

Physiological and biochemical changes in *Matricaria chamomilla* induced by *Pseudomonas fluorescens* and water deficit stress

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ABSTRACT

Environmental stresses and rhizosphere microorganisms affect growth parameters and accumulation of active ingredients especially in plants with medicinal properties. The present study examined the effects of chamomile (*Matricaria chamomilla* L.) seedling inoculation with *Pseudomonas fluorescens* PF-135 strain on its growth parameters, photosynthetic pigments, proline, malondialdehyde (MDA), and hydrogen peroxide (H₂O₂) content, and essential oil concentration at both regular watering and water deficit experiments. Based on the obtained results, water deficit stress reduced root dry mass, and flower fresh and dry mass as well. However, amount of H₂O₂ and MDA in root and shoot tissues were considerably lower in inoculated plants compared to non-inoculated ones under both normal watering and water deficit regimes. It indicates that lipid peroxidation and production of reactive oxygen species has been diminished in inoculated plants. Also, essential oil content in inoculated plants significantly increased compared with that of non-inoculated ones under water deficit stress condition. It can be concluded that *P. fluorescens* PF-135 strain has an outstanding potential to alleviate adverse effects of water deficit on plant growth, and hence can be used as an excellent PGPR in order to boost chamomile productivity especially under water deficit stress condition.

Key words: PGPR; chamomile; essential oil; injury indices; water deficit stress

IZVLEČEK

FIZIOLOŠKE IN BIOKEMIČNE SPREMEMBE PRAVE KAMILICE (*Matricaria chamomilla* L.) VZPODBUJENE Z BAKTERIJO *Pseudomonas fluorescens* IN POMANKANJEM VODE

Okoljski stresi in mikroorganizmi v rizosferi vplivajo na rastne parametre in na kopičenje aktivnih snovi, še posebej v rastlinah z zdravilnimi lastnostmi. V raziskavi so bili preučevani učinki inokulacije sejank kamilice (*Matricaria chamomilla* L.) s sevom bakterije *Pseudomonas fluorescens* PF-135 na njene rastne parametre, vsebnost fotosinteznih barvil, prolina, malondialdehida (MDA), vodikovega peroksida (H₂O₂) in eteričnih olj v razmerah rednega zalivanja in ob pomanjkanju vode. Rezultati so pokazali, da je pomanjkanje vode zmanjšalo suho maso korenin in svežo ter suho maso cvetov. Količini H₂O₂ in MDA v tkivih korenin in poganjkov sta bili manjši pri inokuliranih rastlinah v primerjavi z neinokuliranimi pri obeh vodnih režimih. To kaže, da je bila peroksidacija lipidov in tvorba reaktivnih vrst kisika manjša pri inokuliranih rastlinah. Tudi vsebnost eteričnih olj se je pri inokuliranih rastlinah značilno povečala v primerjavi z neinokuliranimi v razmerah pomanjkanja vode. Zaključili bi lahko, da ima sev bakterije *P. fluorescens* PF-135 izjemen potencial za odpravljanje škodljivih učinkov pomanjkanja vode na rast rastlin in bi bil lahko uporabljen kot odlična PGPR snov za povečanje pridelka kamilice, še posebej v razmerah pomanjkanja vode.

Ključne besede: PGPR; kamilica; eterična olja; indeksi poškodovanosti; sušni stres

1 INTRODUCTION

Chamomile (*Matricaria chamomilla* L.) is a well-known herbaceous annual medicinal plant belonging to Asteraceae family. It is widely used in traditional medicine and pharmaceutical and cosmetic industries around the globe (Frank and Schilcher, 2005). Chamomile possesses antimicrobial and antioxidant

properties and significant antiplatelet and anticancer activities (Srivastava et al., 2010; Charousaei et al., 2011; Roby et al., 2013). Flower of chamomile contains essential oil and flavonoids, which contribute to its medicinal properties (Srivastava and Gupta, 2015).

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Water stress adversely affects physiological aspects of plants (Farooq et al., 2009). One of the inevitable consequences of water deficit is emergence of various reactive oxygen species (ROSs) in different cellular compartments (Cruz de Carvalho, 2008). If amount of ROSs inside the cell exceed the certain threshold, they become extremely deleterious, initiating uncontrolled oxidative cascades that damage photosynthetic pigments, enzymes and other cell constituents (Gill and Tuteja, 2010; Mittler, 2002). ROSs may also attack cellular membrane phospholipids, leading to production of malondialdehyde (MDA). Since MDA is one of the resultants of cellular lipid peroxidation, less amount of MDA production is a sign of more cell membrane integrity. Generally, plants employ various strategies to manage ROSs, including increase in antioxidant activity and production of various compatible organic solutes such as proline (Ashraf and Foolad, 2007).

Plant growth promoting rhizobacterias (PGPRs) are sort of beneficial bacteria predominantly living in the soil around the plant root surface (rhizosphere). These kinds of bacteria stimulate growth and development of the plants via production and exudation of several regulatory chemicals (Ghorbanpour et al., 2015). PGPRs increase plant yield and can improve plant tolerance to abiotic stresses as well, and hence are

considered as an excellent alternative for chemical fertilizers which threaten environment and human health (Vessey, 2003). The effects of PGPRs on plant performance are complex. They make many positive morphological and physiological changes in plants (Sánchez-Blanco et al., 2004; Glick et al., 2007 and 1995). For example, PGPRs induce synthesis of osmo-protectants such as proline and help the plants to maintain cell membrane integrity especially under stress conditions. Therefore, PGPRs can alleviate adverse effects of environmental stresses on the plants (Christians et al., 2009; Glick et al., 2007; Chauhan et al., 2015).

Among the PGPRs, *Pseudomonas fluorescens* (Flügge 1886) Migula, 1895 is a dominant Gram-negative, rod-shaped bacterium in the soil that contributes to stimulation of plant growth as well as inhibiting growth of pathogens (Mehrabi et al., 2016). There is little information on physiological responses of chamomile to inoculation with these bacteria especially under water deficit stress condition. Therefore, the main objective of present investigation was to evaluate changes in stress related physiological parameters, biomass and essential oil content of chamomile (*Matricaria chamomilla* L.) in response to *P. fluorescens* inoculation under both water deficit stress and non-stress conditions.

2 MATERIALS AND METHODS

2.1 Plant Growing condition, Treatments and Sampling

Chamomile (*Matricaria chamomilla* L.) seeds were purchased from Research Institute of Forests and Rangelands, Karaj, Iran. Sterilized seeds with 1 % sodium hypochlorite were washed three times with distilled water and put on filter paper (Whatman no. 1) in Petri dishes for initiating seed germination.

In order to identify the best *fluorescent pseudomonads* strain, 20 PGPR strains (belonging to the fluorescent pseudomonads group) were obtained from Department of Plant Protection, Azarbaijan Shahid Madani University, Tabriz, Iran. A single colony of each PGPR

strain transferred to 100-ml flasks containing 25 ml of tryptone soybean broth (TSB) and grown aerobically in the flasks on a rotating shaker (120 rpm) for 72 h at 28 °C. The bacterial suspension centrifuged (6000 rpm for 15 min) and then washed and diluted in sterile 0.85 % NaCl (saline solution) to attain a final concentration of 109 CFU.ml⁻¹. The PGPR strains then were evaluated based on their ability to increase chamomile seedling growth index in a sand culture assay (data are not shown). Based on the results of this assay, the *Pseudomonas fluorescens*-135 (PF-135) was determined as the best *pseudomonads* strain (Mohammadi et al., 2017). Plant growth promoting activities of this strain are shown in Table 1.

Table 1: Multiple plant growth promoting activities of *Pseudomonas fluorescens*-135 (PF-135).

<i>Pseudomonas</i> strains	PGPR activities					Ecological site of strains isolation (rhizosphere type)
	P solubility (µg ml ⁻¹)	Siderophore production	IAA production (mg l ⁻¹)	ACC deaminase activity	<i>PhlD</i> gene	
PF-135	328.35	0.42	2.46	1.02	active	Wheat (cv. Azar2)

PGPR: Plant growth promoting rhizobacteria, P: phosphorus, IAA: Indole-3-acetic acid (without presence of tryptophan), ACC: 1-aminocyclopropane-1-carboxylic acid.

Part of germinated seeds was inoculated with the inoculums (10^9 CFU.ml⁻¹) of the promising selected strain (i.e. *P. fluorescens* PF-135). Then, all of the seeds (inoculated or non-inoculated) were sown in pots containing sandy loam soil. Pots were placed in greenhouse with 16 h light period per day and 28/18 °C day/night temperatures. Supplementary light ($250 \mu\text{mol m}^{-2} \text{s}^{-1}$) was provided if necessary.

A factorial experiment in randomized complete block design with three replications was performed. Factors were inoculation (i.e. inoculation with *P. fluorescens* PF-135 strain and non-inoculation) and watering regime (i.e. well-watered and watering up to 50 % of soil field capacity, representing non-stress and water deficit stress treatments, respectively). Water deficit stress treatment exerted on the corresponding pots at the initiation of chamomile flowering stage and continued afterward. However, non-stressed pots were continually irrigated normally through the entire experimental period. Plant samples from each treatment were taken at complete flowering stage and kept at -70 °C for measuring physiological parameters. Also, part of samples was shade dried for a week and used for extraction of essential oils. Plant morphological traits including shoot and root dry mass and flower fresh and dry mass were assessed at full flowering stage.

2.2 Plant Physiological Parameters Assays

2.2.1 Plastid pigment measurements

Fifty milligram of fresh shoots were grounded in 0.5 ml of acetone (80 % V/V) in order to extract photosynthetic pigments (i.e. chlorophyll (Chl.) a, Chl. b and carotenoids). The absorbance of the samples was measured at 645, 663, and 470 nm in a T80⁺ UV-Vis spectrophotometer (PG Instrument Ltd., UK). Photosynthetic pigment contents were calculated using the following equations as described by Lichtenthaler and Wellburn (1983):

$$\text{Chl a (mg/g FM)} = 11.75 \times A_{663} - 2.35 \times A_{645}$$

$$\text{Chl b (mg/g FM)} = 18.61 \times A_{645} - 3.96 \times A_{663}$$

$$\text{Carotenoids (mg g}^{-1} \text{ FM)} = 4.69 \times A_{470} - 0.268 \times (20.2 \times A_{645} + 8.02 \times A_{663})$$

2.2.2 H₂O₂ content determination

In order to determine H₂O₂ content in shoot and root of chamomile plants, 0.5 g fresh tissues were homogenized with 5 ml of 0.1 % w/v trichloroacetic acid (TCA) and centrifuged (12,000×g for 15 min). Then supernatant (0.5 ml) was supplemented to 0.5 ml of potassium phosphate (KHPO₄) buffer (10 mM, pH 7.0) and 1 ml of

potassium iodide (1 M). The upper phase was aliquoted to read its absorbance at 390 nm. H₂O₂ was used for graphing calibration curve in order to calculate H₂O₂ concentration (Velikova et al., 2000). The content of H₂O₂ was expressed as $\mu\text{mol g}^{-1}$ FM based on the standard curve.

2.2.3 Assessment of MDA content

Shoot and root fresh tissues (0.5 g each) were crushed and blended in 5 ml of TCA solution (0.1 % w/v) and centrifuged (12,000×g for 15 min). Two milliliters of supernatant was added to 2 ml of TBA (0.6 % w/v). The mixture incubated at 95 °C for 30 min; cooled down on ice and the samples were centrifuged (4,000 × g for 20 min). Absorbance of supernatant was measured at 532 nm. The amount of MDA calculated based on Heath and Packer (1968). The MDA content was calculated using a correction factor of 155 mM⁻¹ cm⁻¹ and expressed in terms of nmole g⁻¹ FM.

2.2.4 Determination of proline content

In order to determine amount of proline free amino acid content in shoot and root, 0.5 g of fresh tissues were homogenized with 10 ml of 3 % aqueous sulfosalicylic acid and briefly centrifuged. Two milliliters of the supernatant was blended with acid ninhydrin and glacial acetic acid (two milliliters of each). The mixture in test tube was put in a water bath for 1 h at 100 °C. The reaction mixture was extracted with toluene (four milliliters). Absorbance of the mixture determined at 520 nm after being cooled down to room temperature. Standard calibration curve was graphed using appropriate proline concentrations (Bates et al., 1973). Finally, based on the standard curve obtained from different concentrations of proline in terms of $\mu\text{mole g}^{-1}$ FM was calculated.

2.2.5 Determination of essential oil yield

Plant aerial parts (15 g) were shade-dried for a week. In order to extract their essential oils, the dried samples were hydro-distilled in Clevenger apparatus for 4 hours (Letchamo, 1993). The obtained aqueous essential oil was dehydrated by sodium sulfate, then its value was calculated according to its volume to the dry mass of the plant sample (v/w %).

2.3 Statistical Analysis

The obtained data were subjected to analysis of variance by SAS statistical software. Means were compared by Duncan's Multiple Range Test at 0.01 probability level ($P \leq 0.01$).

3 RESULTS

3.1 Plant morphological traits

Significant differences were observed between treatments in the case of root and shoot dry mass and flower fresh/dry mass ($p < 0.01$) (Table 2). Compared to well-watered condition, all of the morphological traits

were reduced significantly under water deficit stress condition (Table 3). Compared to non-inoculated plants, inoculated ones possessed highest amount of foregoing traits under both watering conditions (Table 3).

Table 2: Analysis of variance (ANOVA) for different studied traits. Mean squares are shown for main factors and their interactions

Traits	Mean squares for sources of variations				
	block	Factor a (Inoculation)	Factor b (Watering regime)	Interaction a×b	Error
Root dry mass (g)	0.00298**	0.00019*	0.0072**	0.01599**	0.00002
Shoot dry mass (g)	0.2856**	0.7793**	1.6339**	0.0025 ^{ns}	0.0019
Flower fresh mass (g)	0.3295**	1.1439**	2.2995**	0.0647**	0.0028
Flower dry mass (g)	0.0315**	0.1863**	0.1938**	0.0137**	0.0003
Essential oil yield (v/w) %	0.0008 ^{ns}	1.235**	0.261**	0.126**	0.0016
Chlorophyll <i>a</i>	0.0034 ^{ns}	0.1145**	0.228**	0.0114 ^{ns}	0.0026
Chlorophyll <i>b</i>	0.00124 ^{ns}	0.01628*	0.0678**	0.00002 ^{ns}	0.00142
Total chlorophyll	0.0073 ^{ns}	0.2174**	0.5449**	0.0104 ^{ns}	0.0068
Carotenoids	0.0015 ^{ns}	0.049**	0.09**	0.0027 ^{ns}	0.0023
Root MDA contents	0.601 ^{ns}	26.883**	28.296**	14.434**	0.566
Shoot MDA contents	0.081 ^{ns}	22.509**	35.271**	15.872**	0.367
Root H ₂ O ₂ contents	0.005 ^{ns}	1.3068**	2.5595**	1.0585**	0.0032
Shoot H ₂ O ₂ contents	0.0022*	1.261**	2.3039**	0.7752**	0.0003
Root proline contents	212.861*	4170.178**	36259.641**	1652.076**	21.021
Shoot proline contents	21.923 ^{ns}	3078.596*	28419.581**	5344.461**	384.557

*: Significant at the 0.05 probability level, **: Significant at the 0.01 probability level, and ^{ns}: Not significant.

Table 3: Mean comparison of morphological traits in non-inoculated and inoculated chamomile plants with PGPR under different watering regimes

Traits	Non-inoculated		Inoculated	
	Well-watered	Water deficit stress	Well-watered	Water deficit stress
Root dry mass (g)	0.308 ^c ± 0.04	0.267 ^d ± 0.02	0.389 ^a ± 0.05	0.332 ^b ± 0.04
Flower fresh mass (g)	3.448 ^b ± 0.05	2.719 ^d ± 0.04	4.212 ^a ± 0.06	3.19 ^c ± 0.03
Flower dry mass (g)	1.016 ^b ± 0.04	0.829 ^c ± 0.03	1.333 ^a ± 0.05	1.011 ^b ± 0.06

Values with the same letters in each row are not significantly different at $P < 0.05$.

3.2 Plant physiological traits

The photosynthetic pigments were significantly affected by watering regimes and inoculation factors, and there were not significant interaction among levels of the two factors in the case of these traits (Table 2). As shown in table 4, these traits were decreased in response to water deficit stress treatment. Maximum content of photosynthetic pigments were observed in the inoculated plants (Table 4). In the other hand, amount of H₂O₂ in inoculated plants was lower than that of non-inoculated plants in both watering conditions.

Significant differences were also observed among different treatments in the case of root and shoot MDA contents (Table 1 and Figure 1). The minimum and maximum amount of MDA in both shoot and root tissues were observed in well-watered inoculated plants and water-stressed non-inoculated plants, respectively (Figure 1). Under water deficit stress condition, proline content of root and shoot were significantly ($P < 0.01$) augmented in bacterial-inoculated chamomiles (Figure 1).

Table 4: Mean comparison of some traits in chamomile plants under water deficit stress conditions

Irrigation	Shoot dry mass (g)	Chlorophyll a (mg g ⁻¹ FM)	Chlorophyll b (mg g ⁻¹ FM)	Total chlorophyll	Carotenoids (mg g ⁻¹ FM)
Well-watered	2.904 ^a ± 0.15	0.629 ^a ± 0.04	0.406 ^a ± 0.03	1.035 ^a ± 0.06	0.583 ^a ± 0.03
Water deficit stress	2.79 ^b ± 0.12	0.589 ^b ± 0.05	0.368 ^b ± 0.04	0.957 ^b ± 0.05	0.561 ^b ± 0.02
Non-inoculated	3.413 ^b ± 0.05	0.824 ^b ± 0.03	0.48 ^b ± 0.02	1.305 ^b ± 0.05	0.711 ^b ± 0.04
Inoculated	3.528 ^a ± 0.06	0.865 ^a ± 0.04	0.519 ^a ± 0.03	1.384 ^a ± 0.04	0.734 ^a ± 0.03

Values with the same letters in each row are not significantly different at $P < 0.05$.

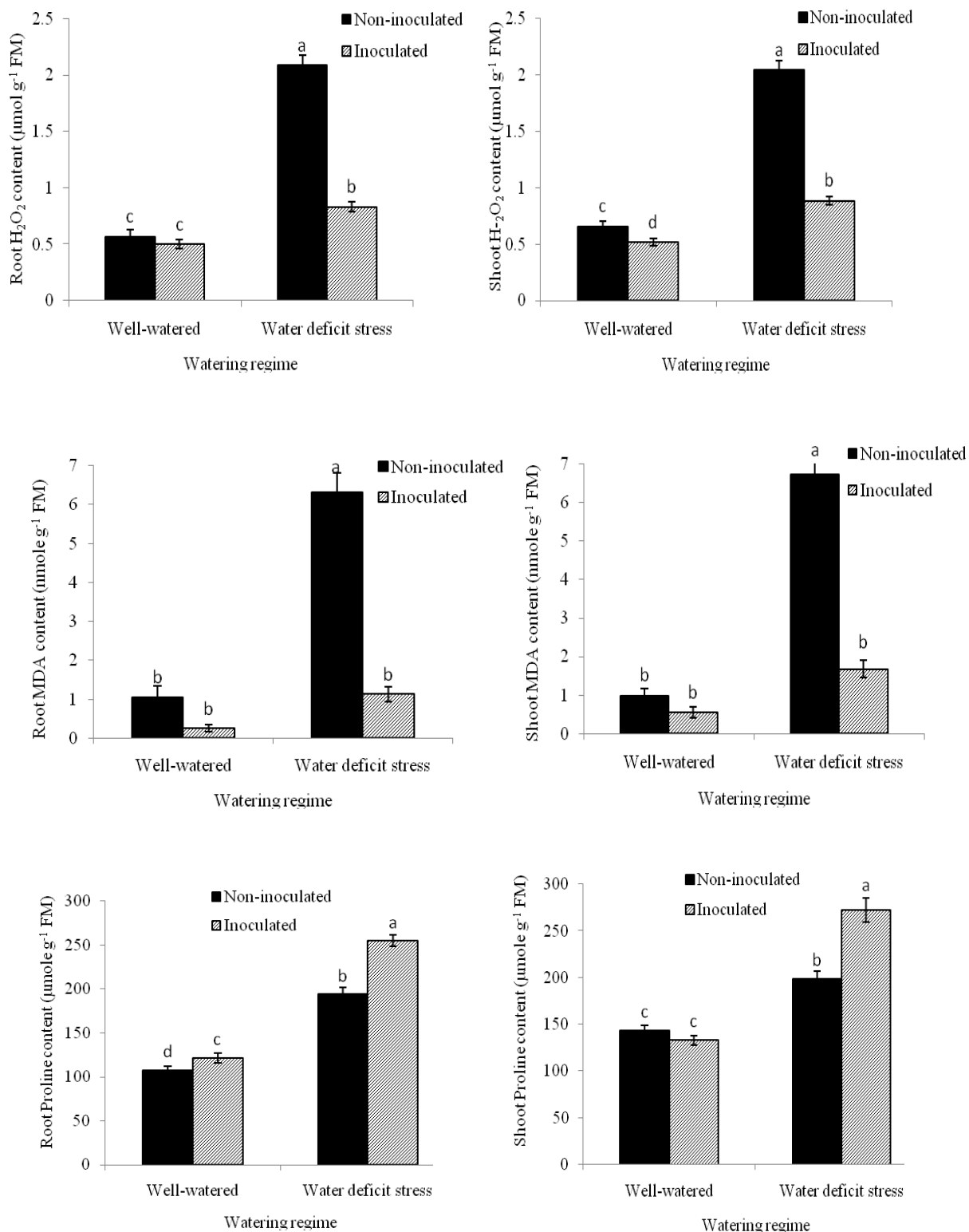


Figure 1: The content of root and shoot H₂O₂, MDA and proline in non-inoculated and inoculated chamomile plants with PGPR under well-watered and water deficit stress conditions. Columns with the same letters are not significantly different at P < 0.05. Vertical bars are ± SD of three independent replicates

3.3 Essential oils yield

The results showed that there were considerable variations among treatments in the case of essential oil yield (Table 1). Generally, water-stressed plants had

more essential oil than well watered plants regardless of inoculation status. In the other hand, inoculated plants had more essential oil than non-inoculated ones in both watering conditions (Figure 2).

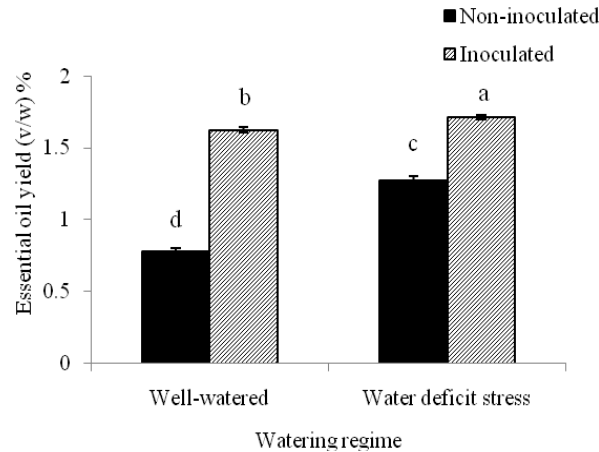


Figure 2: Essential oil yield in non-inoculated and inoculated chamomile plants with PF-135 under well-watered and water deficit stress conditions

4 DISCUSSIONS

It is well accepted that PGPRs ameliorate plant growth and productivity by numerous diverse mechanisms. Generally, these mechanisms include: a) producing various plant hormones; b) asymbiotic nitrogen fixation; c) having antagonistic activity against many plant pathogens; d) converting the nutrients into the more accessible forms to be readily absorbed by plant roots, and e) inhibiting synthesis of ethylene precursor in root cells (Yasmin et al., 2007).

In the present study, inoculation of chamomile with *P. fluorescens* PF-135 strain increased flower fresh and dry mass and root dry mass under both water deficit stress and non-stress conditions (Table 3). Mohammadi et al. (2017) reported that inoculation of *Satureja hortensis* L. with *P. fluorescens* (PF-135) not only significantly increased the plant biomass and essential oil yields under water deficit stress condition but also improved the activity of antioxidant enzymes and synthesis of proline. Moreover, an investigation on corn inoculated with *Pseudomonas* spp. under water deficit stress condition also revealed that inoculated plants had higher plant biomass, relative moisture content, proline, sugar, and amino acids compared to that of non-inoculated plants. Besides, inoculated plants had lower electrolytic leakage and higher antioxidant enzymes activity (ascorbate peroxidase, catalase, and glutathione

peroxidase) under drought stress condition (Sandhya et al., 2010). These observations showed that inoculated plants compared to non-inoculated ones are less susceptible to stress. It can be attributed to improved plant performance which brought about by *P. fluorescens* inoculation.

Fluorescent pseudomonads are important rhizobacteria which can considerably enhance plant productivity especially under stressful environment. It has been reported that fluorescent pseudomonas are able to synthesize ACC-deaminase enzyme (Saravanakumar and Samiyappan, 2007). This enzyme plays a vital role in ameliorating environmental stresses through minimizing ethylene production and stimulating plant root and shoot growth (Glick, 2014; Saleem et al., 2007). These bacteria also inhibit growth of plant pathogenic microorganisms through producing antibiotics. In addition, *P. fluorescens* sustain plant growth via increasing phosphorus solubility, and secreting iron chelating siderophores and indole-3-acetic acid in the soil (Mehrabi et al., 2016).

In this study, leaf MDA and H₂O₂ content (as classical markers of oxidative stress) were decreased more than 2-folds in inoculated plants in comparison with non-inoculated ones under water deficit stress condition.

Inoculated plants also synthesized much more amount of proline compared to non-inoculated plants in response to water deficit condition (Figure 1). It has been reported that plants accumulate nitrogen bearing compounds such as amino acids to be able to cope with drought stress (Amunda and Balasubramani, 2011). In addition, proline is considered to be a compatible solute serve to conserve macromolecular structures and cellular membrane integrity as well as tuning osmotic adjustment especially in plants grown under stressful environment (Ashraf and Foolad, 2007; Maggio et al., 2002). Therefore, it can be extrapolated that application of *P. fluorescens* PF-135 strain decreased lipid peroxidation probably through improving plant enzymatic and/or non-enzymatic antioxidant activities.

Plants secondary compounds have variety of ecological functions such as assisting plants to cope better with harsh environments. It has been demonstrated that when

plants exposed to water deficit, they produce and accumulate much more secondary compounds compared to normal watering (Selmar and Kleinwachter, 2013). Under water deficit stress condition, plants produce higher amount of terpenes, since photo-assimilates are less allocated to current plant growth demand, resulting in considerable accumulation of essential oils (Turtola et al., 2003). In the other hand, biotic elicitors which are produced and secreted by microorganisms such as bacteria can induce production of plant secondary metabolites. They altogether give details why the plants inoculated with the fluorescent pseudomonads synthesis much more amount of essential oils compared to non-inoculated plants especially under water deficit stress condition (Figure 2). Thus, biological elicitors which are also found in *P. fluorescens* can be used for inducing synthesis of secondary metabolites especially in medicinal plants (Ghorbanpour et al., 2016).

5 CONCLUSIONS

Based on the results of the present research, inoculation of chamomile with *P. fluorescens* PF-135 strain significantly boosted essential oil content and plant biomass especially under water deficit condition. Inoculation with *P. fluorescens* PF-135 not only decreased cellular lipid peroxidation but also induced accumulation of proline. Therefore, inoculation of chamomile with *P. fluorescens* alleviated adverse effects of water deficit stress through inducing antioxidant activity, decreasing amount of MDA and H₂O₂ as well as increasing proline content. It can be

concluded that *P. fluorescens* PF-135 strain is as an excellent PGPR for improving chamomile drought tolerance and boosting its growth and essential oil content especially under water deficit stress condition. Since chemical fertilizers are seriously threatening environment and human health in the globe, therefore PGPRs such as *P. fluorescens* (PF-135 strain) can be considered as one of the outstanding alternative for chemical fertilizers in order to develop sustainable farming especially in dry regions of the world where agricultural ecosystems are fragile.

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