

Influence of Organics and Bio Fertilizers on Biochemical Properties of *Calendula officinalis* L.

Shiva Zaferanchi^{1*}, Saeid Zehtab Salmasi¹, Seyed Yahya Salehi Lisar² and Mohammad Reza Sarikhani³

1. Department of Plant Ecophysiology, Faculty of Agriculture, University of Tabriz, Iran.

2. Department of Plant Physiology, Faculty of Natural science, University of Tabriz, Iran.

3. Department of Soil Science, Faculty of Agriculture, University of Tabriz, Iran.

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Abstract

The present study was carried out to investigate biochemical responses of marigold (*Calendula officinalis* L.) to Bio-organic fertilizers. Effects of co-inoculation of two plant growth-promoting rhizobacteria (PGPR) (*Azotobacter* sp.145PI and *Azospirillum* sp.AC49I), humic acid (HA) (10 kg ha⁻¹), vermicompost (VC) (7 T ha⁻¹) and combinations of these treatments were evaluated in two marigold cultivars [Isfahan double flower (DF) and single flower (SF)]. The biosynthesis of leaf protein, soluble and insoluble carbohydrate contents were increased in treated plants, while total free amino acid content was not influenced by treatments. Fertilizers also had positive impact on leaf and flower pigments, total flavonoid content, and total phenolic contents. Maximum amounts of anthocyanins were obtained in the DF cultivar treated by VC+PGPR and VC, which were 11.414 and 11.192 μmol g⁻¹ FW, respectively. The SF cultivar treated by PGPR (36.11 %) and also the same cultivar treated by VC (33.39 %) had the highest antioxidant activities. In general, simultaneous application of fertilizers and also vermicompost were recognized as the best treatment for marigold plants. In conclusion, the findings of the current study confirmed that chemical composition and antioxidant activity of marigold can be positively influenced by Bio-organic fertilizers; therefore they can be used to obtain medicinal plants with improved quality during sustainable agricultural practices.

Keywords: Antioxidant activity, Biofertilizer, Marigold, Carbohydrates, Humic acid, Protein, Vermicompost.

Abbreviations: VC, Vermicomposts; HA, Humic acids, PGPR, Plant growth-promoting rhizobacteria; DF, Isfahan double flower; SF, single flower.

Introduction

Calendula officianilis L. commonly known as calendula or marigold is cultured in both field and greenhouse for ornamental and medicinal purposes for centuries. Marigold typically grows 20-50 cm tall, and its yellow and orange flowers is 4-7 cm in

diameter. It is a member of Asteraceae family and is native to North Africa and Southern Europe (Aliniaiefard et al., 2018). It grows in most parts of Iran, especially west part of the country. Marigold's main commercial value lies in its flowers that are used in ornamental, pharmaceutical and cosmetic industries. Its pharmacological importance is due to its antioxidant, anti-

* Corresponding Author, Email: shzafaranchi@yahoo.com

inflammatory, antibacterial, antifungal and antiviral activities (Baskaran, 2017). Marigold can grow in moderate to relatively poor soils (Naderi and Fallahzade, 2017), which makes it more suitable for studies in organic systems. Since agronomic and economic sustainability is threatened by the lack of agricultural diversity, there is an increasingly focus on organic and low-input systems for production of medicinal plants.

The use of organic substances such as vermicomposting can be considered as the key component of sustainable agriculture. Vermicomposts (VC) are effective organic sources and biocontrol agents that have ability to enhance crop quality and safety (Simsek-Ersahin, 2011). Moreno-Reséndez et al. (2010) reported that vermicomposts are able to supply adequate nutrients to meet the needs of muskmelon (*Cucumis melo* L.) without using of chemical fertilizers. They concluded that vermicompost has potential to support the development of the vegetable species when they are used as part of the potting media.

To apply eco-friendly plant nutritional materials according to the requirements of environmental sustainability, humic acids (HA) appear to be attractive substances for crop production, which can be derived from vermicompost or other natural sources and may have various effects on plant growth. Vermicompost have direct and indirect effects on plant growth and chemical composition: Indirect effects include improvements of soil properties, enhancement of water holding capacity and micronutrient availability for the plants (Tan, 2003). Direct effects are related to the improvements in biochemical and physiological properties of the plants as a result of the uptake of humic substances by crops (Chen and Aviad, 1990). Khan et al. (2013) found that humic acid application in soil or its foliar applications resulted in increased growth, yield, nutrients, chlorophyll, carotenoid and total sugar concentrations of pea (*pisum sativum* L.) plants.

Gutiérrez-Miceli et al. (2017) indicated

that vermicompost leachate contains micronutrients, humic and fulvic acids that promote growth of sugarcane (*Saccharum officinarum*).

Some strains of rhizosphere bacteria that act through different mechanisms and have positive role in plant production are generally called plant growth-promoting rhizobacteria (PGPR), which assist in mineralization and make nutrients available to accelerate plant productivity (Ansari et al., 2017). He et al. (2017) reported that PGPR induced a progressive increase in protein concentration and maintained relatively higher chlorophyll a and b, and carotenoid contents in cotton (*Gossypium* sp.). Ipek et al. (2014) also reported increase in leaf area, root growth, improved mineral uptake and hormonal metabolism by PGPR applications.

The basic processes of producing primary metabolites are also involved in the synthesis of secondary metabolites through more complex mechanisms than those of primary metabolites. The amounts of secondary metabolites in plants are quite variable and can be influenced by different environmental and genetic factors.

Nowadays, more attention is being paid to phenolic acids and flavonoids because of their protective roles, which may be attributed to their antioxidant activity against reactive oxygen species. Organic fertilizers have a role in promoting the acetate shikimate pathway, which results in higher production of flavonoids and phenolics (Sousa et al., 2008). Therefore, in the present study, we hypothesized that organic fertilizers can influence the levels of primary and secondary metabolites production in marigold cultivars.

Materials and methods

Experimental conditions

The present study was conducted in 2015 and 2016 on a sandy loam soil at the field research station of the Faculty of Agriculture, University of Tabriz (38° 05'N, 46° 17'E, and 1360 m above sea level), Tabriz, Iran.

The climate is characterized by mean annual precipitation of 245.75 mm per year and mean annual temperature of 10°C. The experimental design was a factorial randomized complete block with three replications. Bio-organic fertilizers and marigold cultivars were two factors of this study. Fertilizer treatments included: plant growth promoting rhizobacteria [(*Azotobacter* sp.145PI and *Azospirillum* sp.AC49I, (PGPR)] humic acid [Mobicel-H (HA)] (10 kg ha⁻¹), vermicompost [Tak Vermicompost Azerbaijan (VC)] (7 t ha⁻¹), HA + PGPR, VC + PGPR, and control. The two marigold cultivars used in this research were Isfahan double flower (DF) and single flower (SF). The vermicompost was applied uniformly into the top 10 cm of the rows by hand one week before planting and incorporated into the soil. Bacteria were obtained from the laboratory of soil biology, Faculty of Agriculture, University of Tabriz. Perlite and bagasse were mixed in 1:1 ratio and added 20% initial moisture and sterilized in the autoclave. Bacteria consist of

Azotobacter sp.145PI and *Azospirillum* sp.AC49I grown separately on Nutrient Broth (NB) media were added to the solid carrier and mixed to reach the final moisture content of 50%. Population of living bacteria was about 10⁹ CFU g⁻¹. Prepared bacteria with carrier were applied at a rate of 400 g per hectare, and finally were inoculated with the seeds before planting. The HA was applied with the first irrigation immediately after planting. HA powder was mixed with the irrigation water at the rate of 10 kg ha⁻¹.

Individual plot size was 3 m × 2 m and consisted of six rows. Seeds were obtained from Pakan Bazr Company (Isfahan, Iran) and planted by hand on 18 May 2015 and 8 May 2016. Weeds were also controlled manually during the growth season. Biochemical properties analyses were performed on one sample of the last harvest, and for all 72 treatments in two years. The pH and EC of irrigation water were 7.63 and 0.00571 dS.m⁻¹ respectively. Some properties of the soil and the vermicompost are provided in Tables 1 and 2.

Table 1. Some physicochemical properties of the field soil

Sand %	Silt %	Clay %	K mg kg ⁻¹	P mg kg ⁻¹	N %	OC %	EC dS.m ⁻¹	PH	Profile (cm)
74	14	12	257	4.8	0.04	8.4	1.08	7.7	0-30

Table 2. Chemical analysis of vermicompost

pH	EC dS.m ⁻¹	K ₂ O%	P ₂ O ₅ %	N%	OC%	C/N
8.01	2.68	2.06	2.06	1.27	20.38	16.04

Measurements of chlorophyll, carotenoid and anthocyanin contents

The chlorophyll and total carotenoid contents were determined by using 80% acetone extracts of the fresh plant material, according to the spectrophotometric methods of Porra (2002) and Lichtenthaler and Welburn (1983). Homogenates were centrifuged for 15 min (D.T.A.P, Instrument T16) (3000 rpm). Absorbance of the extracts were determined at 645, 662 and 470 nm (Analytic Jena, specol 200), respectively and pigment contents were evaluated and expressed in mg g⁻¹ fresh

weight (FW) of leaves and flowers.

The anthocyanin content was measured as described by Mita et al. (1997) with a slight modification. Plant materials (100 mg) were homogenized with 4 ml methanol containing 1% hydrochloric acid. After storage at 4°C for one night, each sample was centrifuged (Eppendorf MiniSpin plus) (13,000 rpm) for 10 min at room temperature. The absorbance of the supernatant was measured at wavelengths of 530 and 657 nm using spectrophotometer (Analytic Jena, specol 200).

Measurements of soluble and insoluble carbohydrates, soluble protein and free amino acid contents

Total soluble carbohydrate (SCH) and insoluble carbohydrate (ICH) contents in dried leaves was determined by the method of Kochert (1978) and absorbance was recorded at 485 nm. Glucose was used for standards and carbohydrate levels expressed as mg glucose g⁻¹ dry weight (DW). Total soluble protein (TSP) content of leaves was determined by the method of Bradford (1976). This assay is referring to the binding of Coomassie Brilliant Blue G-250 at aromatic amino acid radicals and measuring the colour at 595 nm. Calibration curves were made with bovine serum albumin (BSA) and concentrations were expressed as mg BSA g⁻¹ FW. The contents of total free amino acids (AAs) were assayed using ninhydrin Colorimetric method and glycine as a standard according to Hwang and Ederer (1975). Absorbance recorded at 570 nm and values are expressed as mmol glycine g⁻¹ FW.

Measurements of Total phenolics and total flavonoids

Total phenolic contents (TPC) were measured by Folin–Ciocalteu method as described by Meda et al. (2005) with some modifications. Plant extracts (100 µl) were mixed with 2.8 ml distilled water, 100 µl of Folin–Ciocalteu reagent (50%), and 2.0 ml of sodium carbonate (2%) were added. Samples were kept at room temperature for 30 min. The absorbance of samples was measured at 720 nm against the blank with the spectrophotometer. The results were calculated on the basis of the calibration curve of gallic acid (GA) and expressed as gallic acid equivalents (mg g⁻¹ FW).

Total flavonoid contents (TFC) were determined by the aluminum chloride colorimetric method as described by Chang et al. (2002) with some modifications. Briefly, 500 µl of each sample mixed with 2.8 ml of distilled water and 100 µl of potassium acetate (1M), 100 µl of

aluminum chloride solution (10%) and 1.5 ml methanol. After 40 min, the absorbance was measured against the blank at 415 nm. A calibration curve was constructed by preparing quercetin solutions and total flavonoid values were expressed as quercetin equivalents (mg g⁻¹ FW).

Measurement of DPPH radical-scavenging activity

The method described by Miliauskas et al. (2004) was used to assess the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of methanolic extract. The DPPH solution was freshly prepared by mixing 0.004 gr DPPH with 100 ml methanol. About 2.0 mL of DPPH solution was added to 2.0 mL of extracts. The absorbance of the mixture was recorded at 517 nm after reacting for 30 min in the dark. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity.

Statistical analysis

Analysis of variance (ANOVA) and mean separations were performed using the general linear model (GLM) procedure of SAS 9.1 program. Duncan Multiple Range Test at a probability level of 0.05 was used to determine statistically significant differences among treatment means.

Results

Soluble protein, free amino acid, soluble and insoluble carbohydrate contents

Summary of ANOVA for the studied parameters is indicated in Tables 3 and 4. The changes in TSP of marigold leaves were indicative of the positive effects of treatments used in this study (Table 5). VC+PGPR treatment caused the maximum amount of TSP (36.5 mg g⁻¹ FW), followed by HA+PGPR, VC and HA (34.97, 33.49, 31.82 mg g⁻¹ FW, respectively) Treatments. PGPR caused an increase in TSP compared to the control, although the recorded difference was not significant (P>0.05). Among the two cultivars, DF had higher TSP than the SF (Table 5).

Table 3. A summary of ANOVA of the effects of different fertilizers and cultivars on some parameters of marigold (Mean squares)

Source of variation	df	TSP	AAs	SCH	ICH	TPC	TFC	Anthocyanin	Antioxidant
Year (Y)	1	336.077**	0.407 ^{ns}	1834.252**	473.306**	257.609 ^{ns}	112.319*	41.644*	18.673 ^{ns}
Fertilizers (A)	5	145.463**	0.519 ^{ns}	480.027**	252.373**	1721.978**	116.333**	24.815**	44.835**
Cultivars (B)	1	200.000**	0.063 ^{ns}	287.001**	170.670**	858.163 ^{ns}	72.494 ^{ns}	1.057 ^{ns}	51.869**
Replicates	2	46.155 ^{ns}	0.533 ^{ns}	52.474 ^{ns}	71.989*	323.444 ^{ns}	25.630 ^{ns}	1.473 ^{ns}	7.049 ^{ns}
Y * A	5	5.274 ^{ns}	0.069 ^{ns}	21.528 ^{ns}	6.763 ^{ns}	180.814 ^{ns}	11.877 ^{ns}	8.207 ^{ns}	12.505 ^{ns}
A * B	5	5.782 ^{ns}	0.262 ^{ns}	8.307 ^{ns}	8.939 ^{ns}	207.521 ^{ns}	24.483 ^{ns}	11.351*	34.681**
Y * B	1	9.071 ^{ns}	0.004 ^{ns}	1.903 ^{ns}	3.101 ^{ns}	178.395 ^{ns}	0.327 ^{ns}	0.051 ^{ns}	0.688 ^{ns}
Y * A * B	5	3.556 ^{ns}	0.353 ^{ns}	5.596 ^{ns}	0.882 ^{ns}	85.818 ^{ns}	6.415 ^{ns}	0.457 ^{ns}	8.786 ^{ns}
Error	46	19.638	0.229	31.916	18.547	441.384	19.608	4.518	6.530

Total soluble protein (TSP), total free amino acids (AAs), total soluble carbohydrate (SCH), insoluble carbohydrate (ICH), total phenolic contents (TPC), and total flavonoid contents (TFC). * ** Significant at $p \leq 0.05$ and $p \leq 0.01$ levels respectively; ns: Not significant.

Table 4. A summary of ANOVA of the effects of different fertilizers and cultivars on some parameters of marigold (Mean squares)

Source of variation	df	Chlorophyll a	Chlorophyll b	Chlorophyll a + b	Leaf carotenoid	Flower carotenoid
Year (Y)	1	0.081 ^{ns}	0.251**	0.674**	0.005 ^{ns}	0.160**
Fertilizers (A)	5	0.112**	0.550**	1.090**	0.035**	0.089**
Cultivars (B)	1	0.307**	0.005 ^{ns}	0.389**	0.043**	0.177**
Replicates	2	0.102*	0.009 ^{ns}	0.137*	0.006 ^{ns}	0.074*
Y * A	5	0.034 ^{ns}	0.019 ^{ns}	0.016 ^{ns}	0.009 ^{ns}	0.026 ^{ns}
A * B	5	0.001 ^{ns}	0.005 ^{ns}	0.007 ^{ns}	0.005 ^{ns}	0.031 ^{ns}
Y * B	1	0.000073 ^{ns}	0.0000083 ^{ns}	0.000028 ^{ns}	0.004 ^{ns}	0.001 ^{ns}
Y * A * B	5	0.003 ^{ns}	0.000 ^{ns}	0.001 ^{ns}	0.002 ^{ns}	0.025 ^{ns}
Error	46	0.028	0.016	0.039	0.005	0.015

* ** Significant at $p \leq 0.05$ and $p \leq 0.01$ levels respectively; ns: Not significant

Table 5. Total soluble protein (TSP), total free amino acids (AAs), soluble carbohydrate (SCH), insoluble carbohydrate (ICH) contents of marigold cultivars as affected by organic and bio fertilizers

Treatments	TSP (mg g ⁻¹ FW)	AAs (mmol g ⁻¹ FW)	SCH (mg g ⁻¹ DW)	ICH (mg g ⁻¹ DW)
Control	26.91 ± 2.99 ^d	1.04 ± 0.29	49.00 ± 4.87 ^d	31.30 ± 3.99 ^c
PGPR	30.01 ± 3.54 ^{cd}	1.22 ± 0.33	53.93 ± 5.64 ^c	35.66 ± 3.24 ^b
HA	31.82 ± 4.18 ^{bc}	1.27 ± 0.18	54.58 ± 5.25 ^c	37.49 ± 3.56 ^b
VC	33.49 ± 3.58 ^{abc}	1.39 ± 0.37	60.58 ± 2.28 ^b	41.69 ± 3.30 ^a
VC+PGPR	36.50 ± 2.65 ^a	1.52 ± 0.48	67.02 ± 3.03 ^a	43.49 ± 3.08 ^a
HA+PGPR	34.97 ± 2.25 ^b	1.61 ± 0.29	60.02 ± 4.43 ^b	41.52 ± 2.77 ^a
DF	33.95 ± 3.35 ^a	1.37 ± 0.32	59.51 ± 6.63 ^a	40.06 ± 4.80 ^a
SF	30.62 ± 0.41 ^b	1.31 ± 0.41	55.52 ± 7.25 ^b	36.99 ± 5.37 ^b

Within columns values followed by the same letter are not significantly different at the $P \leq 0.05$ level. Plant growth promoting rhizobacteria (PGPR), humic acid (HA), vermicompost (VC), double flower (DF) and single flower (SF).

Although there were slight increases in HA+PGPR and VC+PGPR compared to the other treatments, bio-organic treatments of marigold plants were not caused any significant ($P > 0.05$) differences regarding AAs of leaves compared with non-treated

plants. Furthermore, there was not a significant ($P > 0.05$) difference among cultivars (Table 5).

SCH (67.02 mg g⁻¹ DW) was in highest amount when the plants were treated with VC+PGPR while its lowest value (48.99

mg g⁻¹ DW) was obtained in control plants. SCH caused significant ($P < 0.01$) differences among cultivars and was higher for the DF (59.51 mg g⁻¹ DW) than the SF cultivar (55.52 mg g⁻¹ DW) (Table 5). The same trend was found for ICH of marigold leaves between the cultivars. There was a variation in plant responses to the applied fertilizers (Table 5). VC+PGPR, VC and HA+PGPR treatments (43.49, 41.69 and 41.51 mg g⁻¹ DW, respectively) had the maximum values, while the lowest ICH was obtained in control plants.

Chlorophyll and carotenoid contents

Based on the results of analysis of variance, the main effect of fertilizers and cultivars on chlorophyll and carotenoid contents was significant except for chlorophyll b content, which was not different between the cultivars (Table 6). Chlorophyll a content was significantly ($P < 0.01$) increased due to application of HA+PGPR, VC+PGPR and PGPR compared to the control, while Chlorophyll b content was increased by all of the fertilizers except for HA. HA+PGPR and VC caused increase in Chlorophyll b content by 67.58% and 53.98% respectively.

HA+PGPR treatment had the highest total chlorophyll content, which showed 22.87% increase in comparison with its

content in control plants, while, in PGPR treatment there was only 10.09% increase in total chlorophyll content. HA was not significantly ($P > 0.05$) different from control plants, which is indicative of synergistic effect of the fertilizers.

VC+PGPR resulted in an increase in the leaf carotenoid content by 45.12% followed by HA+PGPR and VC. Carotenoid content in the flowers increased by 24.39% in HA treatment, followed by VC+PGPR, HA+PGPR and VC.

Anthocyanin content

The results showed significant ($P < 0.01$) differences among Bio-organic fertilizers, while no significant ($P > 0.05$) differences were observed between the cultivars for anthocyanin and total flavonoid contents (Table 7). VC+PGPR and VC had the maximum anthocyanin content, while it was not significantly ($P > 0.05$) different from HA+PGPR and control plants. The amount of anthocyanin in HA treatment decreased by 22.7%.

Maximum amounts of anthocyanin was detected in the DF cultivar treated by VC+PGPR and VC, which were 11.414 and 11.192 $\mu\text{mol g}^{-1}$ FW, respectively. The minimum amount of anthocyanin was found in the SF cultivar treated by PGPR (6.561 $\mu\text{mol g}^{-1}$ FW) (Fig. 1).

Table 6. Chlorophyll a, b, a + b and carotenoid contents of marigold cultivars as affected by organic and bio fertilizers.

Treatments	Chlorophyll a (mg g ⁻¹ FW)	Chlorophyll b (mg g ⁻¹ FW)	Chlorophyll a + b (mg g ⁻¹ FW)	Leaf carotenoid (mg g ⁻¹ FW)	Flower carotenoid (mg g ⁻¹ FW)
Control	2.742 ± 0.13 ^b	0.765 ± 0.08 ^d	3.536 ± 0.18 ^d	0.308 ± 0.07 ^d	1.054 ± 0.09 ^b
PGPR	2.956 ± 0.17 ^a	0.900 ± 0.09 ^c	3.893 ± 0.25 ^c	0.378 ± 0.06 ^{bc}	0.947 ± 0.19 ^c
HA	2.867 ± 0.13 ^{ab}	0.770 ± 0.06 ^d	3.664 ± 0.15 ^d	0.344 ± 0.06 ^{cd}	1.178 ± 0.14 ^a
VC	2.884 ± 0.1 ^{ab}	1.178 ± 0.08 ^a	4.122 ± 0.1 ^b	0.410 ± 0.02 ^{ab}	1.131 ± 0.06 ^{ab}
VC+PGPR	2.991 ± 0.13 ^a	1.026 ± 0.12 ^b	4.064 ± 0.21 ^b	0.447 ± 0.02 ^a	1.151 ± 0.23 ^{ab}
HA+PGPR	2.997 ± 0.18 ^a	1.282 ± 0.15 ^a	4.345 ± 0.2 ^a	0.433 ± 0.07 ^{ab}	1.143 ± 0.12 ^{ab}
DF	2.972 ± 0.15 ^a	0.995 ± 0.23 ^a	4.011 ± 0.32 ^a	0.411 ± 0.06 ^a	1.150 ± 0.13 ^a
SF	2.841 ± 0.14 ^b	0.979 ± 0.22 ^a	3.864 ± 0.33 ^b	0.362 ± 0.07 ^b	1.051 ± 0.19 ^b

Within columns values followed by the same letter are not significantly different at the $P \leq 0.05$ level. Plant growth promoting rhizobacteria (PGPR), humic acid (HA), vermicompost (VC), double flower (DF) and single flower (SF).

Table 7. Anthocyanin, total phenol (TPC), total flavonoid (TFC) contents and antioxidant activity of marigold cultivars as affected by organic and bio fertilizers.

Treatments	Anthocyanin ($\mu\text{mol g}^{-1}\text{FW}$)	TPC ($\text{mg g}^{-1}\text{FW}$)	TFC ($\text{mg g}^{-1}\text{FW}$)	Antioxidant activity (%)
Control	$8.97 \pm 1.23^{\text{ab}}$	$88.33 \pm 9.49^{\text{c}}$	$39.454 \pm 55.67^{\text{b}}$	$28.92 \pm 3.13^{\text{bc}}$
PGPR	$8.11 \pm 2.17^{\text{bc}}$	$79.44 \pm 20.23^{\text{c}}$	$45.046 \pm 42.39^{\text{a}}$	$33.17 \pm 3.73^{\text{a}}$
HA	$6.93 \pm 0.97^{\text{c}}$	$107.5 \pm 21.34^{\text{ab}}$	$39.460 \pm 47.99^{\text{b}}$	$29.56 \pm 2.21^{\text{bc}}$
VC	$10.72 \pm 1.16^{\text{a}}$	$95.63 \pm 14.82^{\text{abc}}$	$39.249 \pm 56.91^{\text{b}}$	$32.13 \pm 1.86^{\text{a}}$
VC+PGPR	$10.45 \pm 2.02^{\text{a}}$	$90.79 \pm 9.4^{\text{bc}}$	$43.603 \pm 45.12^{\text{a}}$	$28.21 \pm 1.94^{\text{c}}$
HA+PGPR	$9.55 \pm 1.45^{\text{ab}}$	$111.03 \pm 13.85^{\text{a}}$	$46.030 \pm 47.15^{\text{a}}$	$30.98 \pm 2.09^{\text{ab}}$
DF	$9.00 \pm 2.36^{\text{a}}$	$98.91 \pm 18.42^{\text{a}}$	$41.137 \pm 54.03^{\text{a}}$	$29.65 \pm 2.34^{\text{b}}$
SF	$9.24 \pm 1.54^{\text{a}}$	$92.01 \pm 17.78^{\text{a}}$	$43.144 \pm 55.86^{\text{a}}$	$31.34 \pm 3.38^{\text{a}}$

Within columns values followed by the same letter are not significantly different at the $P \leq 0.05$ level. Plant growth promoting rhizobacteria (PGPR), humic acid (HA), vermicompost (VC), double flower (DF) and single flower (SF).

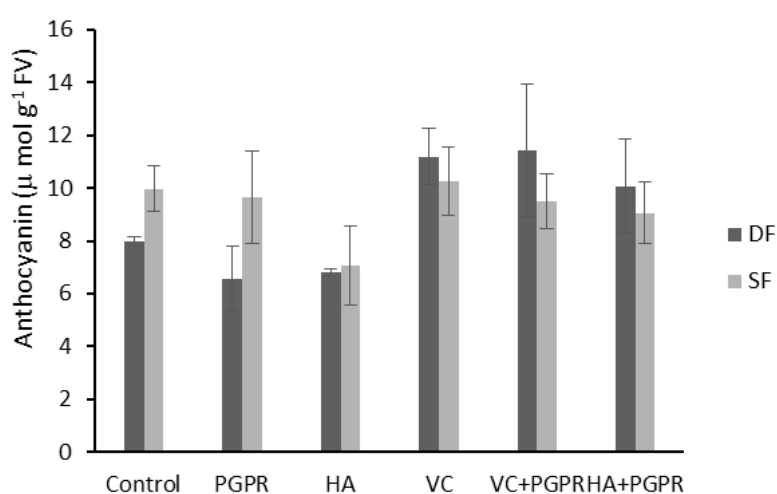


Fig. 1. Changes in anthocyanin content of marigold cultivars in response to different fertilizers in Marigold plants. Plant growth promoting rhizobacteria (PGPR), humic acid (HA), vermicompost (VC), double flower (DF) and single flower (SF).

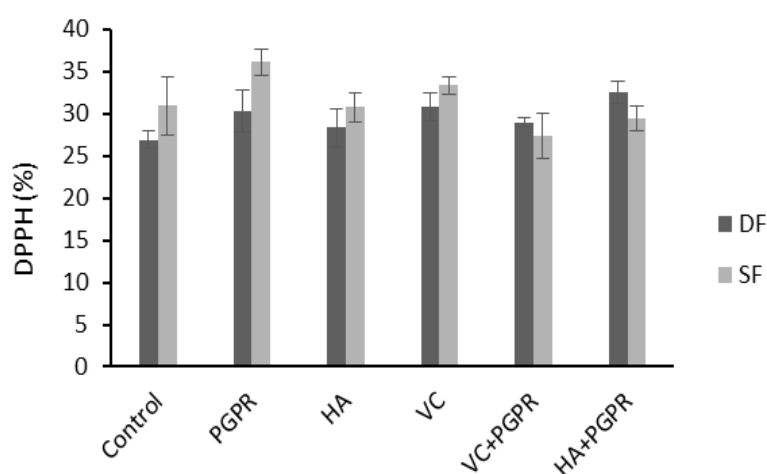


Fig. 2. Changes in antioxidant activities of marigold cultivars by the assay of DPPH in response to different fertilizers in Marigold plants. Plant growth promoting rhizobacteria (PGPR), humic acid (HA), vermicompost (VC), double flower (DF) and single flower (SF).

Phenol and flavonoid contents

Bio-organic fertilizers caused increase in phenol content of marigold plants, but only HA+PGPR had a significant ($P<0.01$) difference by 25.69% with the control plants (Table 7). HA+PGPR, PGPR and VC+PGPR increased flavonoid content by 16.66, 14.17 and 10.51%, respectively. The difference among cultivars was not significant for phenol and flavonoid contents (Table 7).

Antioxidant activity

The results of DPPH assay are shown in Table 7. Antioxidant capacities of the SF cultivar were significantly ($P<0.01$) higher than that of the DF cultivar. The DPPH radical scavenging effects of PGPR and VC were significantly ($P<0.01$) higher than that of the control. Interaction effects also showed that the SF cultivar treated with PGPR (36.11 %) had the highest values, while it was not significantly ($P>0.05$) different when the same cultivar treated with VC (33.39 %). The lowest value was found in the DF cultivar without fertilizer treatments (Fig. 2).

Discussion

Soluble protein, free amino acid, soluble and insoluble carbohydrate contents

Carbohydrates and proteins are two major classes of biochemical compounds that are important for structural support and physiological functions. In this study we found some increases in carbohydrate and protein contents of plants that were supplemented with organic and bio fertilizers. According to previous studies, Bio-organic fertilizers can enhance mentioned biochemicals due to high availability of nutrients.

Omara et al., (2017) reported increase in total carbohydrate and protein contents in seeds of soybean with the application of different microbial strains. They also found elevated activities of soil enzymes, especially dehydrogenase, urease and phosphatase, which lead to enhancement in vegetative growth and seed yield.

Another study by Salehi et al., (2016) showed increase in sugar concentrations in leaves of German chamomile (*Matricaria chamomilla* L.) treated with vermicompost. They have linked the effects to the increase in leaf area and photosynthetic capacity. There are some other reports that introduce HA as a fertilizer and activator that increases carbohydrate content in turn leading to higher yields or improving product qualities. According to El-Shabrawi et al., (2015) humic acid increased the activity of plastid enzymes involved in photosynthesis, sucrose biosynthesis and starch accumulation.

Song et al., (2015) stated that PGPR only in combination with vermicompost significantly increased soluble protein in spinach (*Spinacia oleracea* L.). Hosseinzadeh et al., (2018) also reported increase in protein content, by the application of vermicompost in chickpea.

Amino acids have a role as intermediates in metabolism and apart from being bound as proteins, also exist in the free form in different parts of plant and are known as free amino acids. Measurement of AAs can show the physiological and health status of the plants. In this study we did not find significant differences among treatments in AAs. According to a study by Ekinici et al., (2014) on cauliflower (*Brassica oleracea* L.) although, the amount of amino acids did not change compared to the control, PGPR applications increased varying in proportions of different amino acids.

Synergistic effects between biological and organic fertilizers can increase PGPR activity in the soil. It seems, effects were even higher for VC+PGPR treatments in this study. Bio-organic treatments enhance the absorption of nutrients by plants, especially availability of nitrogen, which leads to higher levels of proteins. Moreover, increase in photosynthetic pigments that we measured in this research, can accordingly strengthen photosynthesis. Because of the marigold's indeterminate growth, it can be speculated that starch synthesis and degradation are extremely

active at the same time. Continuously growth and flowering needs extra energy and nutrition consumption. Hydrolysis of starch provides soluble sugars for supporting the plant. Therefore, Bio-organic fertilizers which poses more balanced source of nutrients than common fertilizers, can help this conversion process and sink-source relations.

Chlorophyll, carotenoid anthocyanin contents

Carotenoids are considered as a class of antioxidant compounds in plant cells which act through a non-enzymatic pathway to reduce oxidative damage to the plant. In addition, marigold as a valuable source of carotenoid compounds is one of the key alternatives for replacing chemical dyes in order to produce natural and environmentally friendly dyes (Guinot et al., 2008). Therefore, it is important to know the factors affecting the amount of these compounds.

Chlorophyll content is an index of plant health. The results of present study pertaining to the effectiveness of applied fertilizers on pigments are in line with some previous findings. For instance, Gholami et al., (2018a) reported that the HA and VC improves nutrient uptake, yield and photosynthetic pigment contents in chicory (*Cichorium intybus* L.). Hosseinzadeh et al., (2018) also reported the same results on photosynthetic pigments by the application of VC in chickpea. There are some feasible ways to increase photosynthetic pigments by applying the organic fertilizers. Increase in the RUBISCO activity that participate in Calvin cycle is one of the basic ways. Moreover, Bio-organics increase pigment contents through stimulating absorption of nutrients such as nitrogen, which are necessary for chlorophyll biosynthesis and other substantial products with a protein structure.

Previous studies also reported increase in anthocyanin contents by application of organic fertilizers on plants. Theunissen et al., (2010) reported that use of VC due to a

high amount of HA can cause the synthesis of phenolic compounds such as flavonoids and anthocyanins. Trinh et al., (2018) found a higher expression of many key genes regulating anthocyanin and flavonoid biosynthesis pathways in *Arabidopsis thaliana* seedlings treated by PGPR. Furthermore, increase in the content of anthocyanins with an increase in alpha-amylase and soluble sugars, was reported by Parandian and Samavat, (2012). In an organic production, plants may face with some stresses, which induce plants to adapt and synthesize compounds with a protective role to modify their growth conditions. Therefore, it is understandable, why plants do some decomposition in carbohydrates, which leads to increases in anthocyanin levels.

Phenol and flavonoid contents

In this study the results showed an increase in phenol and flavonoid contents of treated plants but in the case of flavonoids, the significant difference was only for HA+PGPR treatments.

Onofreia et al., (2017) and Gholami et al., (2018b) reported increase in phenolic and flavonoid contents by the use of organic fertilizers in marigold and Chicory. Moreover, according to Schiavon et al., (2010) changes in the metabolism of phenylpropanoic acid lead to decrease in phenylalanine and tyrosine levels and increase in phenolic compounds.

Garcia-Seco et al., (2015) reported increase in gene expression of the flavonoid biosynthesis pathway by using PGPRs. These microorganisms are able to produce siderophores, which induce a defense mechanism to produce secondary metabolites.

It is well known that, the higher concentrations of phenolic compounds in plants can be a distinct role of organic fertilizers, which induce the acetate shikimate pathway, resulting in higher production of secondary metabolites. In addition, promoting effects of organic

treatments, lead to high potential of antioxidant activity in medicinal plants.

Antioxidant activity

There are different methods for measuring the antioxidant activity. In the present study, antioxidant activity of marigold extracts was analyzed by DPPH method, which is one of the most sensitive and widely used methods for the determination of antioxidant activity.

The results of this study confirmed the positive effects of bio and organic fertilizers on antioxidant activity, although the differences were significant only for VC and PGPR treatments. In another study, Onofreia et al., (2017) reported increase in free radical scavenging activity by the application of organic fertilizers in marigold. Fallah et al., (2018) also reported the same effects of organic manure on dragonhead (*Dracocephalum moldavica*) in an intercropping system.

Increase in antioxidant capacity, can be linked to the high quantities of some major secondary compounds. It could be the result of Bio-organic compounds application in the overall promotion of the secondary metabolic pathways in the plant. These compounds, also provide balanced levels of nutrients, promote photosynthesis of the plant, which supplies starting materials for other substances.

Therefore, despite the limitations in existing studies and the need for further research, existing evidence reinforces the hope that the move to organic farming will increase the quality of crops, including the amount of antioxidants in many foods and herbs.

Conclusion

Applied Bio-organic fertilizers in this study caused positive responses in primary and secondary metabolism. Co-application of Bio-organic fertilizers comparatively improved crop quality parameters better than using them individually. VC+PGPR induced the maximum increase of TSP (36.5 mg g⁻¹

FW), SCH (67.02 mg g⁻¹ DW) and ICH (43.49 mg g⁻¹ FW). HA+PGPR treatment had the highest total chlorophyll content, which showed an increase of 22.87%. VC+PGPR increased leaf carotenoids content by 45.12% followed by HA+PGPR and VC. All mentioned attributes were high for the DF cultivar. Treatments did not induce any significant differences regarding AAs of marigold leaves. The interaction effects of Bio-organic fertilizers and cultivars showed maximum amounts of anthocyanin in the DF cultivar treated by VC+PGPR and VC, which were 11.414 and 11.192 (μmol g⁻¹ FW) respectively. Bio-organic fertilizers led to increase in phenol content, but only HA+PGPR had a significant difference by 25.69% in comparison with phenol content in control plants. SF cultivar treated with PGPR (36.11 %) had the highest level of total antioxidant activity, which did not show significant difference when the same cultivar treated by VC (33.39 %).

Therefore, it is concluded that, application of Bio-organics in this study can promote the production of primary and secondary metabolites of marigold. Moreover, increase in amount of active compounds with protective roles cause elevated antioxidant activities. In total, it is feasible to achieve marigold plant with high medicinal values without using chemical fertilizers, which have toxic and detrimental effects for the environment.

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