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Plant growth promoting rhizobacteria enhance oil content and physiological status of *Thymus daenensis* Celak. under drought stress

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ABSTRACT

Background & Aim: Currently, the use of beneficial microorganisms with the hope to reduce the adverse effects of drought has been the focus of attention. In present study, the effects of plant growth promoting rhizobacteria (PGPR) and drought stress on essential oil (EO) and physiological status of *Thymus daenensis* Celak, were investigated.

Experimental: The experiment was arranged as a factorial pattern in a randomized complete design with three replications. Factors were four irrigation regimes included: well-watered (A: absence of stress), irrigation after depletion of 20-25% of field capacity (L: low stress), irrigation after depletion of the 35-40% of field capacity (M: mild stress) and irrigation after depletion of the 55-60% of field capacity (S: severe stress). Also, two PGPR treatments, non-inoculation (C: control) and inoculation with PGPR were conducted.

Results: The results showed that drought stress reduced root and shoot dry weight, relative water content, photosynthetic pigments and gas change parameters but PGPR inoculation improved all of them. Proline, malondialdehyde, electrolyte leakage and stomatal resistance increased with increasing water stress, but PGPR inoculation ameliorate these increases in corresponding treatments. PGPR inoculation increased essential oil production although this increase was not statistically significant but water stress decreased it.

Recommended applications/industries: The results suggest that PGPR inoculation could be an excellent strategy to alleviate adverse effects of water stress in *Thymus daenensis* cultivation in drought stress conditions. Therefore, farmers in semiarid regions could produce *T. daenensis* by using of PGPR at low water stress for the highest economic amount of extracted essential oil.

1. Introduction

Drought stress is one of the major constraint on agricultural production and is expected to increase its intensity and cause serious plant growth problems for more than 50% of arable lands by 2050 (Vurukonda *et al.*, 2015). Drought stress interrupts normal growth of plants by disturbing water potential and turgor of the plants and can induce secondary stress (Khan *et al.*,

2018). Drought stress alters morphology, physiology, plant development and finally reduces crop production.

Recently, it has been shown that one strategy to cope with drought stress is to use of plant growth promoting rhizobacteria (PGPR) (Nadeem *et al.*, 2014; Mohammad *et al.*, 2016; Enebe and Babalola, 2018; Khan *et al.*, 2018). PGPR induced drought tolerance in plants through several mechanisms including producing exopolysaccharides, phytohormones, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, volatile compounds, inducing accumulation of osmolytes, antioxidants, upregulation or down regulation of stress responsive genes and alteration in root morphology (Vurukonda *et al.*, 2015).

The use of the PGPR in improving growth of some medicinal plants under drought stress include basil plants (*Ocimum basilicum* L.) (Heidari *et al.*, 2012; Agami *et al.*, 2016), chamomile (*Matricaria chamomilla* L.) (Mohammadi *et al.*, 2018), *Catharanthus roseus* (Abdul Jaleel *et al.*, 2007), *Satureja Hortensis* (Mohammadi *et al.*, 2016), Dill (*Anethum graveolens* L.) (Mirmozaffari Roudsari *et al.*, 2015), Cumin (Seghatoleslami, 2013) have been well studied.

Thymus daenensis Celak, one of the *Thymus* species, is a medicinal plant endemic to Iran. The aromatic and medicinal properties of the genus *Thymus* have made it one of the most important medicinal plants (Nickavar *et al.*, 2005). According to our literature review, there is little information on physiological responses of *Thymus daenensis* to inoculation with PGPR especially under water deficit stress condition. Therefore, the main objective of the present study was to assess the changes in stress related physiological parameters, biomass and essential oil content of *Thymus daenensis* in response to *Pseudomonas fluorescens* and Pseudomonas *aeruginosa* inoculation under both water deficit stress and non-stress conditions.

2. Materials and Methods

After obtaining *Thymus daenensis* seed from Pakan Bazr Seed Company, the viability of seeds was measured as approximately 90–95%. In the last week of April 2017, seeds were sown in plastic tray (Sterlized Plantflora substrate) and after four weeks, seedlings were transplanted to the pots (26 cm diameter top, 20 cm diameter base and 20 cm depth) filled with farm soil (an autoclaved farm soil at 121˚ C and 0.11 MPa for 2h)) in Shahrekord, Chaharmahal & Bakhtiari province (Iran) on fourth week of May. Physical and chemical properties of the soil used in this study are presented in Table 1. Neither inorganic fertilizer nor systemic pesticide was used during the experiment. Weeds were manually controlled as needed.

Table 1. Physical and chemical properties of the soil in the research area

Soil properties	Texture	pH	EC (dS/m)
Values	Loam	8.26	1.3
Total nitrogen (%)	**Organic carbon (%)**	**Available (Olsen's method) phosphorus (ppm)**	**Available potassium (ppm)**
0.025	1.23	55.87	606.80

The experiment was arranged as factorial pattern in randomized complete design with three replications. Factors were consisted of four irrigation regimes and two inoculum. The first factor included four irrigation regimes, consisted of well-watered (A: absence of stress), irrigation after depletion of 20-25% of field capacity (L: low stress), irrigation after depletion of the 35-40% of field capacity (M: mild stress) and irrigation after depletion of the 55-60% of field capacity (S: severe stress). In cultivated treatments, for the first 4 month (established phase), non-water stress was performed. Time-domain reflectometry (TDR) using probes and access tube (TRIME-FM, England) were used to measure soil water content (θ_v) in experimental pots at the depths of 0–20 cm and irrigation treatments were designed to keep the soils at four different moisture levels. Data on soil volumetric water content were collected daily during the growing seasons. The second factor included two PGPR treatments, non-inoculation with PGPR (C: control) and inoculation with PGPR (P: *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*). The PGPR inocula (10^8 CFU mL^{-1}) were obtained from Soil Microbiology Department, Soil and Water Research Institute, Tehran, Iran and because of their availability and previous literatures (Ndeddy Aka and Babalola, 2016; Mohammadi *et al.*, 2017). In inoculation treatments, seeds were dipped for 1 h in bacterial culture (10^8 CFU mL^{-1}) before sowing and after transplanting to pots, 20 mL of mixed inoculum suspension per pot (10 mL of

each strain) was applied as a soil drench around the plant base stem.

The aerial and root parts of the *Thymus daenensis* were harvested at full flowering stage (October, 2017). Shoot and root dry weight (DW) were determined. Harvested tissues were dried at 40 ◦C in the dark until it reached a constant weight. For essential oil (EO) isolation thirty gram powdered plant material was subjected to hydro-distillation (500 ml distillated water) for 3 h using a Clevenger-type apparatus. Relative water content (RWC) was estimated according to method described by Levitt (1980). Electrolyte leakage (EL) was defined as method described by Lutts *et al.* (1996). The method of Bates *et al.* (1973) was used for estimation of proline content in leaf tissues. For malondialdehyde (MDA) content estimation in the leaves method of Velikova *et al.* (2000) was performed. Photosynthetic contents were estimated according to Lichtenthaler (1987). Gas exchange characteristics (net photosynthesis rate (P_n), stomatal resistance (rS), transpiration rate (E)) were measured using a LCA4 (ADC, Bioscientific, Ltd Hoddesdon, UK) portable photosynthesis system.

Statistical analysis

The data was statistically analyzed by two-way analysis of variance using SPSS (19.0) software. The significance of differences among treatment means was tested using Duncan's multiple range test at p≤0.01 levels.

3. Results and discussion

3.1. Growth characteristics

Results indicated that inoculation with PGPR had significant positive differences on root DW and shoot DW ($p \leq 0.01$) and significantly increased them compared to control. Also, water stress had significant effects on them ($p \leq 0.01$) (Table 2). Interaction of AMF inoculation and water stress was not significant in two parameters (Table 2). Results showed that drought stress decreased root and shoot DW parameters but PGPR inoculation to some extent compensate this decrease. Improved plant growth under drought condition by PGPR has already been reported (Van Loon, 2007; Agami *et al.*, 2016; Cappellari *et al.*, 2015). Drought stress causes reduction in water content of plant tissues and thereby, reduce enlargement and cell division and finally reduce plant growth (Agami *et al.*, 2016). Naderifar and Daneshian (2012) stated that PGPR enhance plant growth by increasing water and nutrients absorption. Moreover, PGPR increase root weight due to increase in number of lateral roots (Banchio *et al.*, 2008) and leads to increase in root surface area and water and nutrients uptake (Zhang *et al.*, 2007). Production of growth-promoting substances such as indole acetic acid (IAA) and cytokinins is another cause of improving plants growth by PGPR (Banchio *et al.*, 2008). The roles of auxins and cytokinins in promoting plant growth have been well documented (Dey *et al.*, 2004; Gray and Smith, 2005).

Table 2. Effects of PGPR inoculation, drought stress and their interaction on shoot dry weight (DW), root DW, essential oil (EO), relative water content (RWC), electrolyte leakage (EL), proline and malondialdehyde (MDA) of *T. daenensis*

Treatment	Shoot DW (g)	Root DW (g)	EO (mL/100 g DM)	RWC (%)	EL (%)	Proline (µmol/g FW)	MDA (µmol/g FW)
Inoculum							
C	10.59 ± 1.57 ^{b}	14.52 ± 1.82 ^{b}	0.26 ± 0.04	73.02 ± 6.27 ^{b}	27.75 ± 7.04 ^{a}	8.39 ± 5.34 ^{a}	1.41 ± 0.45 ^{a}
P	12.13 ± 1.80 ^{a}	16.56 ± 2.39 ^{a}	0.27 ± 0.04	74.68 ± 5.64 ^{a}	24.38 ± 6.80 ^{b}	6.66 ± 4.47 ^{b}	1.28 ± 0.51 ^{b}
ANOVA	p ≤ 0.01	p ≤ 0.01	n.s	p ≤ 0.01	p ≤ 0.01	p ≤ 0.01	p ≤ 0.01
Water Stress							
A	13.09 ± 1.01 ^{a}	16.24 ± 1.09 ^{b}	0.27 ± 0.01 ^{b}	81.73 ± 1.13 ^{a}	18.33 ± 0.57 ^{d}	1.98 ± 0.33 ^{d}	0.72 ± 0.18 ^{d}
L	12.17 ± 1.83 ^{a}	17.73 ± 2.36 ^{a}	0.31 ± 0.02 ^{a}	75.74 ± 1.08 ^{b}	21.56 ± 3.30 ^{c}	5.07 ± 0.95 ^{c}	1.14 ± 0.03 ^{c}
M	10.06 ± 1.20 ^{b}	15.30 ± 1.12 ^{b}	0.28 ± 0.01 ^{b}	71.37± 1.55 ^{c}	29.42 ± 2.51 ^{b}	8.60 ± 0.84 ^{b}	1.61 ± 0.05 ^{b}
S	10.13 ± 1.17 ^{b}	12.88 ± 1.37 ^{c}	0.20 ± 0.01 ^{c}	66.58 ± 2.34 ^{d}	34.93 ± 1.74 ^{a}	14.53 ± 1.73 ^{a}	1.92 ± 0.08 ^{a}
ANOVA	p ≤ 0.01	p ≤ 0.01	p ≤ 0.01	p ≤ 0.01	p ≤ 0.01	p ≤ 0.01	p ≤ 0.01
Interaction							
ANOVA	n.s	n.s	n.s	n.s	p ≤ 0.01	p ≤ 0.01	p ≤ 0.01

FW: fresh weight, DW: dry weight, C: control, P: PGPR inoculation, A: absence of water stress, L: low water stress, M: medium water stress, S: severe water stress, n.s: not significant, Means (±SD) accompanied by the same letter do not differ significantly at P ≤ 0.05 and P ≤ 0.01 by Duncan's multiple range test.

3.2. Essential oil (EO) content

Results showed that there was a significant difference between irrigation regimes for EO content (p≤0.01). PGPR inoculation increased EO but this increase was not statistically significant. By increasing drought stress, EO content increased in low drought stress and then decreased by increasing drought stress severity. Interaction of PGPR inoculation and water stress was not significant for EO content (Table 2). In line with our results, Cappellari *et al.* (2013) reported that PGPR inoculation lead to increase in EO production. Some researchers stated that increases in total EO yield in response to inoculation PGPR were not due to increased biomass. They concluded that it may have resulted from increased biosynthesis of terpenes (Banchio *et al.*, 2008; Cappellari *et al.*, 2015). Since several EOs have antimicrobial properties, increases in synthesis of EOs can be considered as a defensive response to colonization by microorganisms (Sangwan *et al.*, 2001; Cappellari *et al.*, 2015). Also, Biological elicitors which are also found in *PGPR* can be used for inducing synthesis of secondary metabolites especially in medicinal plants (Ghorbanpour *et al.*, 2016)

3.3. RWC

One of the easiest agricultural parameters that can be used to consider plants drought tolerance is RWC (Agami *et al.*, 2016).The results revealed that increasing of drought stress significantly (P<0.01) decreased the RWC. PGPR plants possessed a higher RWC than non-inoculated plants and this difference was statistically significant (P≤0.01). Interaction of PGPR and drought stress on RWC was not significant (Table 2). The result of this study is in accordance with those reported by Agami *et al.*, (2016) and Kordi *et al.*, (2013) who reported that PGPR inoculation compensate RWC reduction by increasing drought intensity.

3.4. Proline and MDA

For proline and MDA contents, results show that water stress significantly elevated them compared to plants under the normal irrigation condition (P≤0.01). All PGPR inoculations resulted in lower proline and MDA accumulation in plant tissue (Table 2). The highest proline and MDA content were recorded when non-inoculated plants were grown under the severe water deficit condition (Figure 1). Irrespective to PGPR inoculation, Proline content increased by increasing drought stress as confirmed by Agami *et al.* (2016). Proline reduced deleterious effects of drought stress by increasing the osmotic potential, detoxification of ROS produced as a result of water deficit and physically quench singlet oxygen or react directly with hydroxyl radicals (Siripornadulsil *et al.*, 2002, Khalil *et al.*, 2016). Since MDA is one of the resultants of cellular lipid peroxidation, less amount of MDA production is a sign of more cell membrane integrity (Mohammadi *et al.*, 2018). In this study, leaf MDA (as classical markers of oxidative stress) were decreased in inoculated plants in comparison with non-inoculated ones under water deficit stress condition. It has been reported that PGPR may improve the membrane stability through the decrease in lipid peroxidation (Khalil *et al.*, 2016).

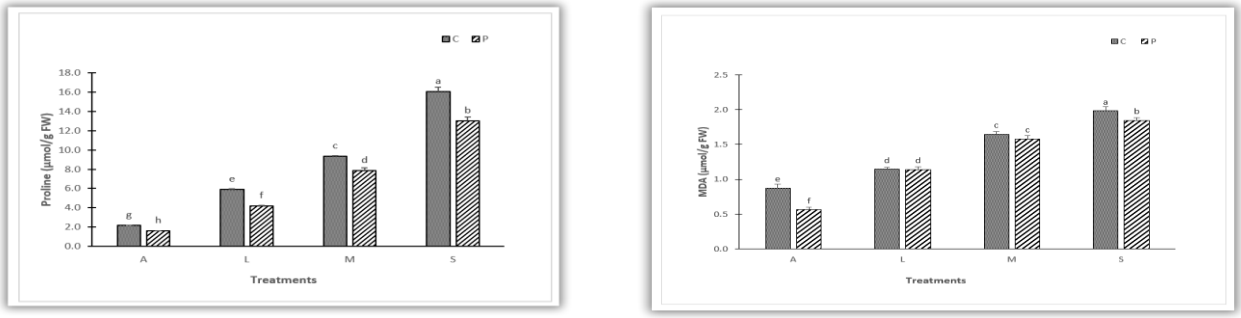

Figure 1. Interaction effect of Plant Growth Promoting Rhizobacteria (PGPR) species application (C: control; non-inoculation with PGPR and P: inoculation with PGPR (*Pseudomonas fluorescens* and *Pseudomonas aeruginosa*) under different drought stress: well-watered (A: absence of stress), irrigation after depletion of 20-25% of field capacity (L: low stress), irrigation after depletion of the 35-40% of field capacity (M: mild stress) and irrigation after depletion of the 55-60% of field capacity (S: severe stress) on proline an dmalondialdehyde (MDA). Data (means ±SD, n=3) followed by different small letters above the bars indicate a significant difference at p≤ 0.01.

3.5. electrolyte leakage (EL)

Leakage of electrolytes increased significantly under drought stress conditions. All PGPR inoculations resulted in lower EL accumulation in plant tissues compared to non-inoculated plants (P≤0.01). The highest EL content was recorded when non-inoculated plants were grown under the severe water deficit conditions (Table 2, Figure 2). In drought stress condition, the increase in free radicals that lead to lipid peroxidation is cause of plasma membrane damage and increased EL (Khalil *et al.*, 2016). It has been reported that PGPR had a significant reduction in the EL increase induced by water stress (Agami *et al.*, 2016; Mohammadi *et al.*, 2018). One cause of reduction of EL in inoculated plant may be duo to expression of antioxidant enzyme which reduce EL (Lu *et al.*, 2015). Therefore, inoculation with bacteria seems to ameliorate cell membrane damage from oxidative stress caused by water stress conditions.

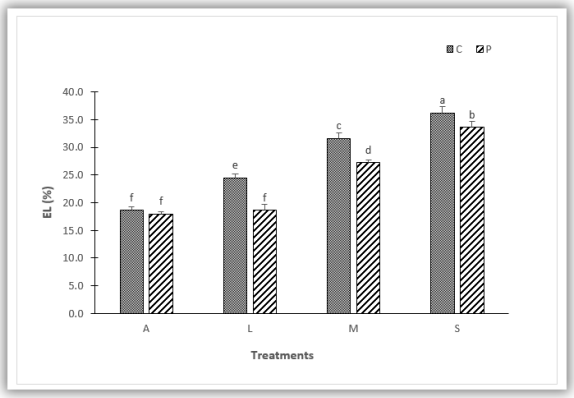

Figure 2. Interaction effect of Plant Growth Promoting Rhizobacteria (PGPR) species application (C: control; non-inoculation with PGPR and P: inoculation with PGPR (*Pseudomonas fluorescens* and *Pseudomonas aeruginosa*) under different drought stress: well-watered (A: absence of stress), irrigation after depletion of 20-25% of field capacity (L: low stress), irrigation after depletion of the 35-40% of field capacity (M: mild stress) and irrigation after depletion of the 55-60% of field capacity (S: severe stress) on electrolyte leakage (EL). Data (means ±SD, n=3) followed by different small letters above the bars indicate a significant difference at p≤ 0.01.

3.6. Photosynthetic pigments

One of the important parameters in evaluation of plant response to environmental stress is chlorophyll concentration (Zhu *et al.*, 2012). Results of this study showed that drought conditions decreased the chlorophyll and carotenoids (P≤0.01), while an increase in Chl a, b, total Chl, and carotenoids was observed in inoculated plant leaves compared to non-inoculated plants (P≤0.01). Interaction of PGPR and drought stress was significant in Chl a and Chl a+b (P≤0.01), but was not significant for Chl b and carotenoids (Table 3, Figure 3). Increase chlorophyll content by PGPR inoculation have already been reported (Cappellari *et al.*, 2015). By increasing water stress photosynthetic pigments decreased and this is may be due to destruction of chlorophyll by increasing the activity of chlorophyll degrading enzymes and chlorophyllase (Baker *et al.*, 2007). As reported by other researchers (Cappellari *et al.*, 2015; Heidari *et al* 2011; Agami *et al.*, 2016) PGPR inoculation increased photosynthetic pigments because PGPR inoculated plants are less susceptible to oxidative stresses that damage the photosynthetic and absorb more water and nutrients than non-inoculated plants**.**

3.7. Gas exchange

The results revealed that increasing of drought stress significantly decreased P_n and E but rS increased with increasing drought stress severity (P≤0.01). PGPR inoculated plants revealed a higher P_n and lower rS compared to non-inoculated plants but, E was not affected by inoculation (P≤0.01). Interaction of PGPR and drought stress was only significant for P_n (Table 3, Figure 4). Increase gas exchange parameters in inoculated plant have already been reported by Nascente *et al.* (2017) who stated that PGPR could positively influence the stomata opening and closing, which lead to a greater accumulation of biomass. It has been stated that the increase in photosynthesis, transpiration rate, and decreased stomatal resistance by PGPR can be attributed to increased leaf area, chlorophyll content and strong source-sink relationship (Semma *et al.*, 2108).

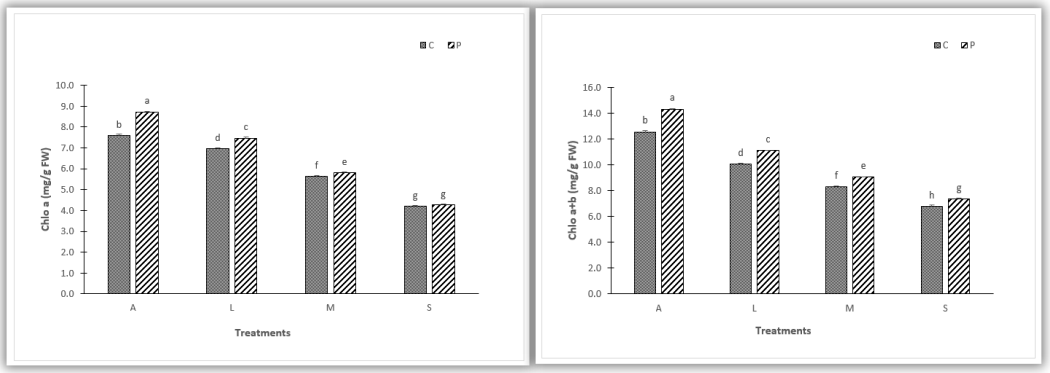

Figure 3. Interaction effect of Plant Growth Promoting Rhizobacteria (PGPR) species application (C: control; non-inoculation with PGPR and P: inoculation with PGPR (*Pseudomonas fluorescens* and *Pseudomonas aeruginosa*) under different drought stress: well-watered (A: absence of stress), irrigation after depletion of 20-25% of field capacity (L: low stress), irrigation after depletion of the 35-40% of field capacity (M: mild stress) and irrigation after depletion of the 55-60% of field capacity (S: severe stress) on chlorophyll (Chl) a and Chla+b. Data (means ±SD, n=3) followed by different small letters above the bars indicate a significant difference at p≤0.01.

Table 3. Effects of PGPR inoculation, drought stress and their interaction on net photosynthesis rate (Pn), transpiration rate (E), stomatal resistance (rS), chlorophyll (Chl) a, Chl b, Chl a+b and carotenoids of *T. daenensis.*

Treatment	P_n (µmol/m^2.s)	E (mmol/m^2.s)	rS (m^2.s/mol)	Chl a (mg/g FW)	Chl b (mg/g FW)	Chl a+b (mg/g FW)	Carotenoids (mg/g FW)
Inoculum							
C	1.26 ± 0.38 ^{b}	0.30 ± 0.11	142.52 ± 39.98 ^{a}	6.09 ± 1.36 ^{b}	3.33± 1.01 ^{b}	9.42 ± 2.24 ^{b}	1.31 ± 0.26 ^{b}
P	1.56 ± 0.46 ^{a}	0.31 ± 0.13	133.97 ± 37.50 ^{b}	6.56 ± 1.76 ^{a}	3.87 ± 1.05 ^{a}	10.44 ± 2.71 ^{a}	1.44 ± 0.28 ^{a}
ANOVA	p ≤ 0.01	n.s	p ≤ 0.01	p ≤ 0.01	p ≤ 0.01	p ≤ 0.01	p ≤ 0.01
Water Stress							
A	2.01 ± 0.31 ^{a}	0.47 ± 0.04 ^{a}	91.61 ± 2.90 ^{d}	8.15 ± 0.63 ^{a}	5.27 ± 0.34 ^{a}	13.42 ± 0.96 ^{a}	1.66 ± 0.06 ^{a}
L	1.49 ± 0.08 ^{b}	0.36 ± 0.04 ^{b}	120.01 ± 6.34 ^{c}	7.21 ± 0.28 ^{b}	3.37 ± 0.28 ^{ab}	10.58 ± 0.56 ^{b}	1.56 ± 0.22 ^{b}
M	1.17 ± 0.10 ^{c}	0.23 ± 0.03 ^{c}	149.99± 5.58 ^{b}	5.72 ± 0.10 ^{c}	2.94± 0.31 ^{b}	8.67 ± 0.40 ^{c}	1.23 ± 0.05 ^{c}
S	0.97 ± 0.22 ^{d}	0.16 ± 0.01 ^{d}	191.36 ± 7.60 ^{a}	4.23 ± 0.05 ^{d}	2.83 ± 0.27 ^{c}	7.05 ± 0.32 ^{d}	1.06 ± 0.05 ^{d}
ANOVA	p ≤ 0.01	p ≤ 0.01	p ≤ 0.01	p ≤ 0.01	p ≤ 0.01	p ≤ 0.01	p ≤ 0.01
Interaction							
ANOVA	p ≤ 0.01	n.s	n.s	p ≤ 0.01	n.s	p ≤ 0.01	n.s

FW: fresh weight, C: control, P: PGPRinoculation, A: absence of water stress, L: low water stress, M: medium water stress, S: severe water stress, n.s: not significant, Means (±SD) accompanied by the same letter do not differ significantly at P ≤ 0.05 and P ≤ 0.01 by Duncan's multiple range test.

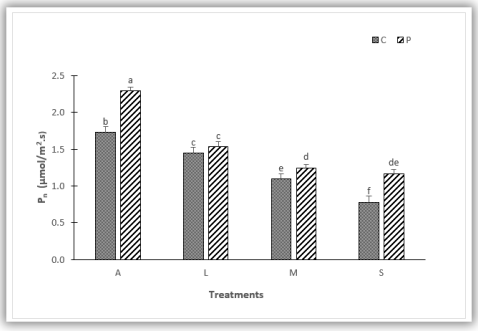

Figure 4. Interaction effect of Plant Growth Promoting Rhizobacteria (PGPR) species application (C: control; non-inoculation with PGPR and P: inoculation with PGPR (*Pseudomonas fluorescens* and *Pseudomonas aeruginosa*) under different drought stress: well-watered (A: absence of stress), irrigation after depletion of 20-25% of field capacity (L: low stress), irrigation after depletion of the 35-40% of field capacity (M: mild stress) and irrigation after depletion of the 55-60% of field capacity (S: severe stress) on net photosynthesis rate (Pn). Data (means±SD, n=3) followed by different small letters above the bars indicate a significant difference at p≤ 0.01.

4. Conclusion

It is well accepted that PGPRs ameliorate plant growth and productivity by numerous diverse mechanisms. The results revealed that drought stress reduced most of growth parameters of *Thymus daenensis* but inoculation with PGPR increased them. The results demonstrated that PGPR inoculation alleviated adverse effects of drought stress in *Thymus daenensis*. From the results of this study, it can be concluded that PGPR inoculation is an excellent strategy to improve cultivation of medicinal plants under drought stress conditions especially in arid and semiarid regions, where water shortage is main obstacle in plants growth.

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