.91^{a}
AV×FC40	21.10±2.83^{c}	84.01±3.85^{c}
(PA+PP)×FC100	20.75±1.83^{d}	80.01±3.80^{d}
(PA+PP)×FC70	19.19±1.83^{d}	74.01±3.72^{d}
(PA+PP)×FC40	17.06±1.81^{e}	65.03±3.60^{e}
(AV+PA+PP)×FC100	20.65±1.83^{d}	79.03±3.73^{d}
(AV+PA+PP)×FC70	19.65±1.83^{d}	73.03 ±3.65^{d}
(AV+PA+PP)×FC40	17.60±1.42^{e}	67.01±3.61^{e}
Control+ FC100	17.01±1.42^{e}	65.03±3.61^{e}
Control+FC70	14.16±1.38^{f}	62.01±3.85^{f}
Control+FC40	13.05±1.64^{f}	60.01±3.87^{f}
F-Ratio	7.28	6.49
P-Value	0.00	0.00

Note: Statistical analyses revealed significant differences among treatments, denoted by distinct alphabetic superscripts ($p < 0.01$). Mean ± standard error.

Mean comparison of bio-fertilizer applications (Table 1) further elucidated that the highest GR and GP (90.24%) were attained with the AV treatment, registering 25.77 Number.d^{-1}. At the same time, the control exhibited the lowest values for both metrics (14.71 Number.d^{-1} and 62.14%). Furthermore, the data showed no statistically significant differences in germination index values between PA+PP and AV ($p>0.01$). It is pertinent to note that the concurrent use of AV and PA+PP (combination) resulted in a marked diminution in GP ($p<0.01$) when contrasted with the application of individual PGPRs.

The data presented in Table 1, concerning the interaction between drought conditions and bio-fertilizer application, revealed that inoculation with AV consistently promoted more robust germination outcomes (maximum) compared to the untreated control under a soil moisture content of 40% field capacity (FC40), with minimal values recorded. Specifically, mono-inoculation with AV led to statistically significant ($p<0.01$) increases in GP and GR of 70% and 55%, respectively. This observed enhancement was more pronounced than that achieved by applying multiple bio-fertilizers simultaneously. Consequently, these findings support the inference that the strategic deployment of bio-fertilizers effectively mitigated the deleterious effects of water deficit on red clover germination. Despite exhibiting diminished efficacy in mitigating drought-induced reductions in germination indices when applied in combination (AV+PA+PP), a synergistic effect emerged under specific drought conditions. Specifically, in the FC40 drought scenario, plant germination showed a statistically significant improvement with the AV+PA+PP treatment compared with the control exposed to FC40 ($p < 0.01$). Analogous findings were documented for the AV+PA+PP treatment under FC70 drought conditions. That is, a notable

elevation ($p<0.01$) in germination indices was observed for the (AV+PA+PP)×FC70 compared to the control+FC70.

Plant Growth

Statistical analysis demonstrated that the examined variables, along with their interactions, had a statistically significant impact on various plant growth attributes ($p < 0.01$). Specifically, the principal influence attributable to drought stress, as delineated in Table 2, showed that the use of FC40 resulted in attenuation of both shoot and root elongation. Conversely, the most pronounced shoot and root lengths were observed under the FC100 condition. The imposition of drought at FC40 levels was associated with reductions in shoot length by 25% and root length by 36% relative to the FC100 treatment.

Correspondingly, the findings concerning the primary effects of drought and PGPRs on plant dry biomass mirrored these observations. An escalation in drought severity was associated with a notable reduction in plant dry biomass characteristics ($p< 0.01$). The primary impact of PGPRs (Table 2) showed that the highest longitudinal root growth and plant weight growth were associated with the AV treatment, whereas the control had the lowest values. Furthermore, analogous trends were observed in shoot length and dry weight.

Analysis of the interaction between bio-fertilizers and drought conditions (Table 2) revealed that singular applications of bio-fertilizers yielded superior outcomes compared to their combined application. Although statistically insignificant differences ($p> 0.01$) were observed in shoot and root longitudinal growth, and plant weight growth in the combinatorial treatments of AV×FC100, AV×FC70, and AV×FC40, these morphological attributes demonstrated a pronounced enhancement following mono-inoculation with AV at FC40, in contrast to combined inoculation strategies.

The findings suggest that *A. vinelandii* demonstrates superior efficacy in mitigating the detrimental consequences of drought stress at the FC40 level, thereby enhancing plant resilience under such conditions. Conversely, the observation of diminished root and shoot length and plant biomass under the combined application of PA and PP at FC40 indicates that this specific bio-fertilizer combination proved ineffective in ameliorating drought-induced impacts. Furthermore, the synergistic application of all bio-fertilizers investigated (AV+PA+PP) revealed that optimal expression of the assessed growth parameters was achieved at the FC100 level. Specifically, root and shoot elongation, along with dry biomass accumulation, were most compromised under the FC40 drought scenario, as detailed in Table 2. Cumulatively, *A. vinelandii* exhibited a more pronounced beneficial influence on plant characteristics and augmented drought tolerance compared to *Pseudomonas* when subjected to drought conditions.

Nutrients Uptake

Drought, in conjunction with bio-fertilizers, resulted in a significant uptake ($p<0.01$) of Zn, Fe, and P. Conversely, K (Figure 1a) and Mn (Figure 2a) absorption remained unaffected by drought conditions, with non-significant effects observed ($p>0.01$). Regarding N content, drought stress primarily elevated N levels at FC100 and reduced them at FC70 (Figure 1a). Analysis revealed that FC100 exhibited the highest P concentration, whereas FC70 displayed the

lowest (Figure 1a). Furthermore, FC70 was correlated with the greatest accumulation of Zn and Fe (Figure 2a).

Analysis of the main effects attributed to bio-fertilizer application revealed significant variations in nutrient uptake. The maximum amount of N (Figure 1b) was quantified in PA+PP. The maximum amount of K was quantified in AV (Figure 1b). Conversely, the PA+PP treatment resulted in diminished P uptake (Figure 1b) and reduced Zn uptake (Figure 2b). The majority of Mn and Fe concentrations were quantified in the PA+PP (Figure 2b). The AV+PA+PP exhibited the lowest Mn concentrations (Figure 2b). A comparison of treatment means indicated that the control treatment had the highest Mn levels (Figure 2b). In contrast, the control yielded the

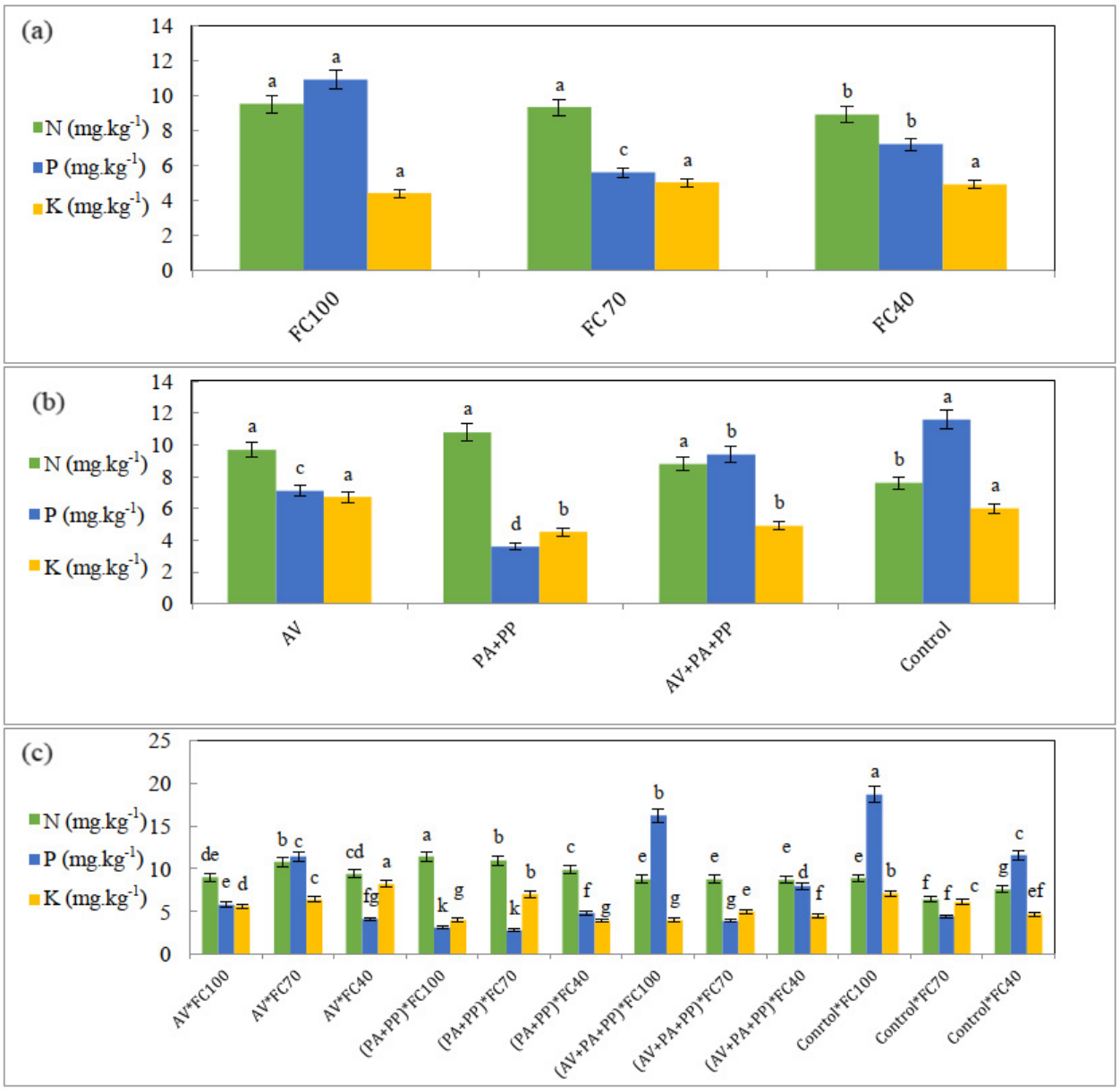

Figure 1) Effects of drought (a), PGPRs (b), and the combined influence of PGPRs and drought (c) on the uptake of N, P, and K (Data ± SE).

lowest N concentration (Figure 1b).

Analysis of interaction effects revealed significant variations in nutrient concentrations. Specifically, the PA+PP treatment with FC100 resulted in an elevated N concentration, whereas the control treatment with FC70 exhibited a reduction in N levels (Figure 1c). Concentration of P peaked under the control+FC100 condition and reached its minimum with the (PA+PP)×FC70 treatment (Figure 1c). The AV treatment at FC40 augmented the k concentration. Conversely, the lowest K levels were observed in (PA+PP)×FC40, AV+PA+PP, and PA+PP, all at FC100 (Figure 1c).

For Fe, the (PA+PP)×FC70 treatment showed the highest concentration, whereas the (AV+PA+PP)×FC70 treatment showed the lowest (Figure 2c). Further examination indicated that the (AV+PA+PP)×FC100 treatment was associated with the most abundant Zn, contrasting with the minimal Zn observed in the (PA+PP)×FC40 treatment

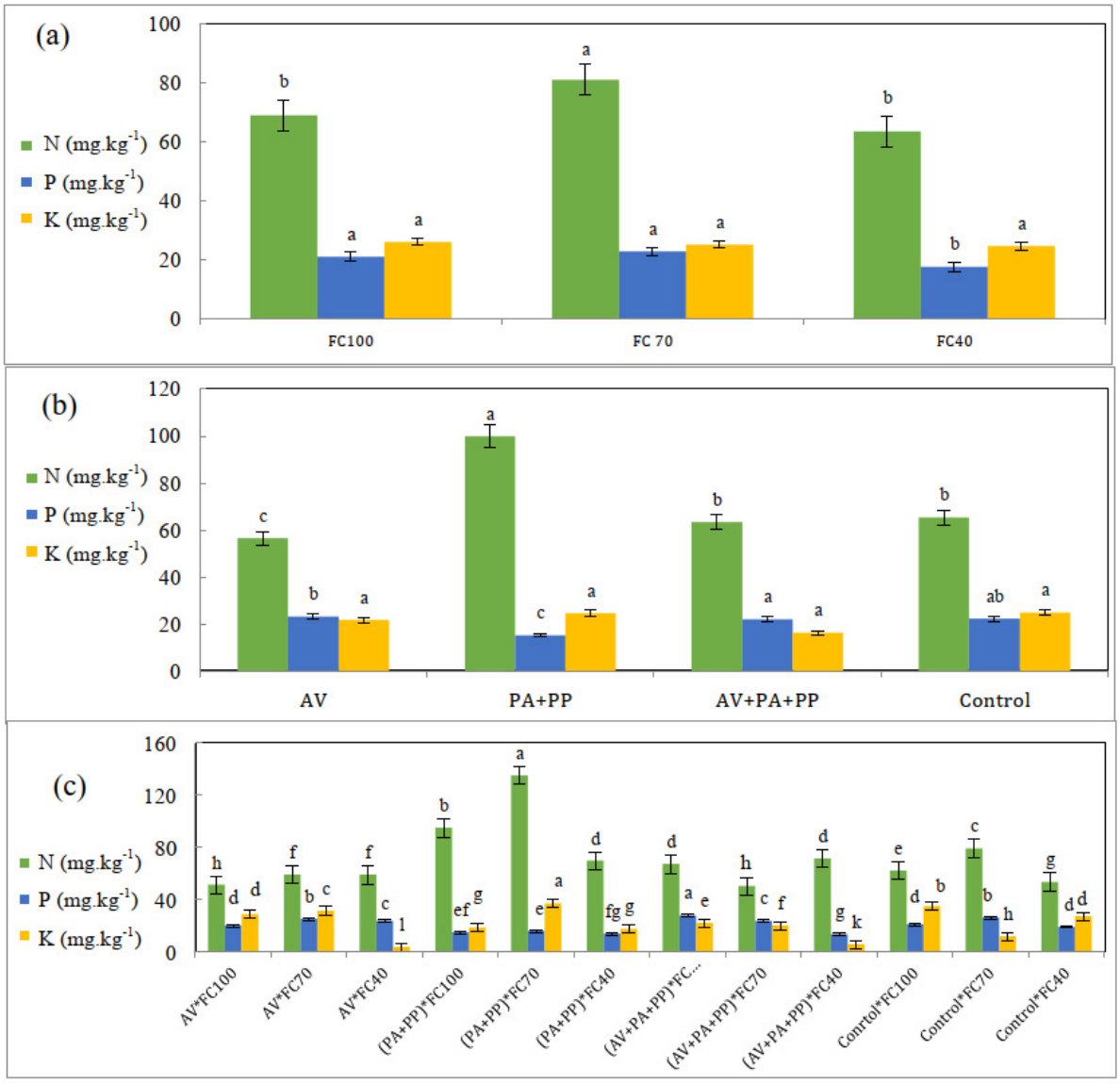

Figure 2) Effects of drought (a), PGPRs (b), and the combined influence of PGPRs and drought (c) on the uptake of Fe, Zn, and Mn (Data ± SE).

Table 2) Effects of drought and PGPRs on morphological traits (main and interaction).

Treatment	Shoot Length (cm)	Root Length (cm)	Aboveground Dry Weight (g.plant^{-1})	Belowground Dry Weight (g.plant^{-1})
FC100	16.32±0.80^{a}	11.13±0.24^{a}	0.72±0.03^{a}	1.62±0.04^{a}
FC70	14.79±0.74^{b}	9.19±0.22^{b}	0.52±0.02^{b}	1.42±0.02^{b}
FC40	12.39±0.72^{c}	7.10±0.19^{c}	0.31±0.02^{c}	1.21±0.02^{c}
F-Ratio	5.18	3.14	0.74	0.69
P-Value	0.00	0.00	0.00	0.00
AV	18.28±0.71^{a}	13.03±0.29^{a}	0.92±0.04^{a}	1.82±0.04^{a}
PA+PP	15.76±0.84^{b}	12.00±0.28^{b}	0.63±0.03^{c}	1.53±0.02^{c}
AV+PA+PP	15.93±0.86^{b}	12.77±0.27^{b}	0.65±0.02^{c}	1.55±0.02^{c}
Control	14.03±0.72^{b}	11.46±0.26^{b}	0.49±0.02^{c}	1.39±0.02^{c}
F-Ratio	5.92	2.49	3.89	2.23
P-Value	0.00	0.00	0.00	0.00
AV×FC100	18.65±0.76^{a}	10.72±0.44^{a}	0.95±0.04^{a}	1.85±0.04^{a}
AV×FC70	18.80±0.78^{a}	10.62±0.43^{a}	0.97±0.04^{a}	1.87±0.04^{a}
AV×FC40	18.89±0.79^{a}	10.79±0.44^{a}	0.98±0.04^{a}	1.85±0.04^{a}
(PA+PP)×FC100	16.10±0.63^{b}	9.89±0.41^{c}	0.70±0.03^{b}	1.60±0.03^{b}
(PA+PP)×FC70	14.50±0.60^{c}	9.76±0.40^{c}	0.50±0.01^{c}	1.40±0.02^{c}
(PA+PP)×FC40	13.24±0.59^{d}	9.00±0.39^{de}	0.43±0.01^{d}	1.33±0.01^{d}
(AV+PA+PP)×FC100	13.11±0.59^{d}	9.17±0.40^{d}	0.40±0.01^{d}	1.30±0.01^{d}
(AV+PA+PP)×FC70	11.43±0.58^{e}	8.15±0.38^{e}	0.20±0.00^{e}	1.10±0.01^{e}
(AV+PA+PP)×FC40	10.42±0.55^{e}	8.98±0.38^{e}	0.10±0.02^{f}	1.00±0.01^{e}
Control+ FC100	14.36±0.59^{c}	10.11±0.40^{b}	0.51±0.02^{c}	1.41±0.02^{c}
Control+FC70	12.42±0.57^{e}	10.00±0.39^{b}	0.29±0.00^{e}	1.19±0.03^{e}
Control+FC40	11.30±0.55^{e}	8.41±0.38^{e}	0.20±0.00^{e}	1.09±0.01^{ef}
F-Ratio	0.76	5.55	4.88	3.11
P-Value	0.00	0.00	0.00	0.00

Note: Statistical analyses revealed significant differences among treatments, denoted by distinct alphabetic superscripts ($p< 0.01$). Mean ± standard error.

(Figure 2c). Under maximum drought stress, the combination of three bacteria exhibited the lowest Zn absorption. The highest efficiency of *A. vinelandii* in Zn absorption occurred under stress condition 70, which was not significantly different from the same condition in the control treatment ($p<0.01$)—regarding Mn, the (PA+PP)×FC70 treatment yielded the highest concentration. In contrast, the AV×FC40 treatment recorded the lowest (Figure 2c).

Discussion

Germination

Drought-induced stress can profoundly modify the structural architecture of seeds, thereby impeding germination. This detrimental effect is primarily mediated by reduced cellular hydration and disruption of key phytohormone signaling pathways crucial for germination, including those that facilitate the catabolism of seed reserves, such as amylase [24]. Furthermore, as drought stress severity increases from FC70 to FC40, the seeds' capacity for water imbibition is significantly impaired. Consequently, a decline in germination rates and root emergence indices is anticipated. Research indicates a correlation between constrained water uptake by seeds and a compromised translocation of stored reserves.

Additionally, diminished synthesis and allocation of organic reserves and proteins within the seed embryo can adversely affect the germination process [20]. A pivotal mechanism underlying enhanced seed germination under bacterial inoculation is increased synthesis of specific phytohormones, notably gibberellins. This plant hormone orchestrates germination by activating enzymes such as amylase, which are instrumental in starch metabolism. The PGPRs can induce seeds to make indole-3-acetic acid (IAA). The auxin derivative promotes cellular expansion and augments germination by engaging with the seed's endogenous amino acid pool [25]. Empirical observations reveal that the concurrent application of multiple PGPRs demonstrated inferior efficacy in mitigating water deficit during germination of plants compared to the use of a solitary PGPR agent.

Morphological Traits

This investigation demonstrates that increased drought conditions detrimentally affect the morphological attributes of red clover. Arid conditions significantly reduce plant output by impairing plant proliferation and maturation (Figure 3). The magnitude of yield deficit and organismal viability is contingent upon the temporal extent and intensity of desiccation [26, 27]. Broadly, our findings suggest that FC40 and FC70 environmental regimes induce the most pronounced reductions in subterranean and aboveground linear dimensions of red clover, respectively. Prior research by De Silva et al. (2023) on red clover indicated that drought-induced water scarcity reduces soil moisture and diminishes water potential in the plant's aerial tissues, including stems and foliage [28]. Restricted moisture availability within the rhizosphere can substantially impede nutrient translocation and bioavailability in the edaphic environment, culminating in nutritional dyscrasia and, consequently, impairing vegetative growth, qualitative characteristics, and overall efficacy. This research elucidated a notable decline in the desiccated growth of plants under water stress. This observation is consistent with prior findings indicating that red clover is more susceptible to arid conditions than timothy [29]. Comparable outcomes were

documented in investigations examining the effects of prolonged drought on the biomass of *Medicago* spp. [30]. In optimal soil hydration, *Medicago* spp. attained maximal dry matter accumulation when cultivated as a sole crop; however, under drought stress, its yield reduction approximated 13.0%. As per Tucak et al. (2016), the pronounced sensitivity of red clover to water scarcity resulted in diminished yields across 23 cultivars [31]. Furthermore, Gaudin et al. (2013) showed the elevated susceptibility of red clover in monospecific stands to water deficit [32]. Küchenmeister et al. (2013) observed that red clover responded to moderate stress by exhibiting an 18% decrease in yield. Drought conditions accelerate the aging of root nodules, concurrently increasing reactive Oxygen generation [33].

This physiological response culminates in a diminution of both nodule and shoot biomass. Water deficit instigates a shift in biomass allocation, manifesting as an augmented root-to-shoot ratio. Under such environmental constraints, a reduced root volume can help mitigate crop yield loss. Furthermore, drought constrains assimilate translocation by compartmentalizing Carbon reserves and impeding gas exchange. This phenomenon may lead to the accumulation of these compounds in leaf tissues, thereby suppressing photosynthetic activity and diminishing overall plant biomass. A decrease in photosynthetic capacity under adverse environmental stimuli, such as drought, can severely degrade a plant's photosynthetic capacity [20, 25].

During periods of water scarcity, plants reduce stomatal conductance as a water-conservation strategy. This physiological response, while preserving hydration, consequently impedes CO_2 assimilation and photosynthetic activity. The resultant decline in photosynthetic output directly impacts the synthesis of vital organic compounds necessary for plant development. Specifically, under arid conditions, the principal impediment to photosynthesis is the augmented resistance to CO_2 diffusion through stomatal pores [20]. Remarkably, investigations revealed that plants subjected to drought conditions at FC40 and inoculated with *A. vinelandii* bacteria demonstrated superior growth compared to other experimental groups. Bio-fertilizers confer drought tolerance through a multifaceted array of mechanisms. These include enhanced nutrient acquisition, stimulation of root proliferation to maximize water uptake, modulation of key phytohormones (gibberellic acid (GA), IAA), and the production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which effectively lowers elevated ethylene concentrations. Furthermore, these beneficial microbes improve root architecture, bolster antioxidant defense systems, synthesize exopolysaccharides (EPS), and reduce the adverse effects of pathogens [25].

In a related study on water stress, Saikia et al. (2018) reported that treating *Pisum sativum* with ACC deaminase-producing PGPRs reduced plant ACC pools and concomitant ethylene biosynthesis [34]. This phenomenon was associated with the upregulation of ACC synthase (ACS) and the downregulation of ACC oxidase (ACO) gene expression [27]. PGPRs possessing ACC deaminase activity have been consistently reported to significantly augment nodule formation in legumes, in the face of water deficit, by facilitating the catabolism of ACC. This enzymatic activity results in the cleavage

of ACC into ammonia and α-ketobutyrate. Ethylene reduction, which is known to suppress nodule development, is effectively ameliorated by these rhizobacteria, thereby increasing nodule number and enhancing the efficiency of symbiotic N fixation [35].

The integration of *A. vinelandii* species can augment plant photosynthetic efficiency, facilitate the biosynthesis of organic acids integral to Carbon frameworks, and furnish the requisite energy for maintaining osmotic equilibrium. Furthermore, enhanced uptake of anionic and cationic species, including Nitrate (NO^{3-}), Ammonium (NH^{4+}), and Potassium (K^{+}), following seed inoculation with diverse *A. vinelandii* strains, may substantially contribute to elevated dry biomass in both aboveground and subterranean plant components. The siderophores and chelators supplied by these microbial fertilizers can bolster plant resilience to a spectrum of environmental stresses and improve micronutrient availability under stress [5]. PGPRs also exert a salutary influence on plant ontogeny by modulating the architecture and functionality of the root system. These microorganisms are instrumental in promoting radicle elongation, the proliferation of adventitious or fibrous roots, and augmenting overall root volume and mass, thereby expanding the root surface area for improved nutrient and water acquisition, ultimately culminating in a more robust plant hydric status. Experimental evidence from inoculation studies shows a simultaneous increase in root height and elongation, an important consequence of PGPRs on root morphology. Specifically, Jabborova et al. (2021) documented a notable increase in soybean dry weight following inoculation with *Pseudomonas putida* under water-deficit conditions [36]. In a related investigation, Rostamikia et al. (2016) observed that PGPR inocula, applied both singly and in consortia, exerted positive modulations on the growth attributes of *Corylus avellana* [37].

Element Absorption

The experimental outcomes demonstrated that the application of PA+PP yielded superior results compared to the control concerning the assimilation of N, Fe, and Mn

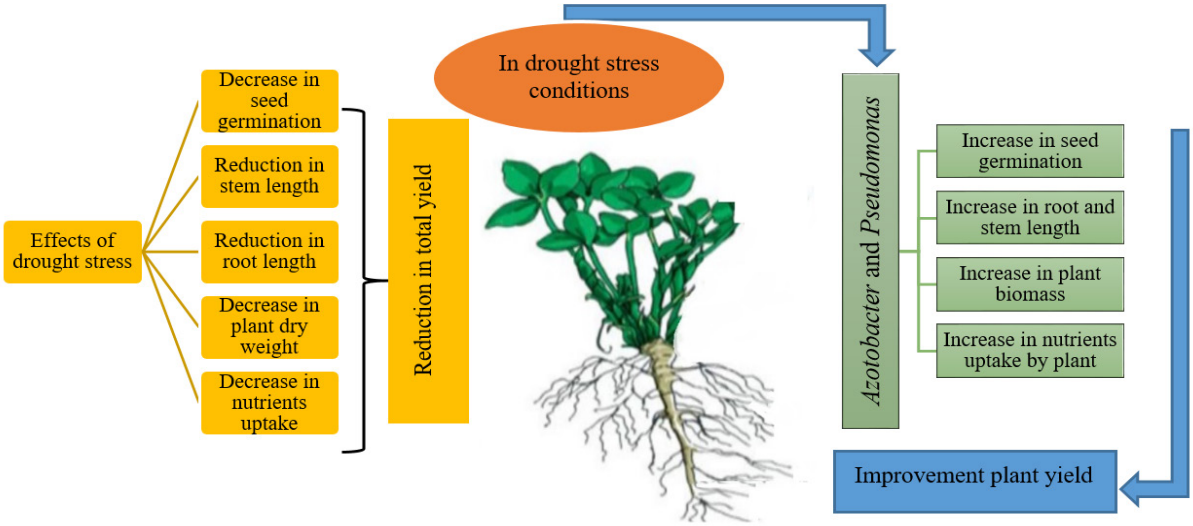

Figure 3) A schematic representation illustrates the synergistic interplay between vegetation and microbial inoculants, designed to promote the augmented development of red clover when subjected to drought conditions.

by plants. Comparable improvements in N, P, and Zn uptake were previously observed in *Bromus tomentellus* [23], and enhancements in N, P, and Zn absorption have been documented in legumes and various crops [27]. The biochemical mechanisms employed by *Pseudomonas* bacteria involve the transformation of recalcitrant P into organic phosphatic acids. This process causes a drop in soil acidity, a rise in soil EC, and the stimulation of enzymatic processes, thereby facilitating the translocation of P within the soil matrix [20].

Existing research suggests that the presence of P-solubilizing microorganisms in soil significantly enhances P uptake by vegetation [5]. This observed enhancement may be attributable to the bio-fertilizer's ability to facilitate N fixation and solubilize P, which are intrinsically linked to improved plant development [27]. PGPRs can also help crop growth by accelerating nutrient uptake, particularly of Phosphorus, in diverse plant organs. The augmented N fixation, coupled with diminished Nitrate assimilation from the soil, culminates in a highly acidic rhizosphere. This acidic environment is hypothesized to play a significant role in mobilizing P from the soil [5].

Within the scope of this investigation, *Azotobacter* was identified as a significant determinant of K absorption. Consequently, increasing plant biomass and maintaining a stable mineral composition, especially Nitrogen, can improve seed germination rates, stimulate shoot elongation, and enhance nutrient uptake. Some of the PGPRs are known to synthesize auxins, gibberellins, and cytokinins. These phytohormones are instrumental in promoting root development and nutrient acquisition, ultimately leading to improved plant performance under water-scarcity conditions [25].

PGPRs alleviate drought-induced reductions in growth by facilitating greater nutrient uptake during periods of water scarcity. The observed improvements in nutrient status and plant biomass suggest that PGPRs counteract drought-induced growth inhibition by promoting nutrient uptake via enhanced root system development, providing a distinct benefit to treated plants. Furthermore, the increase in root biomass stimulated by PGPRs likely enhanced the plant's ability to access water and nutrients in the soil. Elevated nutrient levels under drought conditions are likely attributable to PGPR-driven nutrient mobilization within the rhizosphere, thereby supporting increased nutrient acquisition by plants [27]. Under FC40 stress conditions, the investigation revealed an increase in P concentrations in the plant's aerial vegetative structures. In contrast, K, N, Mn, Fe, and Zn were observed at diminished levels during this FC40 stress regime. The mechanisms governing nutrient acquisition and translocation within plant systems, including mass flow, diffusion, and absorption/transport driven by osmotic gradients, are fundamentally reliant on the soil and root water status. Consequently, these physiological processes are susceptible to alterations in soil and root moisture content [38]. The exogenous application of PA+PP had a pronounced impact on N, Fe, and Mn assimilation, particularly under FC70 stress conditions. Notably, nutrient uptake was most attenuated at FC70 stress levels. The concurrent deployment of these two bio-fertilizers elicited a synergistic response, augmenting microbial biomass and metabolic activity. This enhanced microbial function, in turn, facilitated

greater mineral absorption from the edaphic environment and improved specific plant attributes. The integration of diverse PGPRs appears to engender a cumulative synergistic effect, thereby amplifying the salutary contributions of these microorganisms. Such amplified benefits include an enhanced capacity for water and nutrient uptake from the soil, which ultimately underpins robust plant development [20].

A. vinelandii showed the highest efficiency in K absorption under drought stress of 40. The main effects results also showed that the highest K absorption in red clover occurred in the presence of *A. vinelandii*. K is an essential element in plants, and given its role in conserving plant water and preventing water loss, it helps adjust or reduce the osmotic potential under drought stress [39]. Higher K absorption may be due to the property of *A. vinelandii* to solubilize K in dealing with drought stress. *A. vinelandii* reduces the effects of drought in the plant under drought stress conditions through solubilizing K and greater absorption by the plant [39,5]. Also, the presence of sufficient K maintains photosynthetic activity and promotes the production of photosynthetic materials [5]. Measurement of the photosynthetic performance of red clover in line with this research (data not published) also showed an increase under drought stress. Increasing photosynthetic capacity also increases plant performance.

Conclusion

In conclusion, red clover under water deficit showed significant impairment in seed viability, root and shoot elongation, total vegetative mass, and nutrient composition compared to control treatments receiving adequate hydration. The use of PGPRs reduced the adverse consequences of drought, resulting in improved germination, accelerated growth, and increased plant establishment. *Pseudomonas* compounds, alone or in combination with *A. vinelandii*, were effective at reducing the effects of drought on red clover compared to conditions without bacteria under similar drought stress. However, inoculation with *A. vinelandii* had a stronger effect in reducing the impact of drought on red clover growth parameters under maximum-stress conditions than other treatments. The results obtained in this study support our research hypotheses.

Variability across investigations, including disparities in drought intensity, edaphic composition, botanical taxa, and PGPR strains, could have affected the observed outcomes. This heterogeneity potentially constrains the extensive extrapolation of these findings to all plants and ecological settings. Prior investigations have documented that bacterial genera such as *Pseudomonas* and *A. vinelandii* enhance rhizobial symbiosis across various legumes. This aligns with observations that the markedly higher frequency of nodules on leguminous roots probably results from a cooperative interaction between plant PGPRs and the plants' inherent N-fixing capability, thereby promoting greater nodule development and biomass production. Additionally, PGPR can improve root associations with symbiotic partners, leading to increased nodulation and, consequently, higher rates of N-fixation and nutrient acquisition.

To mitigate these influences, treating red clover seeds with *A. vinelandii* is typically recommended, as these PGPRs can improve growth parameters under arid conditions and attenuate the detrimental effects of drought stress.

A confluence of factors serves as a pivotal modulator of microbial activity and plant physiological responses. While applying PGPR cannot fully mitigate the impacts of extreme water shortages, it serves as an important biological mechanism to shield plant growth, enhance resource efficiency, and maintain high yields under increasingly uncertain weather patterns. The localized nature of these benefits highlights the need for careful strain selection and prudent soil care practices. The observations underscore the paramount importance of ongoing inquiry into bespoke microbial interventions specifically engineered for challenging environmental scenarios to maximize drought tolerance in agricultural output.

Acknowledgments

The authors would like to offer deep acknowledgment to the University of Zabol for its non-economic and logistical assistance.

Authors' Contributions: M Ebrahimi, Introduction author/Original researcher, Methodologist (50%); **M Erfani**, Assistant/ Statistical analyst (50%).

Ethical Permissions: The authors certify that this manuscript is original and that any use of others' work or words has been appropriately cited.

Funding/Support: This study has not received any funding from any organization.

Conflict of Interest: No disputes exist concerning the release of this manuscript.

AI Use Declaration: The authors of this work used ChatGPT solely to enhance the manuscript's language and readability, accessed in January 2025. After employing this tool, the authors took full responsibility for the content and made necessary edits and reviews.

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