Plant growth promoting rhizobacteria (PGPR) improve plant growth, antioxidant capacity, and essential oil properties of lemongrass (*Cymbopogon citratus*) under water stress

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Abstract

Among abiotic stresses, drought is considered as the most important growth limiting factor, especially in arid and semiarid regions. Drought impacts can be adjusted by soil microorganisms. Accordingly, the present study was organized in order to the increase the tolerance of lemongrass (*Cymbopogon citratus*) to drought using plant growth promoting rhizobacteria (PGPR). Treatments were water stress in four levels (100% field capacity (FC), 75% FC, 50% FC, and 25% FC) and inoculation by PGPR in three levels (uninoculated, inoculated with *pseudomonas* sp*.,* and inoculated with *Azotobacter* sp.). Water stress significantly decreased chlorophyll content.Compared to control, severe stress decreased Chlorophylle a+b by 36%. The maximum proline content was accumulated in plants under severe stress and PGPR application. Catalase (CAT) and super oxide dismutase (SOD) activities were increased by 77% and 71%, respectively under severe stress compared to the well-watered condition. The highest total phenol content (TPC) was obtained in the interaction of 50% FC and PGPR application. Moreover, 50% FC induced the maximum total flavonoid content (TFC) by 42% compared to 100% FC. *Pseudomonas* and *Azotobacter* increased the TFC by 6% and 18%, respectively in comparison with uninoculated plants. Essential oil (EO) content and yield were increased under 75% FC, and decreased under 50% and 25% FC. EO percentage in 75% FC and PGPR application was higher than other treatments. Under 75% FC, 14% increase in EO yield was reported for both *Pseudomonas* and *Azotobacter* application. To sum up, PGPR can improve the plant growth and EO properties by increasing antioxidant capacity of lemongrass.

Keywords: antioxidant capacity, *azotobacter*, essential oil, lemongrass, *pseudomonas*

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Introduction

Lemongrass (*Cymbopogon citratus*) is a medicinally perennially plant belonging to the family Poaceae, which mainly grows in tropical regions particularly in Southeast Asia (Vaqar et al., 2007; Babarinde et al., 2016). It is commonly used for human consumption or as cooking ingredients (Ajayi et al., 2016). Due to its lemon scented and

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aromatic attribute, *C. citratus* is widely applied in Asian cooking. It gives strong lemony odor due to high content of citral with two main geometric isomers including geranial and neral isomers (Oliveira et al., 2018). Depending on its habitat, *C. citratus* contains various compounds like terpenes, flavonoids, and alkaloids (Ajayi e al., 2016). The essential oil (EO) of *C. citratus* is used in perfume, cosmetic, and food industries. It is also used to treat cough, cold, rheumatism, digestive problems, bladder problems, and as mouth wash for toothache and swollen gums (Balakrishnan et al., 2014; Ahmad and Viljoen, 2015).

Drought stress is one of the most destructive abiotic stresses, which has been remarkably increased during the recent decades, affecting food security in the world. Drought is anticipated to have destructive effects for more than 50% of the arable lands by 2050 (Kasim et al., 2013). Drought affects plant–water potential and turgor, enough to interfere with normal functions, altering morphological and physiological properties in plants (Rahdari and Hoseini, 2012). It can reduce the plant height and biomass by restricting soil moisture, nutrient uptake, and photosynthesis (Selvakumar et al., 2012; Rahdari et al., 2012). Drought makes oxidative stresses by inducing free radicals such as reactive oxygen species (ROS). High ROS concentrations can damage different aspects of organization like lipid peroxidation, membrane degradation, and nutrients imbalance in plants (Singh et al., 2015).

Inoculation with soil microorganisms can enable plants to be more tolerant in response to drought in arid or semiarid regions (Ullah et al., 2016). Beneficial microorganisms colonize the plant root and promote plant growth through various mechanisms (Heidari and Golpayegani, 2012). Many microorganisms like bacteria, fungi, protozoa, and algae coexist in the rhizosphere, mainly occupied by bacteria. Plants make a symbiosis with mycorrhization helper bacteria (MHB) and plant growth promoting rhizobacteria (PGPR) (Ullah et al., 2016).

PGPRs are known to affect plant growth by direct or indirect mechanisms. PGPRs are responsible for some chemical reactions that occur in soil. It has been reported that PGPRs use an array of strategies to improve plant growth, yield, and nutrient uptake. Some strains of bacteria directly induce the synthesis of plant hormones to adjust plant physiology. In contrast, several strains improve plant growth by increasing the availability of soil nutrients (Heidari et al., 2012).

Recently, the environmental issues and concerns have been globally increased because of the climate variations and human activities such as excessive use of inorganic fertilizers in agricultural production. On the other hand, the global attitude to the production of medicinal plants is designed based on the management strategies such as the use of bio-fertilizers to reduce environmental risks. Accordingly, PGPRs positively mitigate water stress effects via dissolving insoluble phosphates in the soil and accelerating their absorption by the plant. In this regard, the beneficial effect of PGPRs along with or without water stress have been reported on *Mentha x piperita* (del Rosario et al., 2019), *Cannabis sativa* (Pagnani et al., 2018), *Solanum lycopersicum* (Calvo-Polanco et al., 2016), and *Lavandula dentata* (Armada et al., 2016). However, the simultaneous effects of PGPR application and water stress on physiological and biochemical properties of lemongrass are still unknown. Therefore, the aims of the present study were to assess the effect of PGPR application on plant growth, antioxidant capacity, and essential oil percentage and yield of lemongrass under irrigation regimes.

Materials and methods

Growth condition and treatments

Six-month seedlings of lemongrass were provided by the Research Institute of Forests and Rangelands, Iran. The pot experiment was conducted in a greenhouse condition as photoperiod of 16/8 (lightness/ darkness) and relative humidity of 65% - 80% in University of Tehran, Karaj, Iran. The soil used for the study was sandy loam with pH: 7.2, EC:1.1, N: 97%, P: 34 mg/kg; K:832 mg/kg. This work was conducted as factorial based on randomized complete block design (RCBD) with three replications. The treatments were water stress at four levels (100% field capacity (FC), 75% FC, 50% FC, and 25 % FC) and inoculation with PGPRs at three levels

(uninoculated, inoculated with *pseudomonas* sp.**,** and inoculated with *Azotobacter* sp.). The water regimes left for 70 days.

Growth parameters

The plant height was measured at the end of the treatment period. After that, to measure dry weight of shoot and root, the fresh samples were dried in a forced draft oven at 70° C for 72 h.

PGPR

To grow the *Pseudomonas putida* and *azotobacter chroococcum* strains, we used a medium consisting meat and yeast extracts, peptone, and sodium chloride, for 48 h at room temperature on the shaker (Heidolph Unimax1010). The bacterial culture was centrifuged at 2287 × *g* for 5 min at 2° C and the sediment was re-suspended in sterilized tap water. The final bacteria suspension consisted of 10^9 colony forming units (CFU) ml^{-1}.

Irrigation

All plants were normally irrigated one month in order to adapt to the new condition. After that, the four levels of irrigation including irrigation at 100% field capacity (FC), 75% FC, 50% FC, and 25% FC were applied for 80 days. The volume of irrigation water was quantified using the following equation (Benami and Ofen, 1984):

$$Vn= (FC-PWP)/100 *\rho b*Vp*MAD$$

where, Vn is the irrigation water required before irrigation (cm^3), FC is field capacity (%), PWP is the wilting point (%), ρb is bulk density (gr/cm^3), Vp is the pot volume (cm^3), and MAD is the management allowed depletion. FC and PWP were measured by a pressure plate.

Chlorophyll assay

Chlorophyll a and chlorophyll b contents were extracted according to Arnon (1949). For this purpose, 200 mg of fresh samples were homogenized in 8 ml 80% acetone. After that, the mixture was centrifuged at 4° C for 15 min (3000 rpm). Supernatants were used for analyzing chlorophyll content. Absorbance was determined at 645 and 663 nm by a spectrophotometer.

Proline level

Bates et al. (1973) was applied to measure proline content. Briefly, 3% (w/v) sulphosalycylic acid was used to homogenize leaf samples (0.5 g) and the homogenate was filtered via filter paper. Subsequently, the ninhydrin and glacial acetic acids were added. Heated water bath was applied to react the samples at 100° C for 60 min. Reaction was then ceased using an ice bath. Toluene extracted the mixture and the observance was read at 520 nm. Calibration curve was used to determine proline concentration shown as µmol g^{-1} FW.

Catalase (CAT) activity

The measurement of initial rate for H_2O_2 disappearance was applied to measure CAT activity (Bergmeyer, 1970). The reaction mixture contained 3% H_2O_2 and 0.1 mM EDTA in 0.05 M Na-phosphate buffer (pH 7). The reduction in H_2O_2 concentration was determined as a decline in optical density at 240 nm and the activity was measured as µmol H_2O_2 decomposed per minute.

Superoxide dismutase (SOD) activity

The SOD activity was analyzed in a mixture including 50 mmol sodium phosphate buffer (pH 7.0), 10 mmol methionine, 1.2 mmol riboflavin, 55 mmol NBT, and 100 µl enzyme extract. The capacity of inhibiting the photochemical reduction in nitro-blue tetrazolium (NBT) at 560 nm was used to read the solution absorbance. Enzyme activity, decreasing the photo-reduction of nitroblue tetrazolium to blue formazan by 50%, was determined as one unit of SOD (Chen and Pan, 1996). The activity of SOD was defined as enzyme units per gram fresh weight (U/g FW).

Determination of total phenolic content (TPC)

Folin–Ciocalteu reagent was selected to measure TPC spectrophotometrically according to

Heinonen et al. (1998). For this purpose, 100 µl of the MeOH solution of the precisely measured weight of the plant 1-10 (2.54, 2.58, 2.25, 4.03, 4.80, 2.13, 4.62, 1.47, 1.58, 15.05 mg/mL respectively) were mixed with 0.75 mL of Folin–Ciocalteu reagent and allowed to stay at 22° C for 5 min. The mixture was supplied with 0.75 ml of $NaHCO_3$. Absorbance was measured at 725 nm by UV–vis spectrophotometer (Varian Cary 50) after 90 min at 22° C. Standard curve was calibrated by gallic acid (0-100 mg/; r > 0.99). Results were represented as mg gallic acid/g Dry weight.

Determination of total flavonoid content (TFC)

Aluminum chloride method was applied to measure the total flavonoid content (Zhishen et al., 1999). Briefly, the mixture containing 0.5 mL of sample and 300 µL of $NaNO_2$ (1:20 w/v) was vortexed for 10 s and left to stand at 24 °C for 5 min. After that, the reaction mixture was changed by 300 µL of $AlCl_3$ (1:10 w/v), 2 mL of NaOH (1 M) and 1.9 mL of distilled water, and then vortexed for 10 s. The absorbance was determined at 510 nm. Quercetin concentrations ranging from 0 to 1200 µg/mL were prepared and linear fit was used for calibration of the standard curve.

Isolation procedure

Lemongrass EO content was quantified based on the method described by European pharmacopoeia for oil production (European Pharmacopoeia, 1983). Accordingly, 100 g of dried aerial parts were utilized to hydro-distillation for 3 h with application of a Clevenger-type apparatus.

Statistical Analysis

All data were analyzed statistically by analysis of variance using software SAS version 9.3 (Cary, NC: SAS Institute Inc., 2011). The means were separated using Duncan test at $p < 0.05$.

Results

Plant growth

Plant height was influenced by water stress, PGPR, and the interaction of water stress and PGPR ($p \leq 0.05$, Table 1). PGRP significantly increased plant height in 50% and 25% FC. Under severe water stress (25% FC), the plant height increased by *pseudomonas* and *azotobacter* by 15% and 14%, respectively compared with uninoculated plants (Fig. I). Shoot dry weight was significantly changed under water stress and PGPR, and the interaction of water stress and PGPR ($p \leq 0.05$, Table 1). PGPR increased the shoot dry weight particularly in moderate and severe stresses. In 50% FC, shoot dry weight was

Table 1
Analysis of variance for the studied traits of lemongrass under water stress and PGPR application

S. O. V.	df	MS											
		Plant height	Shoot dry weight	Root dry weight	Root /Shoot ratio	Chl a+b	Proline	CAT	SOD	TPC	TFC	EO	EO yield
water stress (W)	3	4430^{**}	247^{**}	34.5^{**}	0.35^{**}	960^{**}	0.11^{**}	0.85^{**}	1925^{**}	1925^{**}	38.3^{**}	0.18^{**}	0.04^{**}
PGPR	2	138^{**}	23.5^{**}	0.96^{ns}	0.18^{**}	0.002^{**}	0.0005^{*}	0.001^{*}	10.5^{*}	29.7^{**}	0.7^{*}	0.005^{**}	0.002^{**}
W×PGPR	6	18.8^{**}	3.1^{**}	1.6^{ns}	0.10^{**}	0.001^{*}	0.0004^{*}	0.0004^{ns}	2.3^{ns}	16.07^{**}	0.26^{ns}	0.001	0.0001^{**}
Error	22	3.7	0.36	1.39	2×10^{-3}	4×10^{-4}	1×10^{-4}	3×10^{-4}	2.31	3.45	0.13	2×10^{-3}	1×10^{-4}
C.V.	-	1.78	2.26	8.67	10.8	1.95	2.66	3.86	6.04	5.06	4.24	2.04	2.2

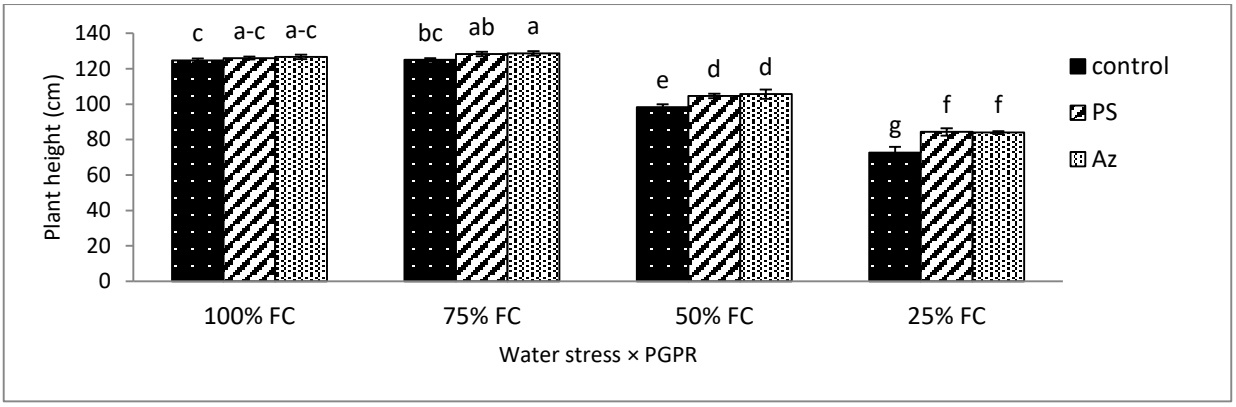

Fig. I. The effect of PGPRs on plant height of lemongrass under irrigation regimes

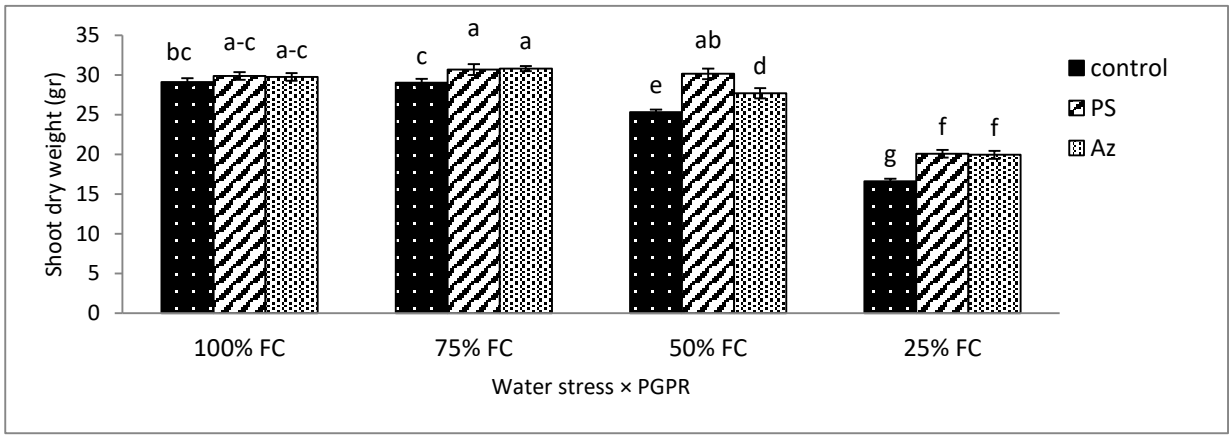

Fig. II. The effect of PGPRs on shoot dry weight of lemongrass under irrigation

Table 2
The effect of water stress on root dry weight, CAT, SOD, and TFC of lemon grass

Irrigation regime	Root dry weight (g)	CAT activity (µmol H_2O_2 decomposed/min/mg protein)	SOD (unit/mg protein)	TFC (mg GA/ g DW)
100% FC	13.21 ± 1.1^{b}	0.22 ± 0.02^{d}	12.77 ± 1.2^{c}	6.55 ± 0.4^{d}
75% FC	15.11 ± 0.8^{a}	0.31 ± 0.02^{c}	14.22 ± 1.7^{c}	7.30 ± 0.5^{c}
50% FC	15.15 ± 0.6^{a}	0.56 ± 0.01^{b}	29.88 ± 2.1^{b}	11.15 ± 0.5^{a}
25% FC	11.01 ± 1.5^{c}	0.92 ± 0.01^{a}	43.77 ± 1.8^{a}	9.23 ± 0.8^{b}

enhanced by 17% and 9% with application of *pseudomonas* and *Azotobacter,* respectively in comparison with uninoculated plants (Fig. II). Root dry weight was affected by water stress ($p \leq 0.05$, Table 1). It was increased by moderate water stress up to 15.15 g (Table 2). Root/shoot ratio was changed by water stress, PGPR, and the interaction of water stress and PGPR application ($p \leq 0.05$, Table 1). The highest root/shoot ratio was observed in the interaction of 25% FC and no application of PGPR (Fig. III).

Chlorophyll and proline contents

Chlorophyll a+b and proline concentrations were influenced by water stress, PGPR, and the interaction of water stress and PGPR ($p \leq 0.05$, Table 1). Water stress significantly decreased chlorophyll content. Under severe stress, 36% reduction of Chlorophyll a+b was observed compared to uninoculated plants (Fig. IV). The maximum proline content was accumulated in plants under severe stress and PGPR application (Fig. V).

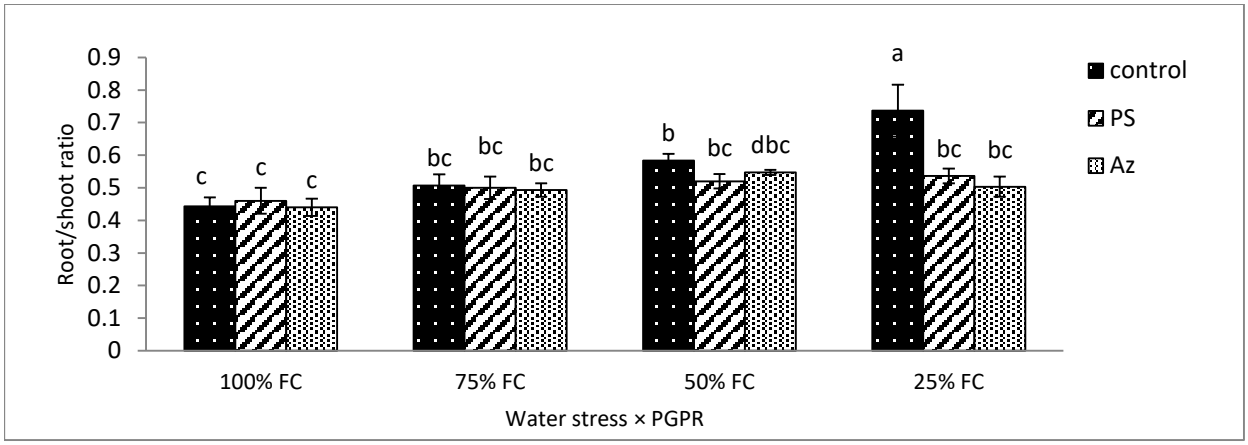

Fig. III. The effect of PGPRs on root/shoot ratio of lemongrass under irrigation regimes

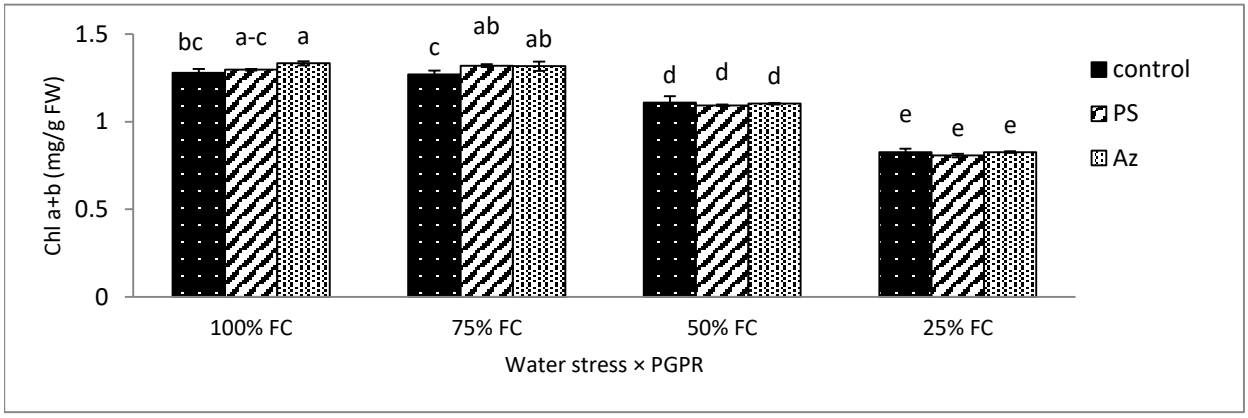

Fig. IV. The effect of PGPRs on chl a+b ratio of lemongrass leaves under irrigation regimes

Table 3
The effect of PGPR on root dry weight, CAT, SOD, and TFC of lemon grass

PGPR	CAT activity (µmol H2O2 decomposed/ min /mg protein)	SOD (unit/mg protein)	TFC (mg GA/ g DW)
no inoculation	0.49 ± 0.01[b]	24.08 ± 2.3[b]	8.18 ± 0.3[b]
pseudomonas	0.51 ± 0.02[a]	25.75 ± 1.8[a]	8.67 ± 0.5[a]
azospirillum	0.52 ± 0.03[a]	25.66 ± 1.8[a]	9.92 ± 0.5[a]

The activity of CAT and SOD

CAT and SOD activity was influenced by water stress and PGPR application (p≤0.05, Table 1). These enzymes' activities were increased by 77% and 71%, respectively, under severe stress compared to the well-watered condition (Table 2). PGPR application increased the activity of antioxidant enzymes (Table 3).

TPC and TFC

TPC was influenced by water stress, PGPR, and the interaction of water stress and PGPR application (p≤0.05, Table 1). The highest TPC was obtained in the interaction of 50% FC and PGPR application (Fig. VI). TFC was affected by water stress and PGPR application (p≤0.05, Table 1). Also, 50% FC induced the maximum TFC by 42% compared to 100% FC. *Pseudomonas* and *azotobacter* increased TFC by 6% and 18%, respectively in comparison with uninoculated plants (Table 3).

EO content and yield

EO content and yield were influenced by water stress, PGPR, and the interaction of water stress and PGPR application (p≤0.05, Table 1). EO content and yield were increased under 75% FC,

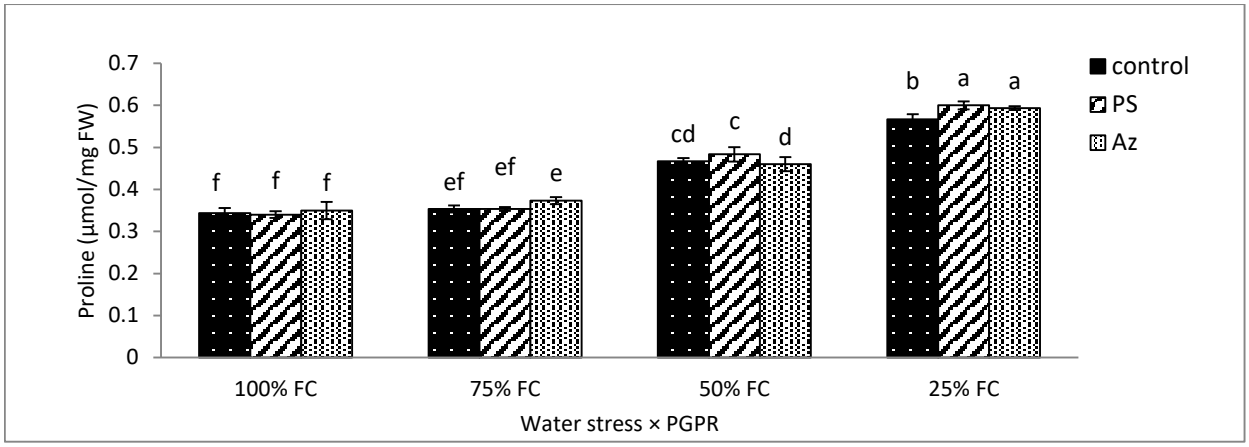

Fig. V. The effect of PGPRs on proline content of lemongrass leaves under irrigation regimes

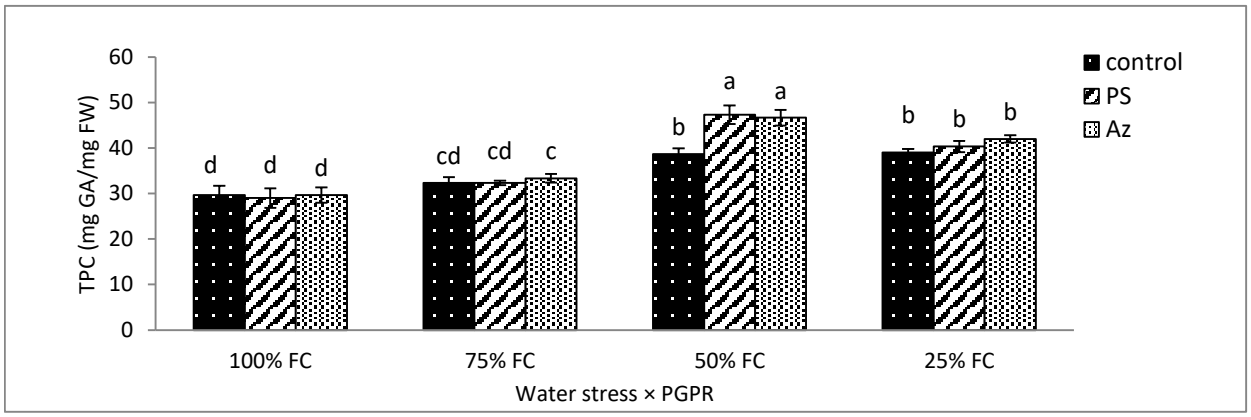

Fig. VI. The effect of PGPRs on proline content of lemongrass leaves under irrigation regimes

and decreased under 50% and 25% FC. EO percentage in 75% FC and PGPR application was higher than other treatments (Fig. VII). Under 75% FC, the 14% increase in EO yield was reported for both *pseudomonas* and *azotobacter* application (Fig. VIII).

Discussion

Plant growth significantly was reduced by water stress, but it was improved by PGPR application. Water stress can affect biochemical activities such as nitrate reductase, because of obstacle in nitrate uptake from the soil (Caravaca et al., 2005). Besides, it simplifies the biosynthesis of several substrates like ethylene, restricting plant growth (Ali et al., 2014). Water stress multidimensionality affects the subcellular organization level up to whole plant (Choluj et al., 2004, Rahdari et al., 2012). Thus it negatively changes plant height and biomass. However, PGPR improved the plant height and biomass of lemongrass particularly in moderate and severe stress conditions. Similarly, the improvement of plant height and biomass with PGPR application were reported on rapeseed **(**Lally et al., 2017), soybean (Xiang et al., 2017), and tomato (Ullah et al., 2016). The function of PGPR in plant growth and nutrient management is well documented. PGPRs colonize the rhizosphere/endo-rhizosphere of plants and induce plant growth via different direct and indirect strategies (Prasad et al., 2015). Hence, the use of PGPR is important in the management of water stress. The mechanisms of PGPR in promoting the plant drought tolerance are (1) the production of phyto hormones like cytokinins and abscisic acid, (2) 1-aminocyclopropane-1-carboxylic acid deaminase to decrease ethylene content in the roots, (3) to induce systemic tolerance through bacterial compounds, and (4) exopolysaccharides of bacteria (Timmusk and Nevo, 2011).

Chlorophyll content was decreased by waters stress especially under 25% FC. There was no significant difference of chlorophyll content between 100% FC and 75% FC. Therefore, it is

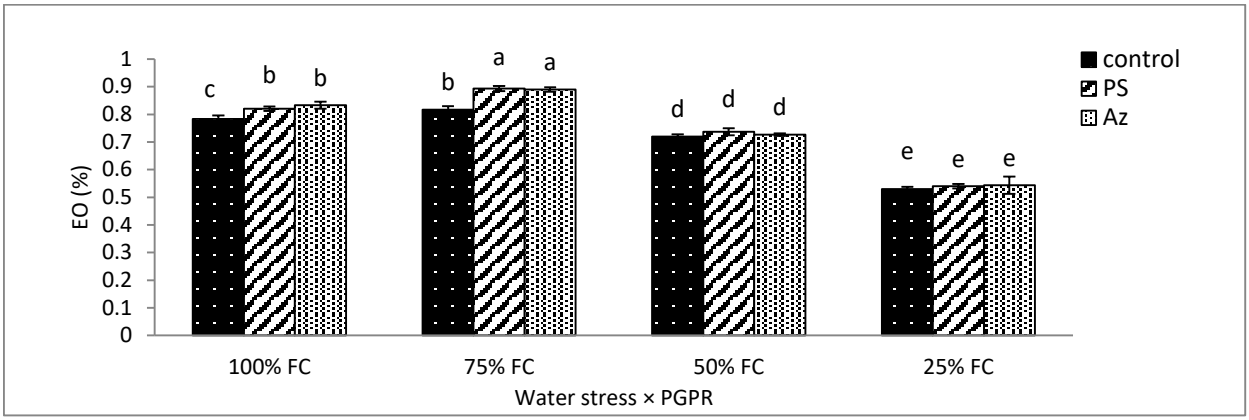

Fig. VII. The effect of PGPRs on EO content of lemongrass leaves under irrigation regimes

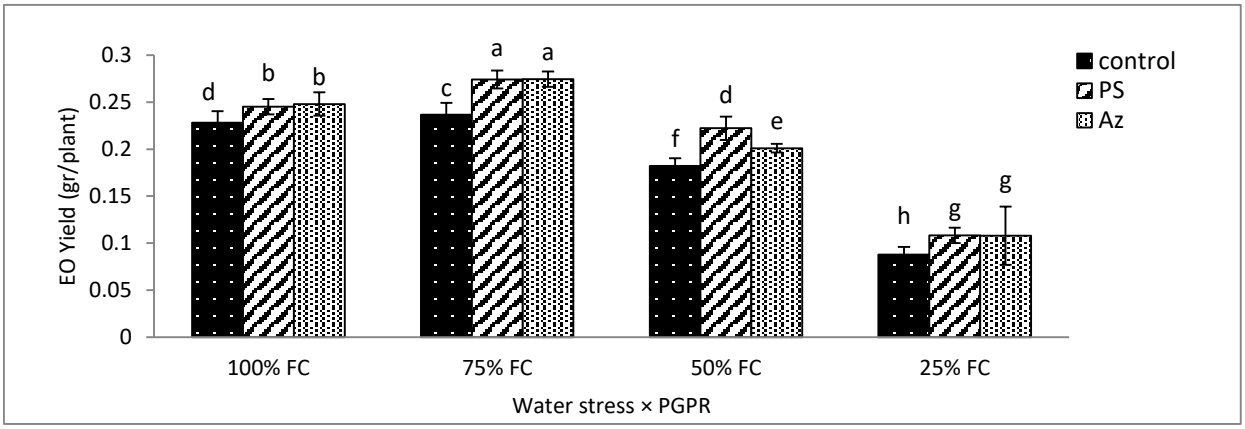

Fig. VIII. The effect of PGPRs on EO yield of lemongrass leaves under irrigation regimes

possible to manage the water content by reducing it to 75% FC with no change in chlorophyll content. The principal benefit of PGPR inoculation for lemongrass plants under water stress conditions was the maintenance of high photosynthetic rates in compression with uninoculated plants. PGPR inoculation improved photosynthetic activity by increasing the stomatal conductance and supporting PSII photochemical processes (Hajiboland et al., 2010, Porcel et al., 2015).

Proline content was increased by interaction of water stress and PGPR application. Water stress decreases water flow toward roots by decreasing both soil water potential and the soil-root gradient of water potential favoring root water uptake. To maintain root water uptake, plants increase their osmotic potential by synthesizing and accumulating compatible osmolytes such as proline (Flowers and Colmer, 2008). The PGPR accelerated the proline accumulation by increasing the osmotic adjustment. Plants have different reactions in proline accumulation in response to water stress and PGPRs (Porcel et al., 2015).

The activity of antioxidant enzymes was enhanced by water stress and PGPRs. Exposure of plants to water stress results in producing the reactive oxygen species (ROS) viz., superoxide anion radicals (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH), singlet oxygen (O_{12}), and alkoxy radicals (RO). ROS induce oxidative damage and diminish the normal functions of plant cells (Vurukonda et al., 2016). To overcome these effects, plants develop antioxidant defense systems to restrict ROS accumulation and to dwindle the oxidative damages appearing under water stress (Vurukonda et al., 2016). Plants have different responses to drought stress and PGPRs in terms of antioxidant defense systems. For instance, the lower activity of antioxidant enzymes in maize plants inoculated with five PGPRs (*pseudomonas* spp. strains namely *P. entomophila*, *P. stutzeri*, *P. putida*, *P. syringae*, and *P. montelli*) was reported under drought stress as compared to uninoculated plants (Sandhya et al.,

2010). However, similar to our results, the significant increase in CAT activity was found in basil plants (*Ocimum basilicum* L.) supplied with *pseudomonas* sp. under water stress. Similarly Heidari and Golpayegani (2011) showed that microbial consortia containing *pseudomonades* sp., *bacillus lentus* and *A. brasilense* induced highest activity of GPX and APX under water stress. These results provide evidence for beneficial and different effects of applying PGPRs in enhancing drought tolerance of plants by changing the antioxidants activity under water stress condition (Gusain et al., 2015).

Phenolic acids are an important group of secondary metabolites found in medicinal plants and they show strong antioxidant activities due to their carboxyl groups and hydroxyl. Hydroxycinnamic acids and hydroxybenzoic acids are two main categories of phenolic acids. Flavonoids via demolishment and detoxification of free radicals have strong impacts on cell biology (Michalak, 2006). The use of soil amendments to increase the biosynthesis of flavonoids and flavonoid compounds in plants has been effective and PGPRs play an important role in this regard. It has been reported that PGPRs induce the synthesis of specific phenolic acids at different growth stages (Kandoliya and Vakharia, 2013). Warwate et al. (2017) reported an increase of TPC and TFC under *pseudomonas and azotobacter.*

Exposing lemongrass plants to moderate water stress led to a significant increase in their EO content and yield. Water stress induced the accumulation of lemongrass EO because of the higher density of oil glands in a given leaf surface (Pirbalouti et al., 2014). Furthermore, EO production under water stress might be due to higher terpene production induced by stress condition. The reduction of soil moisture promoted the amount of EO because more metabolites are produced in plants under water stress to impede cell damage from oxidization stress (Lermen et al., 2015). Recently, the increase in EO content/yield under water stress has been reported in *Calendula officinalis* (Anderson et al., 2016), *Pelargonium graveolens* L. (Amiri et al., 2017), *Salvia officinalis* L. (Vosoughi et al., 2018), and *Thymus Vulgaris* L. (Arpanahi and Feizian, 2019). Under the condition of inoculation with microorganisms, EOs will be more synthesized due to their highly antimicrobial capacity (Sangwan et al., 2001). In analogy, Banchio et al. (2007) reported that herbivore feeding in *M. mollis* induced the synthesis of monoterpenes. The increased lemongrass growth parameters assessed in this study may be attributed to growth-promoting substances that act on plant metabolic processes. Each species of PGPR has particular hosts and can have different reaction based on the rhizosphere condition and plant species (Banchio et al., 2008). PGPRs can improve oil glands and biochemical pathways of EO production. The increase in EO percentage and yield has been found in marjoram (Banchio et al., 2008), sweet basil (Ordookhani et al., 2011), Hyptis suaveolens (Jha and Subramanian, 2016), and safflower (Nosheen et al., 2018).

Conclusions

The present study showed that PGPRs have positive impacts on plant height and biomass, antioxidant potential, and EO quality and quantity of lemongrass. Water stress changed the quality and quantity of lemongrass. In terms of saving water, PGPRs can be applied to reduce irrigation rate by 75% FC with no significant change in plant growth, and to achieve high EO concentration.

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