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Originalarbeit

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Impacts of PGPR, compost and biochar of Azolla on dry matter yield, nutrient uptake, physiological parameters and essential oil of *Rosmarinus officinalis* L.

Einfluss von PGPR, Compost und Biochar von Azolla-Algen auf Ertrag, Nährstoffaufnahme, physiologische Parameter und Gehalt an essentiellen Ölen von Rosmarin (*Rosmarinus officinalis* L.)

Abstract

Rosemary is one of the most important medicinal plants. In order to study the effect of plant growth promoting rhizobacteria (PGPR), Azolla compost and Azolla biochar on dry matter, nutrient uptake, physiological parameters and essential oil of rosemary, a greenhouse experiment was conducted in a completely randomized design with 6 replications. Treatments consisted of T_1 (control), T_2 (1% (1 g 100 g-1 dry soil) Azolla compost), T_3 (1% Azolla biochar), T_4 (PGPR (P. fluorescens)), T_5 (1% compost + PGPR) and T_6 (1% biochar + PGPR). Results indicated a significant enhancement of dry matter, nutrient uptake, photosynthetic pigments, carbohydrate, flavonoid and essential oil contents of rosemary influenced by organic fertilizers compared to control, particularly with co-application of PGPR + compost or biochar. Proline content decreased in all treatments in comparison with control. Results indicated positive impacts of PGPR, compost and boichar of Azolla on rosemary production by increasing nutrient uptake and protecting chlorophyll from degradation and enhancing its content in leaves.

Key words: Rosemary, Photosynthetic pigments, Carbohydrates, Flavonoides, Prolin, Essential oil

Zusammenfassung

In einem Gewächshausversuch wurde der Einfluss von Bio-Düngern wie PGPR, Compost und Biochar aus Azolla-Algen auf Ertrag, Nährstoffaufnahme und diverse Inhaltstoffe der Gewürzpflanze Rosmarin geprüft. Alle Behandlungen zeigten im Vergleich zu den Kontrollen signifikante Effekte auf Ertrag, Nährstoffaufnahme und Gehalte an Chlorophyll, Carotinoiden, Flavonoiden, Kohlenhydraten, Prolin und essentielle Ölen.

Stichwörter: Rosmarin, Photosynthetische Pigmente, Kohlenhydrate, Flavonoide, Prolin, Essentielle öle

Introduction

Rosemary (*Rosmarinus officinalis* L.) is one of the most important medicinal plants in the world. It is an aromatic plant, grown under a wide range of climates, endogenous to Europe, Asia and Africa, mainly in areas surrounding the Mediterranean Sea (Pintore et al., 2002). In the past few years, rosemary has been successfully cultivated in warm and dry climates of arid and semiarid regions (El-Rjoob et al., 2008). Rosemary and its extracts are the

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first marketed among natural antioxidants (Yanishlieva et al., 2006). Organically grown medicinal plants by using different organic fertilizers, produced best results in many investigations (Hussein et al., 2006; Gharib et al., 2008; Yousefzadeh et al., 2015). Salama et al. (2015) reported that application of organic and bio-organic fertilizers enhanced dry weight, yield, total phenolics, total flavonoids and vitamin C content of fennel (*Foeniculum vulgare* Mill.).

Plant growth-promoting rhizobacteria (PGPR) are a well-known group of naturally occurring microorganisms that have the potential to enhance soil quality and plant yield by the ability to increase the availability of nutrients in the rhizosphere (Choudhary et al., 2011). Some PGPR have the ability to solubilize phosphate, resulting in an increased availability of phosphate ions in soil, which can be easily taken up by plants (Wani et al., 2007). Enhancing P uptake by PGPR is due to hormone releasing, increasing root development and therefore nutrient uptake by plants and also releasing organic acids, H^+, chelating compounds and phosphatase enzymes that cause solubilization of inorganic and organic insoluble phosphates and changing them to available forms (Azcon et al., 1976). The other mechanisms of PGPR that can increase crop yields include regulating hormonal and nutritional balance, and inducing resistance against plant pathogens (Vejan et al., 2016). Among the Gram-negative soil bacteria, *Pseudomonas* is the most abundant genus in the rhizosphere soil and the PGPR activities of some of these strains have been known in different regions (Kumar et al., 2015).

The benefit effects of applying various organic composts are widely investigated (Brown and Cotton, 2011; Roghanian et al., 2012), and it has been found to increase soil organic matter content, soil biological activity and nutrient supply to plants (Bouajila and Sanaa, 2011; Agegnehu et al., 2014). Biochar is an organic material containing high levels of carbon, and is produced by heating biomass in the absence of oxygen (pyrolysis). It has an aromatic structure that makes it stable and highly resistant to chemical and biological degradation in soil (Atkinson et al., 2010). Biochar is increasingly used as a soil amendment with the aim to improve soil physical, chemical and biological properties, reduce greenhouse gas emissions, and sequester carbon as part of our response to climate change (Lehmann et al., 2011; Abujabhah et al., 2016). Biochar may stimulate plant growth (Vaccari et al., 2015), result in higher leaf number (Carter et al., 2013), plant height (Abdul and Abdul, 2017) and increase plant biomass (Anyanwu et al., 2018). However, the effectiveness of biochar for enhancing plant production depends not only on soil type, climate and type of crop (Blackwell et al., 2009; Obia et al., 2016) but also on the properties of the biochar (Van Zwieten et al., 2009; Cayuela et al., 2014). The structural and physicochemical properties of biochar, such as surface area, pore structures, surface functional groups and element composition, can also be influenced by changing the pyrolysis condition, such as pyrolysis temperature, heating rate and holding time (Shaaban et al., 2014; Guizani et al., 2017). Previous studies indicated that higher temperature resulted in a higher C content (Liang et al., 2016). In addition, increasing the temperature leads to an increase of the ash and fixed C contents, and decreases the content of volatile materials (Tag et al., 2016). The inherent variability of biochars due to different feedstock and production conditions implies a high variability of their effect on soil properties and productivity (Zhao et al., 2013).

Azolla is a floating fern and belongs to the family of Azollaceae. It forms a nitrogen-fixing symbiosis with the cyanobacterium *Anabaena azollae*, which is present in the leaf cavity of the fern (Yousefzadeh et al., 2015). Azolla is a rich source of protein and essential amino acids and contains several vitamins, such as vitamin A, vitamin B_{12} and beta carotene. It is also rich in minerals, such as calcium (Ca), nitrogen (N), phosphorous (P), potassium (K), iron (Fe), manganese (Mn), copper (Cu) and zinc (Zn). The protein composition of Azolla is 25–35% on dry weight basis (Parashuramulu et al., 2013). Azolla is widely used as a biofertilizer for rice in China, Vietnam, Indonesia, Thailand and other East and South Asian countries (Cheng et al., 2010), and has also been used as a source of green manure and compost (Arora and Singh, 2003).

There is a tendency to produce and reproduce medicinal plants in sustainable and low input farming systems. However, we assessed a lack of studies about the response of rosemary to organic fertilizers. Therefore, the presented research was aimed to investigate the effects of the organic fertilizers PGPR, Azolla compost and biochar as important components of sustainable agriculture on rosemary, especially on the dry matter of the plant's aerial parts, nutrient uptake, physiological parameters (chlorophyll a, chlorophyll b and carotenoid, proline, carbohydrate, flavonoid) and essential oil.

Materials and methods

Soil collection and analysis

The calcareous soil used in this study was collected from surface horizon (0–30 cm) of Koye Asatid series uncultivated lands (Loamy skeletal over fragmental, carbonatic, mesic, Fluventic Xerorthents) in Bajgah, Fars, Iran (1852 m above sea level, 29°50′ N, 52°46′ E), in fall 2017. This region has an average annual precipitation of 307 mm and a mean annual temperature of 17.3°C. The soil sample was air dried, passed through a 2 mm sieve, and mixed uniformly. The soil texture was determined by the method of Gee and Bauder (1986), soil pH was determined in a saturated paste (Thomas, 1996), organic matter (OM) content was determined according to Nelson and Sommers (1996), the electrical conductivity (EC) was determined in a saturated paste extract (Rhoades, 1996), calcium carbonate equivalent (CCE) was determined by titration (Nelson, 1982), total N content by the method of Bremner (1996), P was determined by sodium bicarbonate

Table 1. Selected soil physical and chemical properties

Soil texture	pH	EC (dS m^1)	OM %	N_{total} %	CCE %	P_{Olsen} (mg kg^{-1})	K (mg.kg^{-1})	Fe [a]	Zn [a]	Cu [a]	Mn [a]
								(mg kg^1)			
Silt loam	7.72	0.69	1.20	0.14	42.75	15.40	446.53	4.99	0.73	1.79	9.60

a: DTPA-extractable, EC: Electrical Conductivity, OM: Organic Matter and CCE: Calcium Carbonate Equivalent

extraction (Olsen, 1954), ammonium acetate-extractable K by the method of Page et al. (1982), and DTPA-extractable Fe, Zn, Cu, and Mn were determined according to Lindsay and Norvell (1978). These characteristics are shown in Table 1.

Bacterial inoculum preparation

The bacterium *Pseudomonas fluorescens*, isolate P100, was obtained from Soil Biology Lab, Department of Soil Science and Engineering, Tehran University. Pure bacterial culture was grown on nutrient broth (NB) medium in a shaker incubator, at 28 ºC for 24 hrs. The bacterial population was uniformized by using the McFarland method. The bacterial suspension had a population of 10^7 colony forming units (CFU) ml^{-1}.

Analysis of compost and biochar of Azolla

The dried Azolla biomass and Azolla compost used in this study were obtained from Department of Soil Science and Engineering, Rice Research institute of Iran in Guilan province. Two kilograms of Azolla biomass was washed three times with distilled water, and then dried in the oven at 55°C until the moisture content was around 50%. The dried Azolla biomass was placed in a plastic pot and 250 ml of molasses was added and mixed, then the pot was covered with black plastic. The composting process continued for 2 weeks, and once the temperature of the material increased substantially the material was shifted into another pot. The harvested Azolla compost was dried, passed through a 2 mm sieve, mixed thoroughly and was analyzed by standard methods.

To prepare Azolla biochar, the dried Azolla biomass was ground to pass a 2 mm sieve. Then the dried Azolla samples were pyrolysed under oxygen-limited conditions using a muffle furnace (Heraeus, K-1252) at 600°C. The pyrolysis temperature was raised to the selected value at a rate of approximately 15°C/min and held constant for 4 h, and then the biochar was allowed to cool to room temperature and ground to pass a 2 mm sieve (Yuan et al., 2011). Chemical analysis of compost and biochar of Azolla are presented in Table 2. The total N content was determined according to Bremner (1996). Total P was determined by Vanadate-Molybdate yellow method (Chapman and Pratt, 1961). Micro-nutrients such as Fe, Cu, Mn, and Zn were determined using the dry ash, dissolving in 2N HCl and then measuring with atomic absorption Shimadzu-AA670 and total K was determined by the flame photometer in the extraction of the dry ash method (Chapman and Pratt, 1961), pH and EC were measured at 1:10 (compost, biochar: water) weight ratios. Ash content of biochar and compost was measured by the standard ASTMD-2866 method on a weight basis. One gram of oven-dried biochar and compost was briefly heated at 600°C overnight, cooled and weighed again (Rajkovich et al., 2012).

Greenhouse experiment

The experiment was carried out at the research greenhouse of Soil Science Department of Shiraz University in a peroid of 6 months (January to June 2017). It was conducted with six treatments in a completely randomized design including: T_1 (control), T_2 (1% (1 g 100 g^{-1} dry soil) Azolla compost), T_3 (1% Azolla biochar), T_4 (PGPR inoculation), T_5 (1% Azolla compost + PGPR inoculation) and T_6 (1% Azolla biochar + PGPR inoculation). Six replications per treatment were made to give a total of 36 pots. The soil was passed through a 2 mm sieve and 5 kg of soil were transferred into the plastic pots. Essential

Table 2. Selected chemical properties of compost and biochar of Azolla

	Ash %	pH	EC (dS m^{-1})	N %	P (%)	K (%)	Fe	Zn	Cu	Mn
							(mg kg^{-1})			
Azolla compost	23.55	5.86	4.61	3.42	0.27	1.03	192.58	20.10	4.32	23.53
Azolla biochar	47.93	8.82	7.05	3.95	0.93	2.84	578.66	36.30	7.02	28.63

nutrient elements (except P) based on the results of soil analysis were added to all pots uniformly. Treatments of compost and biochar were added to soil of pots and mixed thoroughly.

Similar rosemary (*Rosmarinus officinalis* L.) seedlings were purchased from Gachsaran greenhouse in Kohgiluye and Boyer Ahmad province. One seedling of rosemary was planted in each pot. In bacterial treatments the root of each seedling was inoculated with 2 ml of NB containing 10^7 CFU ml^{-1} of *P. fluorescens*. The seedlings were irrigated with distilled water, maintaining the soil moisture content at field capacity. After 6 months, the plants were harvested.

Plant analysis

Dry matter and nutrient uptake. Plant aerial parts were harvested and weighed, then air-dried in a shaded place at a convenient temperature and in an air-flow during 15 days. Dry matters were recorded. Fresh and dry samples of plant were transferred to phytochemical analysis in the laboratory. To measure the rosemary nutrient concentration, air-dried samples of plants were dried again to a constant weight in an oven at 65 ºC, then ground into powder for chemical analysis. Total N concentration was measured by Micro-Kjeldahl method (Bremner, 1996), Total P concentration by vanadate-molybdate yellow method (Chapman and Pratt, 1961). Total Fe, Zn, Cu and Mn concentrations were determined using the dry ash method, The powdered dried plant samples dissolved in 2N hydrochloric acid (HCl), filtered and then measured with atomic absorption Shimadzu-AA670 and total K concentration was determined by flame photometer CORNING 405, Gallenkamp, London, Ukb (Chapman and Pratt, 1961). The nutrient uptake of aerial parts is calculated by multiplying plant dry weight by nutrient concentration.

Photosynthetic pigments. Samples of fresh leaves were taken for photosynthetic pigments. Chlorophyll a, chlorophyll b and carotenoid were determined spectrophotometrically by Arnon method (Arnon, 1949). Two hundred milligram of the fresh leaves were weighed and 80% acetone was added and centrifuged at 4800 rpm for 20 min. The above solution was used to measure chlorophyll and carotenoids. The UV-VIS spectrophotometer was adjusted at wavelengths of 645 and 663 nm for chlorophyll and at wavelengths of 470 nm for carotenoids.

Proline determination. Proline accumulation was determined by extracting 0.5 g of fresh leaves in 3% sulfosalicylic acid. The extract was heated in water bath for 10 min and then filtered through filter paper. Two milliliters of extract were mixed into 6 ml assay media containing 2 ml ninhydrin solution and 2 ml acetic acid. After that, all samples were incubated at 100°C for 30 min and cooled at room temperature. The colored product was extracted by adding 4 ml toluene. Finally, absorbance of the organic layer was measured at 520 nm. The concentration (µmol proline g fw ^{1}) was determined from a standard curve and calculated on a fresh weight basis (Bates et al., 1973).

Carbohydrate measurement. Carbohydrate measurement was performed according to Dubois et al. (1956). Ten milliliter of 95% ethanol were added to 0.2 g of fresh plant leaves and put in a hot water bath at 80°C for 60 min. One milliliter of extract was reacted with 1 ml of 0.5% phenol and 5 ml of 98% sulfuric acid. The absorption of each sample was measured at 483 nm with a UV-VIS spectrophotometer. The concentration was determined from a standard table and calculated on a fresh weight basis (mg Glucose g fw ^{1}).

Estimation of flavonoid. The aluminum chloride colorimetric method was used to estimate the flavonoids in rosemary. Ten milligrams of dry sample were extracted by using 80% ethanol then a definite volume of solution (0.5 ml) was mixed with 1.5 ml of 95% ethanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30 minutes, the absorbance of the reaction mixture was measured at 415 nm with a UV–VIS spectrophotometer. The amount of 10% aluminum chloride was substituted by the same amount of distilled water in blank. Quercetin was used to make the calibration curve, the reacted flavonoids of the extracts with aluminum chloride were used for the determination of flavonoid contents as mg QU equivalent g dw^{-1} in plant leaves (Chang et al., 2002).

Essential oil content. The shade-dried aerial parts of rosemary were subjected to water distillation (hydro distillation) for 3 h using an all glass Clevenger-type apparatus to extract essential oil according to the method outlined by the European Pharmacopoeia (Anonymous, 1996).The extracted oil was then dried over anhydrous sodium sulphate and stored in sealed vials at 4°C. The measurement of essential oil was presented as oil percentage (V/W) and was calculated on a dry weight basis.

Statistical analysis

All data were processed by Excel 2010 (Microsoft office 2010, Microsoft, USA). Statistical analyses were performed using the SPSS software version 19.0 for Windows. All statistical tests were considered significant at $P \leq 0.01$ *or* $P \leq 0.05$. Comparisons of the means were done by Duncan's Multiple Range Test ($p \leq 0.05$). Pearson's correlation coefficient was used to determine the relationship between different variables.

Results and discussion

Rosemary dry matter and nutrients uptake

The dry matter of rosemary's aerial part (Fig. 1) was significantly enhanced by organic treatments in comparison with control *($p \leq 0.05$)*.

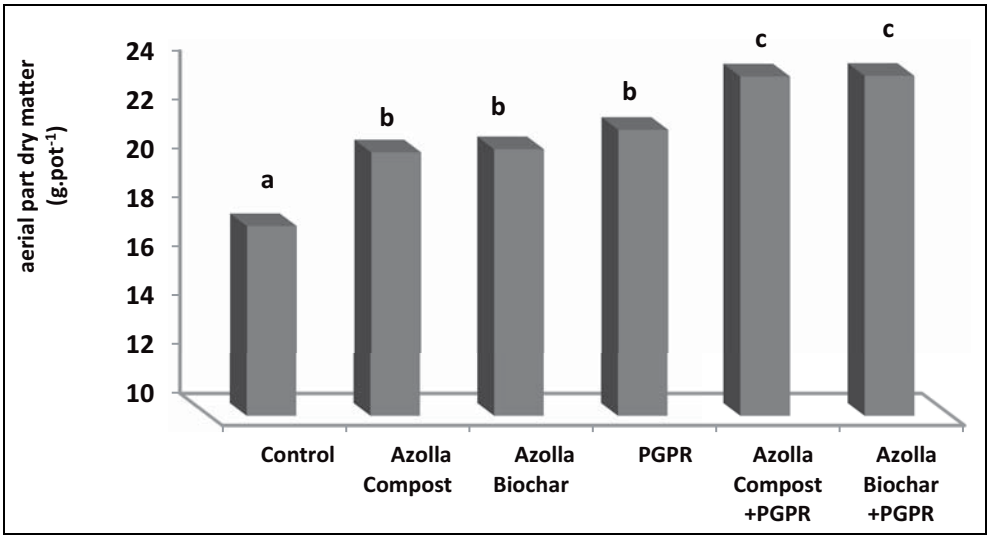

Fig. 1. Effect of treatments on aerial dry matter of Rosemary (Different letters above bars indicate significant difference at $P \leq 0.05$).

The dry matter of aerial parts was increased by 17% using Azolla compost, 18% by using Azolla biochar, 22% by using PGPR, 34.5% by using Azolla compost + PGPR and 34.7% by using Azolla biochar + PGPR compared to control treatment. Jumadi et al. (2014) reported that Azolla compost application increased the height and dry weight of upland kangkong. José et al. (2014) and Vaccari et al. (2015) reported that biochar amendment significantly increased the productivity compared to the unamended pots in alkaline soils. PGPR treatment increased the dry matter of aerial parts compared to control. The highest rosemary dry matter was observed with co-application of compost or biochar of Azolla + PGPR (T_5 and T_6). Nadeem et al. (2017) reported that the synergistic use of biochar, compost and PGPR caused significant increases in aerial part and root biomass of cucumber. The PGPR effects could be attributed to their unique metabolic properties, particularly the ability to produce growth regulators (Mangmang et al., 2015). The nutrient uptake of aerial part of rosemary (Table 3) showed that organic treatments increased nutrient uptake compared to control *($p \leq 0.05$)*. Khosravi et al. (2018) reported that organic fertilizers increased the availability and the uptake of some nutrients, such as N. Plant N, K, Fe, Zn, Cu and Mn uptake were higher in biochar treatment than in treatments with compost and PGPR (T_3> T_2> T_4> T_1).

Due to a higher ash content of Azolla biochar than Azolla compost, biochar contained high levels of both macro- and micro-nutrients (Table 2), which subsequently can be utilized by plants (Tan and Lagerkvist, 2011; Manolikaki et al., 2016). Wang et al. (2014) found that the biochar amended pots had significantly ($P \leq 0.05$) higher P uptake compared to control in a sandy soil. Patten and Glick (2002) reported that PGPR enhanced nutrients uptake by increasing root elongation and growth due to Indoleacetic acid (IAA) production and other PGPR activities. Rosemary P uptake were in the order of T_4> T_3> T_2> T_1. PGPR enhance the supply of P to plants because of their ability to solubilize organic and inorganic P (Richardson and Simpson, 2011). Results in treatments 5 and 6 illustrated the synergistic effects of co-application of compost or biochar of Azolla and *P. fluorescens* on plant nutrients uptake compared to their separate application. The highest nutrients uptake was observed in Azolla biochar + PGPR treatment. Fox et al. (2014) reported high abundance of aromatic sulfonate desulfurizing, phosphonate-mobilizing, phosphate ester-mineralizing, and tri-calcium phosphate-solubilizing rhi-

Table 3. Effect of PGPR, compost and biochar of Azolla on nutrient uptake of aerial parts (mg pot^{-1}) of rosemary (different letters in each column indicate significant difference at $P \leq 0.05$)

Treatments	N	P	K	Fe	Zn	Cu	Mn
T_1 (Control)	292.44 ^{a}	32.43 ^{a}	317.86 ^{a}	6.53 ^{a}	0.49 ^{a}	0.13 ^{a}	0.71 ^{a}
T_2 (Compost)	423.63 ^{bc}	48.75 ^{b}	476.15 ^{bc}	8.03 ^{b}	0.69 ^{b}	0.19 ^{bc}	0.94 ^{b}
T_3 (Biochar)	438.39 ^{bc}	53.32 ^{b}	476.70 ^{bc}	8.06 ^{b}	0.73 ^{bc}	0.21 ^{cd}	0.96 ^{bc}
T_4 (PGPR)	414.92 ^{b}	62.72 ^{c}	450.76 ^{b}	7.77 ^{b}	0.68 ^{b}	0.18 ^{b}	0.93 ^{b}
T_5 (Compost + PGPR)	458.58 ^{c}	65.39 ^{c}	499.00 ^{c}	8.88 ^{c}	0.76 ^{c}	0.22 ^{d}	0.98 ^{c}
T_6 (Biochar + PGPR)	532.39 ^{d}	84.52 ^{d}	603.21^{d}	9.30 ^{c}	0.92 ^{d}	0.27 ^{e}	1.13 ^{d}

Table 4. Pearson linear correlations among dry matter of aerial part, nutrient uptake, physiological parameters and essential oil of *Rosmarinus officinalis*.

	N uptake	P uptake	K uptake	Fe uptake	Zn uptake	Cu uptake	Mn uptake	Chlorophyll a	Chlorophyll b	Carotenoid	Proline	Carbohydrate	Flavonoid	Essential oil
dry matter	0.79 **	0.85 **	0.73 **	0.95 **	0.68 **	0.65 **	0.82 **	0.77 **	0.77 **	0.80 **	- 0.69 **	0.75**	0.71**	0.39 ns a
N uptake		0.92 **	0.99 **	0.88 **	0.97 **	0.96 **	0.98 **	0.90 **	0.83 **	0.86 **	- 0.84 **	0.92**	0.81**	0.85 **
P uptake			0.90 **	0.94 **	0.89 **	0.88 **	0.95 **	0.92 **	0.78 **	0.81 **	- 0.87 **	0.91**	0.80**	0.62 **
K uptake				0.84 **	0.98 **	0.98 **	0.97 **	0.88 **	0.80 **	0.83 **	- 0.83 **	0.90**	0.78**	0.87 **
Fe uptake					0.82 **	0.80 **	0.93 **	0.89 **	0.79 **	0.83 **	- 0.79 **	0.87**	0.80**	0.52 * b
Zn uptake						0.99 **	0.97 **	0.89 **	0.76 **	0.79 **	- 0.82 **	0.89**	0.78**	0.86 **
Cu uptake							0.96 **	0.87 **	0.75 **	0.77 **	- 0.81 **	0.89**	0.77**	0.88 **
Mn uptake								0.92 **	0.81 **	0.84 **	- 0.84 **	0.92**	0.82**	0.76 **
Chlorophyll a									0.87 **	0.90 **	- 0.78 **	0.94**	0.89**	0.70 **
Chlorophyll b										0.98 **	- 0.62 **	0.75**	0.86**	0.71 **
Carotenoid											- 0.68 **	0.80**	0.90**	0.71 **
Proline												- 0.83 **	- 0.65 **	- 0.63 **
Carbohydrate													0.79**	0.73 **
Flavonoid														0.66 **

[a] **: Correlation is significant at $P \leq 0.01$, [b] *: Correlation is significant at $P \leq 0.05$.

zospheric bacteria suggesting that biochar amendment enhances microbially mediated nutrient mobilization of S and P resulting in improved plant growth.

Nutrient uptake is also closely connected to plant growth (Leggett and Frere, 1971). Our results showed significant and positive correlations between the dry matter of aerial parts of rosemary and nutrient uptake (Table 4). Thus, co-application of compost or biochar of Azolla + PGPR (*P. fluorescens*) at early stage of rosemary development could affect rosemary growth and yield probably due to the production of phytohormones by bacteria and enhancement of mineral nutrient uptake.

Physiological parameters

Photosynthetic pigments. Our results show that organic treatments significantly increased photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoid) contents compared to control *($p \leq 0.05$)* (Fig. 2). The highest content of photosynthetic pigments was observed with co-application of compost or biochar of Azolla and PGPR. This might be due to efficient absorption and assimilation of N by plants which serves as a constituent of chlorophyll in plant tissue (Bojović and Marković, 2009).

Significant and positive correlations were observed among chlorophyll a, chlorophyll b, carotenoid and nutrient uptake (Table 4). Compost and biochar of Azolla contained high concentration of N (Table 2), which can be utilized by plants. Gairola et al. (2009) reported that nitrogen is a constituent of chlorophyll, protein, amino acids and photosynthetic activity and chlorophyll is strongly related to nitrogen concentration in the soil. These results are consistent with those reported by Ondieki et al. (2011) who found an increase in chlorophyll 'a' and 'b' concentration after increasing the levels of fortified compost manure on African nightshade species. Vivas et al. (2003) showed that inoculation of PGPR (*Bacillus* sp.) increased stomatal conductance and chlorophyll content of lettuce. Soval-Villa et al. (2002) and Uddling et al. (2007) stated that leaf chlorophyll content is affected by several factors, such as: nutrient concentration, distribution of chlorophyll in leaves, and plant genotype.

Carbohydrate content. Organic treatments significantly increased leaf carbohydrate content (Fig. 3) compared to control ($p \leq 0.05$). Leaf carbohydrate was increased by

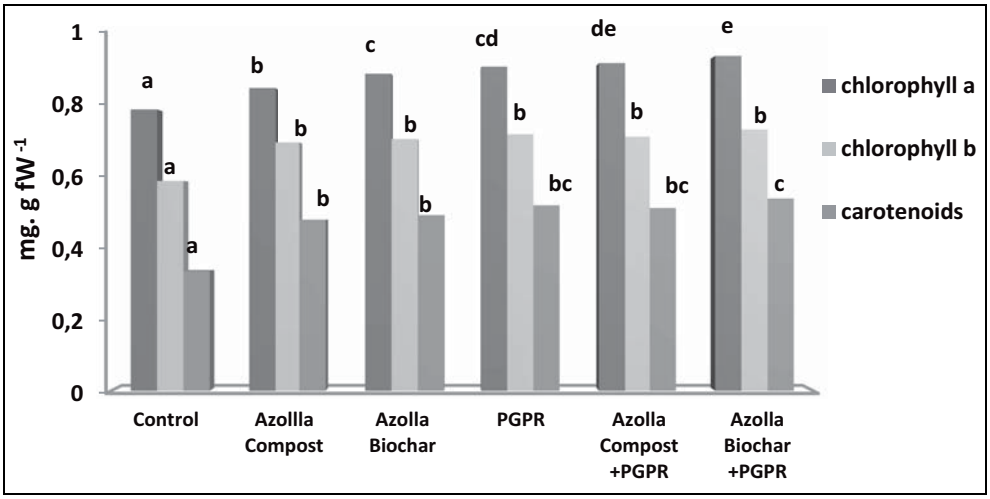

Fig. 2. Effect of treatments on photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids) content of *Rosmarinus officinalis* L. (Different letters above bars indicate significant difference at $P \leq 0.05$).

15% by Azolla compost, 19% by Azolla biochar, 23.3% by PGPR, 33.4% by Azolla compost + PGPR and 35.7% by Azolla biochar + PGPR compared to control. These effects of organic treatments may depend on the appropriate supply of nutrients to growing plants. Our results show significant and positive correlations among carbohydrate content, nutrient uptake and photosynthetic pigments (Table 4). Abdel-Sabour and El-Seoud (1996) showed that the increase in pigment content influenced by compost application was positively correlated with the increase in total carbohydrate content. They stated that the enhancement in chlorophyll and carbohydrate contents can be attributed to an acceleration of metabolic rates related to the synthesis of such constituents. An enhancement of maximum proximate chemical constituents including carbohydrate, proteins and dry matter were recorded by the application of PGPR (Pandey et al., 2018).

Proline content. Application of organic fertilizers significantly decreased proline content in comparison with control ($p \leq 0.05$) (Fig. 3). The lowest proline content was observed in treatments with co-application of compost or biochar of Azolla and PGPR. Proline accumulates in plant cells in response to various stresses (Hayat et al., 2012). When organic fertilizers are applied to soils, the following enhancements can be obtained: increasing the availability of water, proper porosity and availability of nutrients to plants. These positive effects may be decrease stress conditions (HongBo et al., 2005). Our results show significant but negative correlations among proline content, dry matter, nutrient uptake, photosynthetic pigments, carbohydrate, flavonoid and rosemary essential oil (Table 4).

Flavonoid content. Our results (Fig. 3) show that organic treatments significantly enhanced flavonoid content of rosemary in comparison with control *($p \leq 0.05$)*. Flavonoid was increased by 7.2% by using Azolla compost, 8.9% by using Azolla biochar, 11.9% by using PGPR, 12.2% by using Azolla compost + PGPR and 13.9% by using Azolla biochar + PGPR compared to control. Significant and positive correlations were observed among flavonoid content, nutrient uptake, photosynthetic pigments and carbohydrate (Table 4). Chlorophyll and flavonoids are practical indicators of both potential photosynthetic productivity and plant vigor, which are related to the N concentration and serve as a measure of the response of plants to soil nutrient status (Martínez and Guiamet, 2004; Bozzolo et al., 2017). Salama et al. (2015) reported that application of organic and bio-organic fertilizers enhanced total flavonoid content of fennel (*Foeniculum vulgare* Mill.).

Rosemary essential oil content

The results show that all organic fertilizers increased the percentage of essential oil of rosemary compared to control (Fig. 4). The highest content of plant essential oil was observed in treatments with co-application of compost or biochar of Azolla and PGPR. Our results show significant and positive correlations among essential oil content, nutrient uptake, photosynthetic pigments, flavonoid and carbohydrate of *R. officinalis* (Table 4).

The increase in essential oil might be due to either increase in vegetative growth or changes in leaf oil gland population and monoterpene biosynthesis (Gharib et al., 2008). The production of essential oil is dependent on the physiology of the whole plant, particularly the development state of synthesizing tissue and metabolic processes (Tawfeeq et al., 2016). The effects of organic fertilizers may be due to the fact that organic fertilizers contain many different proteins, pigments, minerals and plant growth hormones which are not found in inorganic fertilizers. These contents positively affect cellular metabolism and beneficial conditions of the plants following root elongation and root formation. This has the consequence of improving bud and cell division to give larger vegetative growth and increasing the number of glands. Furthermore, Chojnacka et al. (2012) reported that hormones are largely responsible for plant growth stimulation in terms of increased effectiveness of photosynthesis,

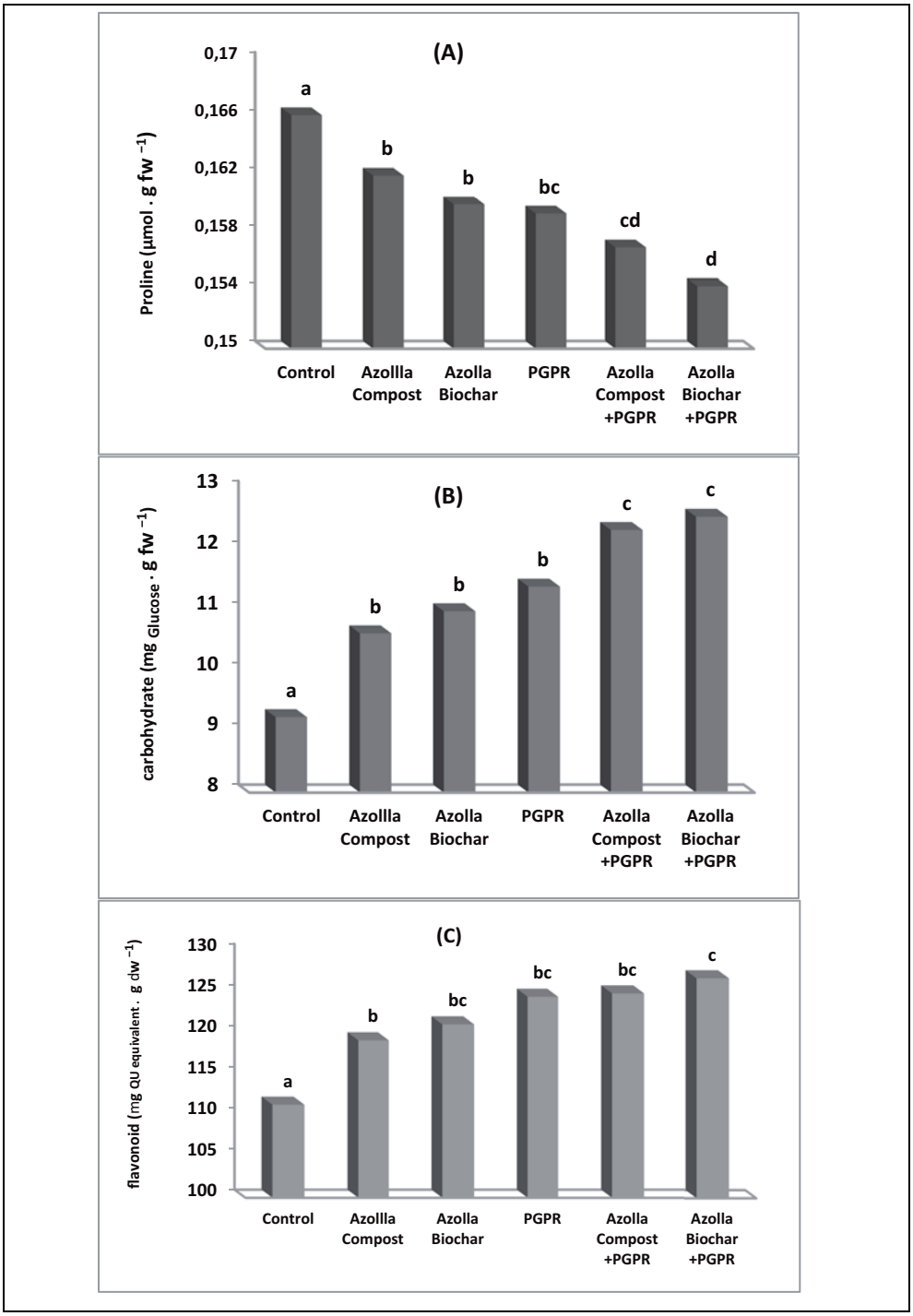

Fig. 3. Effect of treatments on (A): proline, (B): carbohydrate and (C): flavonoid content of *Rosmarinus officinalis* L. (Different letters above bars indicate significant difference at $P \leq 0.05$).

with protecting of chlorophyll from degradation and enhancing its content in leaves. In addition, leaves that contain less chlorophyll and less developed chloroplasts show effective stomatal closure, which restricts gas exchange in the photosynthetic process. Tawfeeq et al. (2016) stated that the volume of essential oil obtained from rosemary plant differs, depending on the type of fertilizer and the method of application. They also reported that seaweed fertilizer treatments showed significantly higher yields of essential oil than the watered inorganic treatment.

Conclusion

Results of the present study indicate a significant enhancement of dry matter yield, nutrient uptake, photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoid), carbohydrate, flavonoid and essential oil content of rosemary induced by Azolla compost, Azolla biochar and PGPR (*P. fluorescens*), particularly with co-application of *P. fluorescens* + compost or biochar of Azolla. However, application of organic fertilizers significantly decreased proline content in all treatments compared to control.

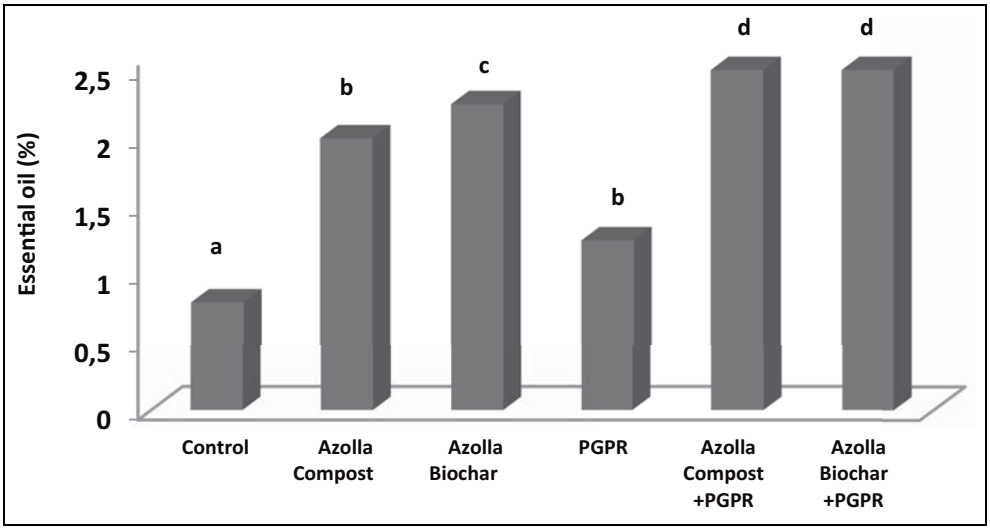

Fig. 4. Effect of treatments on the essential oil content of *Rosmarinus officinalis* L. (Different letters above bars indicate significant difference at $P \leq 0.05$).

These results may be due to the positive impact of organic fertilizers on biomass production via increasing root growth, enhancement of nutrient uptake and a more effective photosynthesis through the protection of chlorophyll from degradation and enhancement of its content in leaves.

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