

CO-APPLICATION OF HUMIC ACID AND *BACILLUS* STRAINS ENHANCES SEED AND OIL YIELDS BY MEDIATING NUTRIENT ACQUISITION OF SAFFLOWER (*CARTHAMUS TINCTORIUS* L.) PLANTS IN A SEMI-ARID REGION

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Abstract. Faced with the deterioration of natural resources, current trends in agriculture are focused on seeking eco-friendly methods to improve plant growth promotion and crop productivity. Plant growth promoting rhizobacteria (PGPR) and humic substances can improve crop production in sustainable farming due to their various features. This study examined how humic acid (HA) and inoculation with PGPR (*Bacillus megaterium* and *Bacillus subtilis*) affect the agronomic performance of safflower (*Carthamus tinctorius* L.) in a semi-arid environment. In the field experiments, PGPR inoculated and non-inoculated safflower seeds were cultivated in soil treated with humic acid (0, 200, 300, and 400 kg ha⁻¹), alone or in combination. It was observed that PGPR and HA improved growth, yield parameters and seed mineral contents. Their plant growth responded positively to inoculation with *Bacillus* sp. strains owing to the N-fixing and P-solubilizing capabilities, whereas greatly positive response in the seed yield and quality was found when applied in combination with humic acids. The combined application of 300 and 400 kg ha⁻¹ HA with *Bacillus subtilis* inoculation enhanced seed and oil yields by an average of 162% and 221% relative to the control plants. This study confirms that integrated management strategies can ensure higher crop productivity of safflower in sustainable and semi-arid environments.

Keywords: *energy crop, humic substances, plant growth promoting rhizobacteria, seed yield, sustainable agriculture*

Introduction

Safflower (*Carthamus tinctorius* L.) is an annual oilseed and energy crop belonging to the *Asteraceae* family. The crop was once grown for colorful petals used in food coloring, fabric dyes, flavoring agent and medicinal use (Ekin, 2005). Nowadays, scientific interest in this species is mainly due to its high quality vegetable oil for nutritional and industrial applications. However, safflower has gained importance in recent years as a result of its vegetable oil for human consumption and biodiesel production in arid and semi-arid regions in the world. Safflower is also one of the most adapted oilseed crops to dryland cropping systems with good drought tolerance due to its deep taproot, but it's sensitive to dry soil condition in which kernel filling (flowering) are affected by water stress conditions (Dajue and Mündel, 2006).

To improve the plant productivity, it is of paramount importance to supply adequate water and nutrient acquisition in semi-arid regions typically characterized by variable and unpredictable rainfall, large diurnal ranges in temperature, frequent strong winds and poor moisture storing capacity of soils (Chavoushi et al., 2019). Moreover, this is crucial to producers in semi-arid regions of world and Turkey, including eastern Anatolia, south eastern Anatolia and central Anatolia. In particular, water resources need to be used efficiently because of the anticipated water scarcity in the face of global warming and the increasing competition between domestic, industrial, and agricultural water consumptions. Therefore, it is imperative to improve the drought tolerance of

crops as well as its salt tolerance, heavy metal stress tolerance and disease resistance, and to protect soil health and fertility under the changing circumstances. Currently, there are no economically viable technological means to facilitate crop production under various stress conditions. Towards a sustainable agricultural vision, the uses of plant growth promoting rhizobacteria and humic acid might also be a promising alternative eco-friendly approach, which helps in protecting the soil health, increasing the nutrient uptake capacity and water use efficiency and decreasing the effects of drought, salinity and heavy metal pollution etc.

Plant growth promoting rhizobacteria (PGPR) are bacteria colonizing rhizospheres of plant that, unlike fertilizers and pesticides, depend on various mechanisms like nitrogen fixation, solubilization of phosphate, production of different phytohormones like indole-3-acetic acid, gibberellic acid and cytokines for its success in promoting crop yield (Calvo et al., 2014). In this sense, plant growth promoting rhizobacteria can be used to enhance plant health and promote plant growth rate without environmental contamination. For decades, varieties of PGPR including the species *Pseudomonas*, *Bacillus*, *Enterobacter*, *Klebsiella*, *Azotobacter*, *Variovorax* *Azospirillum*, and *Serratia* have been well studied in various field crops as rhizosphere-colonizing microorganisms (Glick, 2012), such as wheat (Rosas et al., 2009; Hungria et al., 2010; Rana et al., 2012), sugar beet and barley (Cakmakci et al., 2001, 2006), sugarcane (Silva et al., 2017), sunflower (Shadid et al., 2012), rice (Lucas et al., 2009), bean (Hoyos-Carvajal et al., 2009), canola (El-Howeity and Asfour, 2012), maize (Thonar et al., 2017) and soybean (Cassán et al., 2009). However, only limited data are reported in the beneficial effects of PGPR on safflower growth promotion. The literature on PGPR regarding safflower included mostly *Pseudomonas*, *Azospirillum* and *Azotobacter* spp. inoculation which were used to improve nitrogen uptake (Mirzakhani et al., 2009; Soleymanifard and Sidat, 2011; Sharifi et al., 2017; Nosheen et al., 2018), root morphology (Nosheen et al., 2011) and protein quantity and quality of safflower seeds (Nosheen et al., 2016). The various studies also reported that inoculation of specific PGPR species like N₂-fixing *Bacillus subtilis* and phosphorus solubilizing *Bacillus megaterium* instead of synthetic chemicals may serve as an effective alternative and environmental friendly practice since they improve plant nutrition by increasing N and P uptake by plants (Cakmakci et al., 2001; Esitken et al., 2003). But, up till now no data are available with respect to the use of this genus in safflower plants.

Similarly, humic acid (HA) are naturally-available substance in the soil that are end products of microbial decomposition and chemical degradation of dead biota. Humic substances can promote plant growth through improvement in root architecture and morphology, enhancement of nutrient availability, uptake and translocation, promotion of plant physiology and development due to their hormone-like activities and promotion of soil biological activity (Puglisi et al., 2013; Canellas and Olivares, 2014; Ahmad et al., 2016). In the various experiments, direct effects of humic acid on plant growth were well described in cultivation of various crops, such as potato (Suh et al., 2014), tomato (Olivares et al., 2015), maize (Canellas et al., 2013), Hungarian vetch (Esringu et al., 2016) and blueberry (Schoebitz et al., 2016). However, basic mechanisms and benefits of the combined application of humic substances and plant growth promoting rhizobacteria have been recently demonstrated for several plant species grown under field conditions and have been attributed to an increase in the adaptation of seedlings to stress conditions, which result to cause an increase in the macro- and micro nutrient uptakes and root growth (Nardi et al., 2009; Dobbss et al., 2010; Busato et al., 2012;

Puglisi et al., 2013; Canellas and Olivares, 2014). Olivares et al. (2015) observed that co-application of humates isolated from vermicompost with diazotrophic endophytic *Herbaspirillum seropedicae* inoculation can increase significantly nitrate uptake, nitrate reductase activity, fruit biomass, leading to to increased tomato yield. The researchs previously reported that humic substances have potential as enhancer of plant growth promoting bacteria inoculation benefits (Canellas et al., 2013; Esringu et al., 2016; Schoebitz et al., 2016; Silva et al., 2017).

Keeping in view the characteristics of plant growth promoting rhizobacteria and humic acid as a helping way for crop plants to be grown in arid and semi-arid areas we hypothesized that they can enhance the agronomic performance of safflower through the activation of natural processes like nutrient release and uptake by various mechanisms described above. Extensive root system created by improved root growth and architecture by plant growth promoting rhizobacteria and humic acid can also enhance the seed quality by increasing the uptake of nutrients by exploring more and more soil volume. Stimulation of biological activity by humic acid can further enhance nutrient cycling through the action of microorganisms. In this regard, to evaluate the potential of plant growth promoting rhizobacteria and humic acid for agronomic performance of safflower we planned 2 years field experiment in a semi-arid environment. In this experiments we used *Bacillus megatorium* M3 (P-solubilizing), *Bacillus subtilis* OSU-142 (N₂-fixing) and humic acid (obtained from leonardite).

Materials and Methods

Study Site

Field experiments were carried out under rainfed conditions at a research farm located in Ahlat district (38° 46'N and 42°30'E with an altitude of 1722 m) in Eastern Anatolia region, Turkey during 2010 and 2011 (*Fig. 1*). The climate at this location is classified as continental with a total long-term average (1958-2017) precipitation of 562.6 mm mainly in winter. Annual mean air temperature is 9.3°C, with an average temperature of -2.5°C in January and 21.9°C in July. Annual mean relative humidity is 63.8%. The study site climate variables were collected from weather station and averaged for each month (*Table 1*). In 2010 and 2011, total precipitation during the crop season (from April to August) was 291.0 mm, and 269.4 mm, respectively, and the long-term average for the same period was 136.2 mm (*Table 1*). The mean air temperature was 16.5°C, 16.0°C in 2010, 2011, respectively, and long-term average was 16.1°C.

Table 1. Climate data: Monthly means of climate variables for the crop seasons of 2010, 2011 and long-term average (LTA: 1958-2017) in Ahlat, Turkey

Months	Temperature (°C)			Precipitation (mm)			Relative humidity (%)		
	2010	2011	LTA	2010	2011	LTA	2010	2011	LTA
April	7.3	6.9	6.9	154.4	159.0	87.1	71.0	71.0	69.4
May	11.4	11.2	13.1	106.2	90.0	70.2	65.8	69.1	65.0
June	18.3	17.6	18.9	28.0	15.6	28.7	50.4	52.1	55.6
July	22.8	22.3	21.5	1.8	3.2	8.3	37.3	41.3	49.4
August	22.5	22.0	22.8	0.6	1.6	5.7	35.6	40.4	47.7
Season(M/T)*	16.5	16.0	16.1	291.0	269.4	136.2	52.0	54.8	57.4
Yearly(M/T)	10.9	8.6	9.3	399.0	566.6	562.6	59.6	56.4	63.8

* M: Mean, T: Total

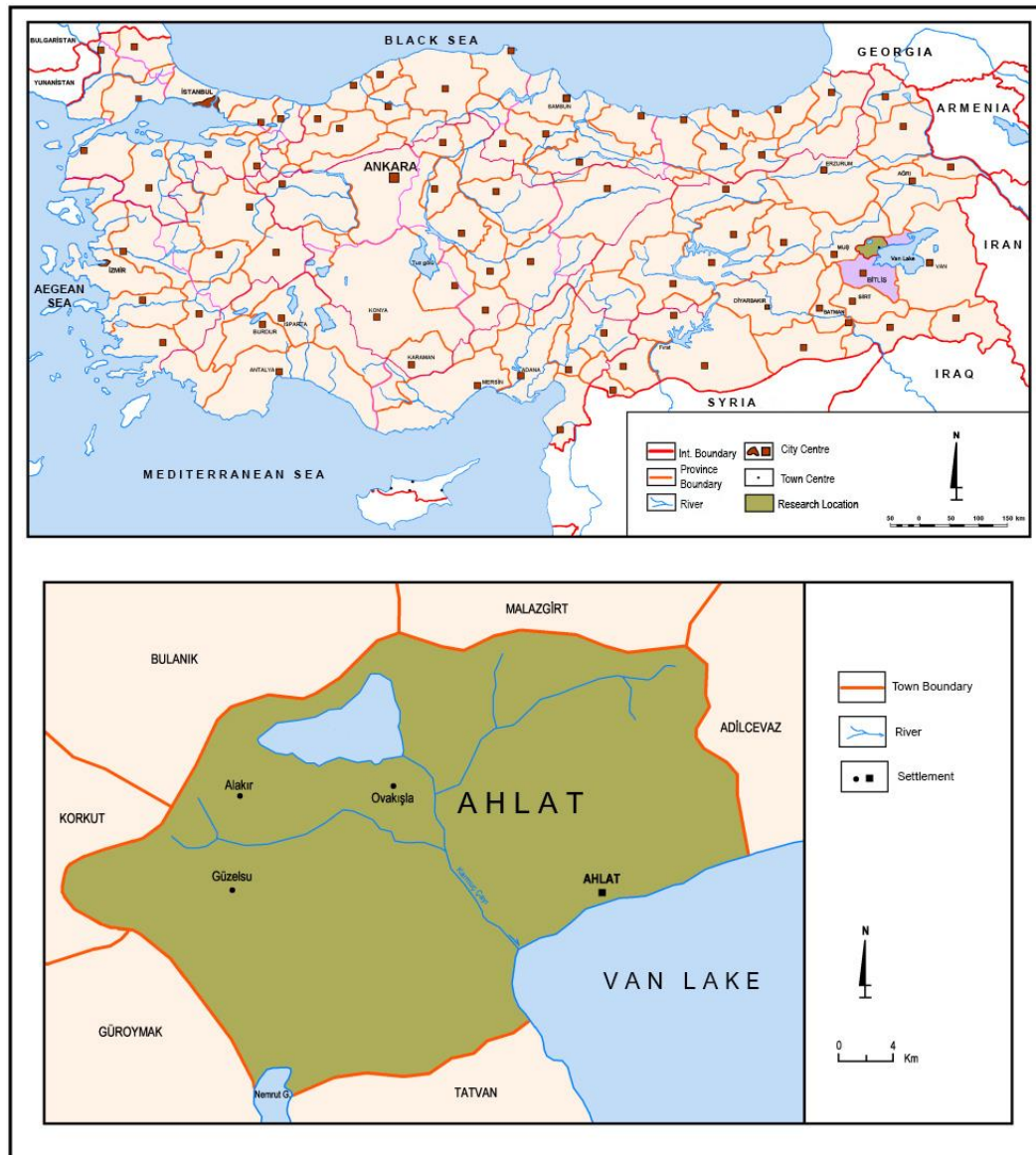


Figure 1. Map of study site

Prior to the experiments, soil samples were collected in the experiment site and analyzed for their physical and chemical properties using the methods described in Soil and Plant Analysis Laboratory Manual by Ryan et al. (2012). Soil samples were collected in the top 30 cm (<2 mm fraction) of soil at different locations of the experiment site. The experiment site soil was silt-clay-loam with a pH of 7.41, total nitrogen content of 0.15 g kg⁻¹, organic matter content of 1.60%, CaCO₃ content of 6.8%, electrical conductivity of 1.16 dS m⁻¹, available phosphorus content of 7.95 mg kg⁻¹, available potassium content of 196 mg kg⁻¹, available manganese content of 3.30 mg kg⁻¹, zinc content of 1.44 mg kg⁻¹, iron content of 5.85 mg kg⁻¹ and copper content of 0.59 mg kg⁻¹.

Plant Material and Experimental Design

Safflower (*Carthamus tinctorius* L.) var. Dincer, Turkey of origin, which exhibits early maturity, spineless, open-pollinated, yellow-orange flower color, white seed color,

28-32% oil content and high-linoleic oil type, was used as plant material. The field trials were set up a factorial design including the two following factors: four HA doses (0= without the addition of HA, 20= 200 kg ha⁻¹ HA, 30= 300 kg ha⁻¹ HA and 40= 400 kg ha⁻¹ HA) and three PGPR applications (B0= no bacterial inoculation, B1: *Bacillus subtilis* OSU-142 and B2: *Bacillus megatorium* M3) with three replications.

Humic Acid Characteristics, PGPR Culture Condition and Seed Inoculation

Agro-Lig, a commercial product, was produced through crude humic acids derived from leonardite and provided by Altintar Chemicals Company (Turkey). Agro-Lig contains total humic acid 85%, total organic matter 75%, pH 3.5-5.5, max moisture 22%, silicon 0.5%, iron 0.5%, magnesium 0.5%, calcium 3.0%, sodium 0.3%, manganese 0.02%, copper 0.0003%, potassium 0.07%, titanium 0.02%, barium 0.03%, boron 0.01%, cobalt 0.0002% dry matter basis. Three HA doses (200, 300 and 400 kg ha⁻¹) were prepared by Agro-Lig in granule form.

The two plant growth promoting rhizobacteria strains (*Bacillus subtilis* strain OSU-142 and *Bacillus megatorium* strain M3) tested were kindly procured at Atatürk University, Department of Plant Protection, Turkey. Currently, these strains are protected in culture collection unit in the Department of Genetic and Bioengineering, Faculty of Engineering at Yeditepe University, İstanbul, Turkey. The choice of these two bacteria is based on their published ability to act as potential biostimulants and biofertilizers. The gram-positive *Bacillus megatorium* M3 has demonstrated abilities to increase the solubilization of inorganic phosphates and to promote plant growth in various plant species (Cakmakci et al., 2001; Orhan et al., 2006). The gram-positive *Bacillus subtilis* OSU-142 is an efficient root colonizer and has been shown to N₂-fixing under field conditions (Cakmakci et al., 2001, 2006). Moreover, it has widely shown the potential to improve plant growth and nitrogen acquisition when inoculated to various plant species (Cakmakci et al., 2001, 2006; Esitken et al., 2003; Orhan et al., 2006; Elkoca et al., 2010; Acikgoz et al., 2016; Ekin, 2019). These strains are indigenous, and are kept in nutrient broth (NB) with 15% glycerol at -80°C for long-term storage, and grown on nutrient agar (NA) for routine use. Single colonies were transferred to 500 ml flasks that contained NB and incubated aerobically on a rotating shaker (150 rpm) overnight at 28°C (Cakmakci et al., 2001, 2006). The bacterial suspension was then diluted in sterilized water to final concentration at cell densities of 10⁹ colony forming units (CFU) ml⁻¹. Seeds were surface sterilized prior to bacterial inoculation with 95% ethanol then sterilized with 10% chlorox for 3 min and washed successively 3–4 times with distilled water. Seeds were inoculated with the liquid cultures of rhizobacteria (*Bacillus megatorium* M3 and *Bacillus subtilis* OSU-142) mixed with 10% sugar solution for 30 min, and were stored overnight to dry under room temperature.

Crop Husbandry and Data Recording

The tillage system was fall plow and spring cultivate. Different doses of humic acid (granule form) were applied to the soil in humic acid treated plots, according to the layout. Humic acid was applied once during planting and then mixed well with the soil. PGPR inoculated and non-inoculated seeds were sown in early April in each year. In the present study, plots were 1.5 m wide and 5 m long and consisted of five rows spaced 0.3 m apart. Plot stands were over sown and hand-thinned approximately at the first four-true leaf stage to 10 cm apart within a row. Throughout the growth season, the production system was

managed based on management practices recommended for the region, which included weed control by hand as required. The safflower plants were manually harvested at the stage of physiological maturity, when most of the leaves turn a brown color and very little green remains on the bracts of the latest flowering heads in late August in each year. Data collecting on the plant height (cm), stem diameter (mm), number of branches per plant, number of capitula per plant, number of seeds per capitulum and capitulum diameter (cm) were recorded for ten randomly selected safflower plants in the central three rows of each plot. The seed yield was expressed in t ha^{-1} . The seed oil and total nitrogen contents were determined by Soxhlet extraction and Kjeldahl methods. Then, total N content was also used for the calculation of the seed protein concentration by multiplication with a conversion factor of 6.25 (Ryan et al., 2012). For mineral content analysis (P, K, Ca, Mg, Fe, Zn, Cu, Mn), safflower seed was ground in a Wiley Mill to pass through an 840- μm screen and wet-digested in $\text{HNO}_3\text{:HClO}_4$ (6:2 v/v) with the Advanced Microwave Digestion System, Ethos Easy, then analyzed with the Inductively Coupled Plasma Optical Emission Spectrometry (iCAP 6000 SERIES, ICP Spectrometer).

Data Analysis

The data were tested for homogeneity and normality of residuals using the Kolmogorov–Smirnov and Bartlett tests, respectively. Afterwards, a combined analysis of variance (ANOVA) was used to compare the effects of humic acid doses and bacterial treatments by field experiment interactions for 2 years using PROC GLM of SAS 9. Mean comparisons were conducted using Fisher's least significant difference ($\text{LSD}_{0.05}$) test.

Results

Morphological and Yield Parameters

The results regarding morphological and yield parameters of safflower in relation to humic acid dose and plant growth promoting rhizobacteria inoculation are presented in Table 2. Highly significant increases were observed in safflower growth and yield parameters after inoculation with PGPR (*Bacillus* strains), and further improved after combined application with humic acid doses. When compared to plant growth promoting rhizobacteria treatments both with and without humic acids, it was observed that inoculation with *Bacillus subtilis* OSU-142 (B_1) was more effective when compared to *Bacillus megatorium* M3 (B_2). Furthermore, a significant interaction between the tested factors was observed in the case of stem diameter, number of branches and capitula per plant, number of seeds per capitulum, capitulum diameter and 1000 seed weight, whereas plant height was highly influenced by humic acid dose and plant growth promoting rhizobacteria inoculation with no significant HA x PGPR interaction (Table 2, Fig. 2). The tallest plant height values were recorded in both *Bacillus subtilis* OSU-142 inoculation and 400 kg ha^{-1} HA dose, while the highest numbers of capitula per plant and seeds per capitulum, largest capitulum diameter and 1000 seed weight values were recorded in co-application of *Bacillus subtilis* OSU-142 with 300 and 400 kg ha^{-1} HA doses ($\text{HA}_{30}+\text{B}_1$ and $\text{HA}_{40}+\text{B}_1$). Likewise, the application of 400 kg ha^{-1} HA dose significantly increased numbers of branch per plant and seeds per capitulum at both B_1 and B_2 inoculation by 81.3% and 90.6% in branch numbers and 85.5% and 83.6% in seed numbers increases when compared to the untreated control plants, respectively (Table 2, Fig. 2).

Table 2. Mean of morphological and yield traits of safflower affected by plant growth promoting rhizobacteria and humic acid doses¹

Treatments	Plant height (cm)	Stem diameter (mm)	No. of branches per plant	No. of capitula per plant	No. of seeds per capitulum	Capitulum diameter (cm)	1000 seed weight (g)
Humic acid (HA)							
0	73.0 d	5.64 c	4.3 d	6.7 d	25.5 d	2.07 b	33.1 d
20	77.4 c	6.70 b	4.9 c	10.4 c	34.8 c	2.39 a	37.0 c
30	82.7 b	6.98 a	5.3 b	10.9 b	35.8 b	2.39 a	38.1 b
40	85.2 a	7.05 a	5.9 a	11.3 a	36.7 a	2.40 a	38.5 a
LSD _{0,05}	2.383	0.118	0.129	0.125	0.627	0.024	0.405
Significance	**	**	**	**	**	**	**
Plant growth promoting rhizobacteria (PGPR)							
B ₀	71.9 c	6.18 c	4.6 c	8.4 c	30.4 c	2.20 c	34.6 c
B ₁	87.6 a	6.91 a	5.6 a	11.3 a	35.6 a	2.41 a	38.5 a
B ₂	79.2 b	6.70 b	5.1 b	10.0 b	33.8 b	2.32 b	37.0 b
LSD _{0,05}	2.064	1.102	0.112	0.108	0.543	0.021	0.350
Significance	**	**	**	**	**	**	**
HA x PGPR							
HA ₀ +B ₀	66.6	4.56 g	3.2 e	4.9 j	20.7 g	1.90 f	29.2 g
HA ₀ +B ₁	79.9	6.40 e	4.9 c	8.6 h	29.0 e	2.20 d	36.0 e
HA ₀ +B ₂	71.8	5.78 f	4.5 d	6.3 i	26.2 f	2.09 e	33.7 f
HA ₂₀ +B ₀	69.7	6.40 e	4.3 d	8.9 g	32.3 d	2.31 c	35.5 e
HA ₂₀ +B ₁	85.3	6.80 d	5.4 b	11.5 b	37.1 ab	2.46 a	38.2 c
HA ₂₀ +B ₂	77.4	6.90 d	4.9 c	10.9 d	34.9 c	2.40 b	37.3 d
HA ₃₀ +B ₀	73.9	6.71 d	4.7 d	9.2 f	33.1d	2.29 c	36.1 e
HA ₃₀ +B ₁	92.9	7.11 b	6.1 a	12.5 a	38.2 a	2.51 a	40.0 a
HA ₃₀ +B ₂	81.4	7.11 b	5.0 c	11.1 c	36.3 b	2.36 b	38.1 c
HA ₄₀ +B ₀	76.5	6.78 d	5.8 a	10.3 e	34.1 c	2.28 c	36.8 d
HA ₄₀ +B ₁	92.5	7.33 a	6.1 a	12.6 a	38.4 a	2.48 a	39.8 a
HA ₄₀ +B ₂	86.6	7.03 c	5.8 a	11.7 b	38.0 a	2.41 b	39.0 b
Significance	ns	**	**	**	**	**	**
CV (%)	4.38	2.62	3.70	1.84	2.79	1.53	1.61

¹20, 30 and 40 treatments refer to safflower plants growing under field condition with 200, 300 and 400 kg ha⁻¹ of HA; B₀ treatment without any application of the bacteria, B₁ treatment with *Bacillus subtilis* OSU-142 inoculation; B₁+20, B₁+30, and B₁+40 with *Bacillus subtilis* OSU-142 and 200, 300 and 400 kg ha⁻¹ of HA, respectively; B₂ treatment with *Bacillus megatorium* M3 inoculation; B₂+20, B₂+30, and B₂+40 with *Bacillus megatorium* M3 and 200, 300 and 400 kg ha⁻¹ of HA, respectively. Means followed by different letters are different by LSD test. *: P < 0.05, **: P<0.01, ns: not significant; CV: coefficient of variations

Seed Yield and Quality Parameters

Seed, oil and protein yields and certain quality parameters of safflower were significantly affected by plant growth promoting rhizobacteria and humic acid treatments based on the two-year average data (Table 3). For most of the measured quality parameters, no significant interaction between plant growth promoting rhizobacteria and humic acid application was recorded, except for the seed yield and fertile and sterile seed ratio (Table 3, Fig. 3).

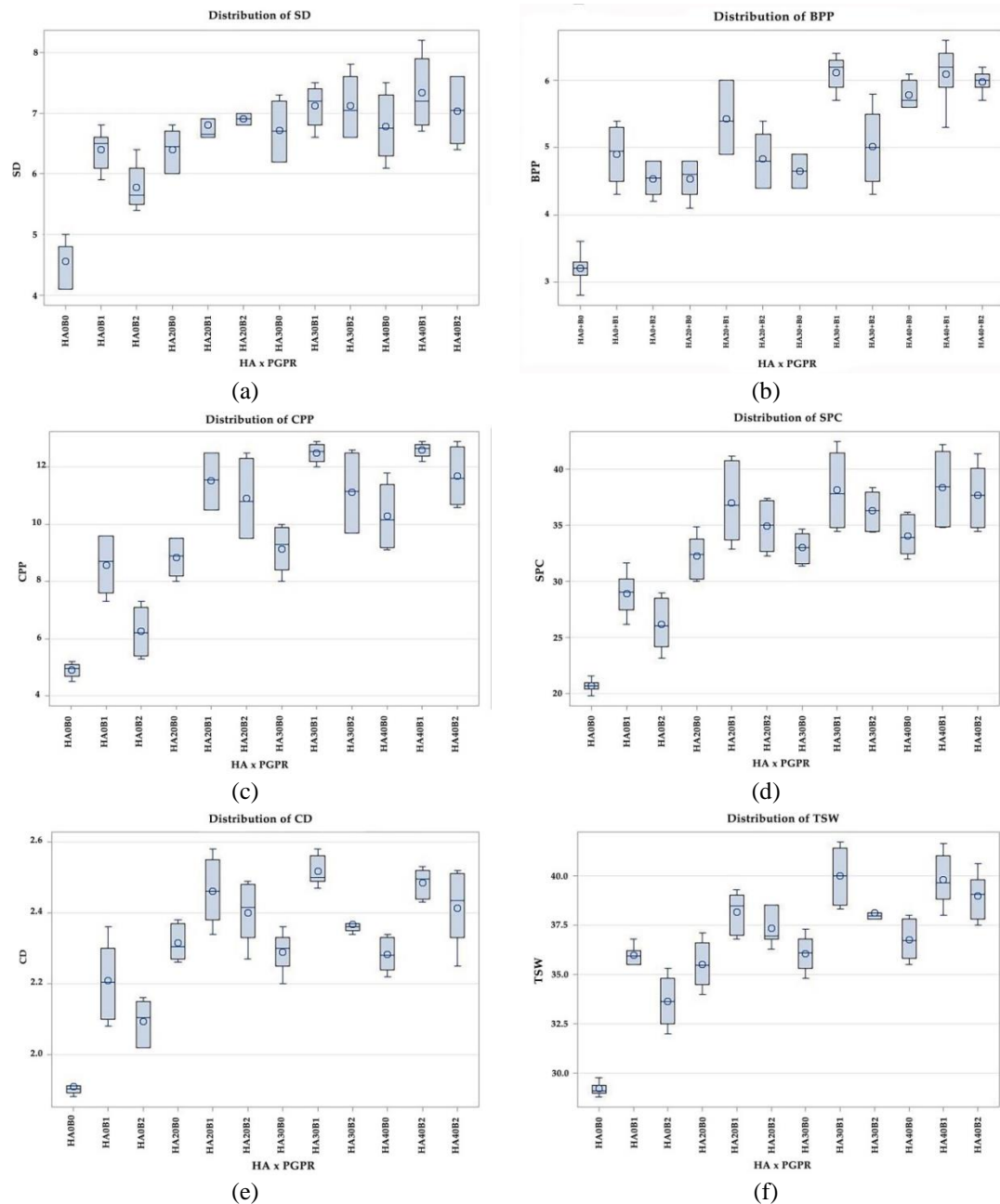


Figure 2. Stem diameter (mm) (a), number of branch per plant (b), number of capitula per plant (c), number of seeds per capitulum (d), capitulum diameter (cm) (e) and thousand seed weight (g) (f) of safflower affected by bacterial inoculation and humic acid. B₀: without bacteria; B₁: *B. subtilis* OSU-142; B₁+20, B₁+30, and B₁+40: *B. subtilis* OSU-142 with 200, 300 and 400 kg ha⁻¹ of HA, respectively; B₂: *B. megatorium* M3; B₂+20, B₂+30 and B₂+40: *B. megatorium* M3 with 200, 300 and 400 kg ha⁻¹ of HA, respectively

Fertile and sterile seed percentages, the most important quality characteristics for safflower, were significantly affected by both plant growth promoting rhizobacteria and humic acid treatments; however, its co-application significantly reduced sterile seed percentage, whereas an opposite trend was observed for the fertile seed percentage (Table 3). The highest fertile seed and lowest sterile seed percentages were recorded in co-application of

both PGPR strains with 300 kg ha⁻¹ humic acid dose (HA₃₀+B₁ and HA₃₀+B₂), whose values were higher by 9.0 and 8.6% and lower by 85.1% and 79.9%, respectively, compared to the untreated control. In the present study, a significant interaction between the tested factors was also observed for the seed yield (Table 3). Inoculation with *Bacillus subtilis* OSU-142 triggered ameliorative effects in terms of seed yield at both 300 and 400 kg ha⁻¹ humic acid doses and led to an increase in seed yield by 163.6% and 161.4%, respectively, when compared to the control (Table 3, Fig. 3). Furthermore, its effect was much more pronounced than that of inoculation with *Bacillus megatorium* M3. Among the humic acid doses, the highest seed yields were also recorded with the application of 300 and 400 kg ha⁻¹ humic acid doses which was statistically similar (Table 3).

Table 3. Mean of seed yield and certain quality traits of safflower affected by plant growth promoting rhizobacteria and humic acid doses¹

Treatments	Seed ratio (%)		Seed content (%)		Yield (t ha ⁻¹)		
	Fertile	Sterile	Oil	Protein	Seed	Oil	Protein
Humic acid (HA)							
0	92.72 d	7.27 a	27.1 c	13.9	1.22 c	0.33 c	0.16 b
20	94.90 c	4.17 c	29.0 bc	13.6	1.74 b	0.51 b	0.24 a
30	95.89 b	2.25 d	30.7 ab	13.2	1.95 a	0.59 a	0.25 a
40	97.75 a	5.10 b	31.3 a	12.8	2.01 a	0.62 a	0.26 a
LSD _{0,05}	0.745	0.751	2.186	1.438	0.149	0.022	0.051
Significance	**	**	**	ns	**	**	**
Plant growth promoting rhizobacteria (PGPR)							
B ₀	94.24 b	5.78 a	27.7 b	12.7	1.40 c	0.39 c	0.19 c
B ₁	95.62 a	4.38 b	30.5 a	13.8	2.06 a	0.63 a	0.28 a
B ₂	96.15 a	3.89 b	30.4 a	13.5	1.73 b	0.52 b	0.22 b
LSD _{0,05}	0.645	0.651	1.892	1.245	0.174	0.052	0.034
Significance	**	**	**	ns	**	**	**
HA x PGPR							
HA ₀ +B ₀	90.37 d	9.85 a	25.7	14.0	0.88 h	0.23	0.12
HA ₀ +B ₁	94.22 bc	5.77 bc	27.1	14.1	1.56 ef	0.42	0.22
HA ₀ +B ₂	93.17 c	6.82 b	28.3	13.5	1.15 g	0.33	0.15
HA ₂₀ +B ₀	93.98 c	6.02 b	26.8	13.9	1.41 f	0.37	0.19
HA ₂₀ +B ₁	95.77 b	4.22 c	30.6	14.0	2.05 b	0.62	0.29
HA ₂₀ +B ₂	97.92 a	2.25 cd	29.8	12.9	1.76 d	0.52	0.23
HA ₃₀ +B ₀	96.70 ab	3.29 c	28.3	13.3	1.60 e	0.45	0.21
HA ₃₀ +B ₁	98.52 a	1.47 d	32.4	13.8	2.32 a	0.75	0.31
HA ₃₀ +B ₂	98.03 a	1.98 d	31.4	12.5	1.93 c	0.59	0.25
HA ₄₀ +B ₀	95.25 b	4.74 c	29.9	13.1	1.64 e	0.48	0.21
HA ₄₀ +B ₁	93.95 c	6.04 b	31.5	13.2	2.30 a	0.73	0.31
HA ₄₀ +B ₂	95.50 b	4.50 c	32.4	11.9	2.06 b	0.66	0.24
Significance	**	**	ns	ns	*	ns	ns
CV (%)	1.15	13.64	5.84	4.80	7.32	4.84	7.50

¹20, 30 and 40 treatments refer to safflower plants growing under field condition with 200, 300 and 400 kg ha⁻¹ of HA; B₀ treatment without any application of the bacteria, B₁ treatment with *Bacillus subtilis* OSU-142 inoculation; B₁+20, B₁+30, and B₁+40 with *Bacillus subtilis* OSU-142 and 200, 300 and 400 kg ha⁻¹ of HA, respectively; B₂ treatment with *Bacillus megatorium* M3 inoculation; B₂+20, B₂+30, and B₂+40 with *Bacillus megatorium* M3 and 200, 300 and 400 kg ha⁻¹ of HA, respectively. Means followed by different letters are different by LSD test. *: P < 0.05, **: P<0.01, ns: not significant; CV: coefficient of variations

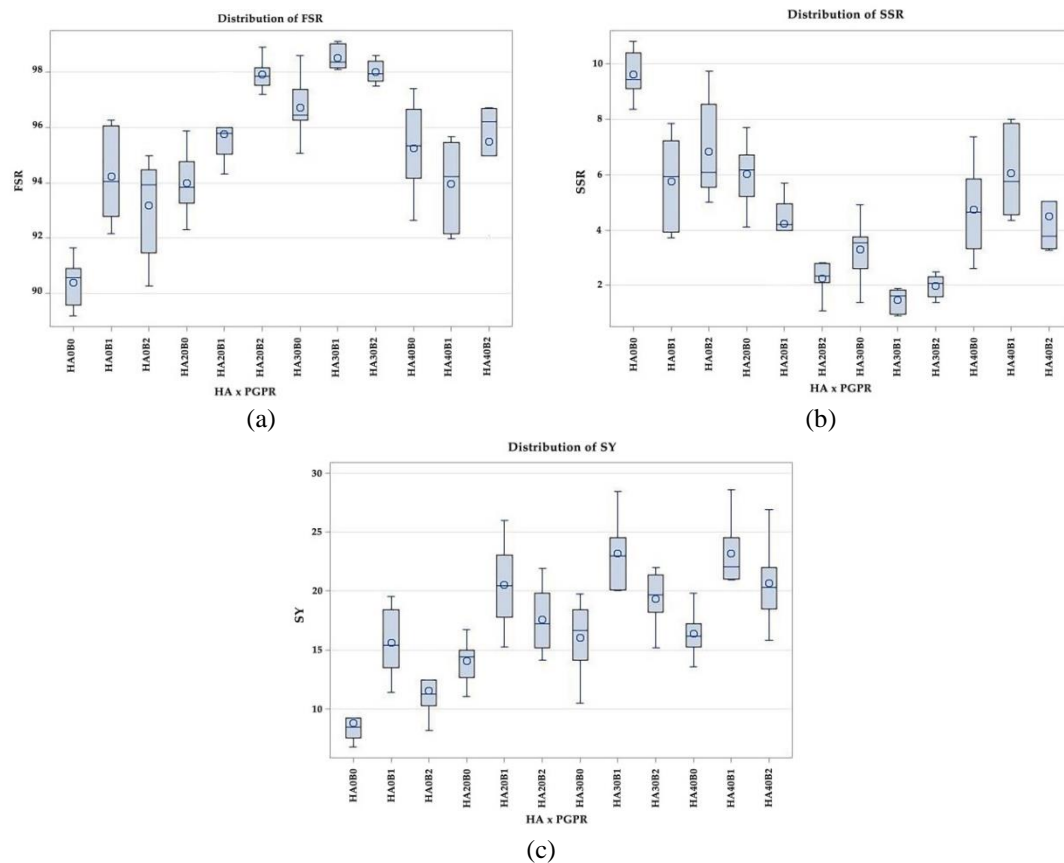


Figure 3. Fertile seed ratio (%) (a), Sterile seed ratio (%) (b) and seed yield ($t\ ha^{-1}$) (c) of safflower affected by bacterial inoculation and humic acid. B₀: without bacteria; B₁: *B. subtilis* OSU-142; B₁+20, B₁+30, and B₁+40: *B. subtilis* OSU-142 with 200, 300 and 400 $kg\ ha^{-1}$ of HA, respectively; B₂: *B. megatorium* M3; B₂+20, B₂+30 and B₂+40: *B. megatorium* M3 with 200, 300 and 400 $kg\ ha^{-1}$ of HA, respectively

Neither humic acid doses nor plant growth promoting rhizobacteria inoculation had a significant influence on protein content in seeds, whereas seed oil concentration, oil and protein yields were highly influenced by humic acid dose and PGPR application with no significant HA x PGPR interaction (Table 3, Fig. 2). The oil concentration in seeds was positively affected by increasing the humic acid doses with no significant effect for inoculation with B₁ and B₂ (30.5% and 30.4%, respectively), and the highest value by 31.3% was recorded with 400 $kg\ ha^{-1}$ humic acid application (Table 3). However, significant increases in oil and protein yields were obtained with separate 300 and 400 $kg\ ha^{-1}$ humic acid dose and *Bacillus subtilis* OSU-142 applications (Table 3).

Mineral Composition of Seeds

The two years long field trials demonstrated that safflower seed mineral composition was significantly affected by both plant growth promoting rhizobacteria and humic acid treatments, however, these integrated administrations significantly increased the mineral concentrations in seeds (except for Cu) when compared to single treatments (Table 4). The Cu concentration in seeds was only negatively affected by both increasing humic acid doses and inoculation with PGPR strains.

Table 4. Mean of mineral composition (mg kg^{-1}) of safflower seeds affected by plant growth promoting rhizobacteria and humic acid doses¹

Treatments	P	K	Ca	Mg	Fe	Zn	Cu	Mn
Humic acid (HA)								
0	3789.5 d	3687.7 d	2026.7 d	1235.1 d	49.21 d	30.81 c	15.59 a	11.09 b
20	4181.7 c	4128.3 c	2384.5 c	1391.9 c	54.14 c	34.66 b	15.42 ab	12.12 a
30	4394.9 b	4299.9 b	2519.5 b	1510.6 a	58.88 b	35.71 a	15.04 b	12.08 a
40	4506.2 a	4374.4 a	2710.3 a	1475.4 b	61.43 a	35.97 a	14.41 c	12.45 a
LSD _{0,05}	21.658	32.421	17.067	5.777	0.506	0.455	0.390	0.399
Significance	**	**	**	**	**	**	**	**
Plant growth promoting rhizobacteria (PGPR)								
B ₀	3456.8 c	3800.9 c	2215.0 c	1194.4 c	51.40 c	31.10 b	16.00 a	11.10 c
B ₁	4161.9 b	4356.6 a	2533.1 a	1593.0 a	60.20 a	35.69 a	14.41 c	12.82 a
B ₂	5042.1 a	4210.1 b	2482.5 b	1422.3 b	56.26 b	36.08 a	14.97 b	11.89 b
LSD _{0,05}	18.757	28.077	14.780	5.003	0.439	0.394	0.338	0.345
Significance	**	**	**	**	**	**	**	**
HA x PGPR								
HA ₀ +B ₀	2743.0 k	2883.3 g	1763.1 j	940.4 j	39.77 h	21.19 d	16.69	9.50 d
HA ₀ +B ₁	3959.1 g	4133.2 d	2094.7 i	1430.9 e	54.61 e	35.66 a	14.74	12.07 b
HA ₀ +B ₂	4693.2 d	4046.6 e	2222.1 g	1334.2 g	51.68 g	34.14 b	15.54	11.44 b
HA ₂₀ +B ₀	3527.7 j	3974.1 f	2166.4 h	1236.5 I	51.10 g	34.83 b	16.29	11.83 b
HA ₂₀ +B ₁	4083.3 f	4264.5 c	2557.8 d	1553.4 c	57.88 d	36.97 a	14.77	12.03 b
HA ₂₀ +B ₂	4933.8 c	4146.3 d	2429.0 f	1385.9 f	53.45 f	36.10 a	15.21	12.49 b
HA ₃₀ +B ₀	3701.5 i	4116.8 d	2441.7 f	1327.4g	54.82 e	34.05 b	15.92	10.98 c
HA ₃₀ +B ₁	4306.4 e	4530.5 a	2607.0 c	1685.4 b	63.14 b	36.38 a	14.40	13.49 a
HA ₃₀ +B ₂	5176.5 b	4252.4 c	2510.1 e	1519.0 d	58.69 d	36.69 a	14.78	11.77 b
HA ₄₀ +B ₀	3854.6 h	4229.5 c	2488.6 e	1273.4 h	57.95d	32.84 c	15.22	11.81 b
HA ₄₀ +B ₁	4298.9 e	4498.4 a	2873.2 a	1702.6 a	65.15 a	33.76 b	13.69	13.68 a
HA ₄₀ +B ₂	5364.8 a	4395.2 b	2769.1 b	1450.3 e	61.20 c	37.38 a	14.32	11.87 b
Significance	**	**	**	**	**	**	ns	**
CV (%)	1.76	2.16	2.07	1.60	2.32	2.94	3.78	4.89

¹20, 30 and 40 treatments refer to safflower plants growing under field condition with 200, 300 and 400 kg ha⁻¹ of HA; B₀ treatment without any application of the bacteria, B₁ treatment with *Bacillus subtilis* OSU-142 inoculation; B₁+20, B₁+30, and B₁+40 with *Bacillus subtilis* OSU-142 and 200, 300 and 400 kg ha⁻¹ of HA, respectively; B₂ treatment with *Bacillus megatorium* M3 inoculation; B₂+20, B₂+30, and B₂+40 with *Bacillus megatorium* M3 and 200, 300 and 400 kg ha⁻¹ of HA, respectively. Means followed by different letters are different by LSD test. *: P < 0.05, **: P<0.01, ns: not significant; CV: coefficient of variations

The study results indicated that increasing the humic acid dose from 200 kg ha⁻¹ to 400 kg ha⁻¹ was accompanied by a linear increase in important mineral contents such as the P, K, Ca and Fe (Table 4). Furthermore, plant growth promoting rhizobacteria inoculation significantly improved in these contents after combined application with humic acid dose. The highest P concentration was obtained with *Bacillus megatorium* M3 bacterial treatment in 400 kg ha⁻¹ humic acid dose by 95.6% increase when compared to control, while the highest K, Ca, Mg, Fe and Mn concentrations were recorded with the 400 kg ha⁻¹ humic acid dose with *Bacillus subtilis* OSU-142 bacterial inoculation (56.1%, 62.9%, 81.1%, 63.8% and 44.0% increases in K, Ca, Mg, Fe and Mn when compared to control, respectively). For Zn concentration, a significant interaction between the HA and PGPR factors was observed, where the inoculation with plant growth promoting rhizobacteria strains in all of humic acid doses significantly increased Zn accumulation in seed about 68.3% - 76.4 %, compared to untreated safflower plants (Table 4, Fig. 4).

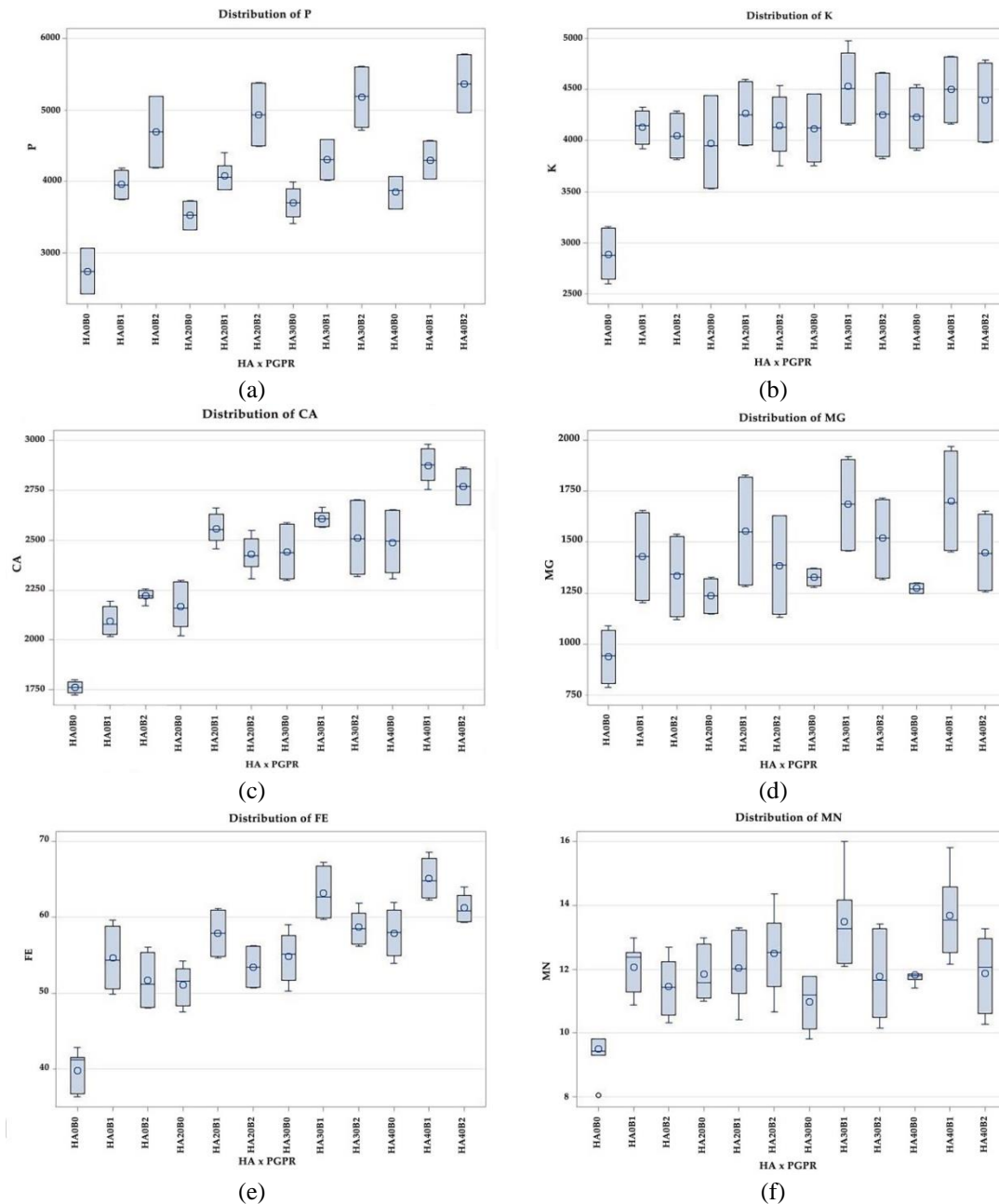


Figure 4. Phosphorus content (mg kg^{-1}) (a), potassium content (mg kg^{-1}) (b), calcium content (mg kg^{-1}) (c), magnesium content (mg kg^{-1}) (d), iron content (mg kg^{-1}) (e) and manganese content (mg kg^{-1}) (f) of safflower seeds affected by bacterial inoculation and humic acid. B₀: without bacteria; B₁: *B. subtilis* OSU-142; B₁+20, B₁+30, and B₁+40: *B. subtilis* OSU-142 with 200, 300 and 400 kg ha⁻¹ of HA, respectively; B₂: *B. megatorium* M3; B₂+20, B₂+30 and B₂+40: *B. megatorium* M3 with 200, 300 and 400 kg ha⁻¹ of HA, respectively

Discussion

In the present study, the plant growth-promoting rhizobacteria (*Bacillus megatorium* and *Bacillus subtilis*) and humic acids isolated from leonardite, individually or in combinations, was applied on safflower plants grown in the field trials under semi-arid conditions. Both plant growth promoting rhizobacteria inoculation and humic acid doses

significantly increased the growth parameters, seed yield and mineral concentrations in seeds (except for Cu) in comparison with control plants (grown in without PGPR and HA application), and further improved when applied in combination with humic acids. Inoculation of N₂-fixing *Bacillus subtilis* strain OSU-142 effectively improved plant growth when compared to P-solubilizing *Bacillus megatorium* strain M3 and control, and increased plant height by 21.8%, stem diameter by 11.8%, numbers of branch and capitula per plant by 22.1 and 34.5%, respectively, number of seeds per capitulum by 17.1, seed and oil yields by 47.1 and 61.5%, when compared to the untreated control plants. Inoculation of *Bacillus megatorium* M3 produced the highest P concentration in seed, while the highest K, Ca, Mg, Fe and Mn concentrations were recorded with *Bacillus subtilis* OSU-142 bacterial inoculation (56.1%, 62.9%, 81.1%, 63.8% and 44.0% increases in K, Ca, Mg, Fe and Mn when compared to control, respectively). The inoculation with PGPR strains in all of humic acid doses significantly increased Zn accumulation in seed about 68.3% - 76.4 %, compared to untreated safflower plants. Nitrogen and water requirements are the major limiting factors in safflower plant growth and productivity (Dajue and Mündel, 2006). Cakmakci et al. (2001) reported that inoculation of N₂-fixing *Bacillus subtilis* OSU-142 may satisfy nitrogen requirements of sugar beet and barley under field conditions in highland areas, and this bacteria inoculant significantly improved seed yields. Another field studies indicated that *Bacillus megatorium* might have phosphorus dissolving ability since it gave similar increases in growth and yield consistently to alone P application (Cakmakci et al., 2006; Orhan et al., 2006; Elkoca et al., 2010). Furthermore, the various studies with *Bacillus subtilis* and *Bacillus megatorium* reported remarkable growth and yield increases in potato (Ekin, 2019), common bean (Elkoca et al., 2010), sour cherry (Karakurt et al., 2011), raspberry (Orhan et al., 2006), apricot (Esitken et al., 2003) and some turfgrass species (Acikgoz et al., 2016). However, no data were reported on growth promotion with inoculation of this *Bacillus* genus in safflower plants. The literature on plant growth promoting rhizobacteria regarding safflower included mostly *Pseudomonas*, *Azospirillum* and *Azotobacter* spp. inoculation which were used to improve nitrogen uptake (Mirzakhani et al., 2009; Soleymanifard and Sidat, 2011; Sharifi et al., 2017; Nosheen et al., 2018), root morphology (Nosheen et al., 2011) and protein quantity and quality of safflower seeds (Nosheen et al., 2016). These researchers mainly observed marked changes in primary safflower metabolism in response to plant growth-promoting bacterial species; there were also strong effects of PGPR applied in improving root morphology and plant growth, and increasing endogenous hormonal levels (indole-3-acetic acid and gibberellic acid) in safflower leaves.

In the present study, humic acid treatment increased growth parameters and yields above values in controls (Tables 2 and 3). The increasing the humic acid dose from 200 kg ha⁻¹ to 400 kg ha⁻¹ generally was accompanied by a linear increase in growth parameters (except capitulum diameter and sterile seed percentage). Humic acid doses affected not significantly capitulum diameter only, while 200 kg ha⁻¹ humic acid dose significantly decreased the sterile seed percentage compared to the control (Tables 2 and 3). However, the highest seed yields recorded with the application of 300 and 400 kg ha⁻¹ humic acid doses which was statistically similar (Table 3). In different studies, safflower crops showed consistent increases with humic acid treatments at the different locations (Basalma, 2014; Mehraban and Miri, 2017). Enhanced plant growth and seed yield were also suggested to explain the beneficial effects of humic acid. Hajghani et al. (2016) similarly promoted the safflower growth and development as well as seed and oil

yields by humic substance application. Previous reports had shown that the beneficial effect of humic substances on plant growth may be related to increased fertilizer and water use efficiency, reducing soil compaction, enhancing total root area (especially lateral root emergence and root hairs) and plant biomass (Canellas and Olivares, 2014) and increasing soil microflora (Puglisi et al., 2013). In various field experiments, direct effects of humic acids on plant growth were well described in cultivation of various crops, such as potato (Suh et al., 2014), tomato (Olivares et al., 2015), maize (Canellas et al., 2013) and Hungarian vetch (Esringu et al., 2016). However, Canellas and Olivares (2014) recently reviewed the effects on plant metabolism of humic substance alone and in combination with plant growth promoting rhizobacteria, and their agronomical relevance.

Humic acid and plant growth promoting rhizobacteria application significantly affected plant growth development and production of safflower seed; however, their co-application further increased these agronomical parameters. Inoculation with *Bacillus subtilis* OSU-142 triggered ameliorative effects in terms of seed and oil yields at both 300 and 400 kg ha⁻¹ humic acid doses; these effects enhanced seed and oil production by average 162% and 221%, respectively, relative the control plants in semi-arid conditions (Table 3, Fig. 3). Previous reports had shown that integrated effects of plant growth promoting rhizobacteria and humic acid further increased plant growth, seed yield and quality in safflower (Mirzakhani et al., 2009; Sharifi et al., 2017), probably because of synergisms between improved nutritional status and plant stimulation factors; the net effects increased branch numbers, capitulum and stem diameters, 1000 seed weight, seed oil percentage, seed and oil yields and mineral compositions of seeds (Figs. 2, 3 and 4). Furthermore, humic substances are the most complex and biologically active organic matter compounds in the soil and stimulates both plants and microbial activities including plant growth promoting rhizobacteria through a number of mechanisms (e.g. through humic extracts of leonardite, compost or other organic fertilizer) (Nardi et al., 2009; Puglisi et al., 2013; Canellas and Olivares, 2014). PGPR enhancement of soil is largely attributable to biological N₂ fixation, phosphorus solubilization and increases in the availability of nutrients in the rhizosphere. Availability of nutrients increases in response to root surface area enhancement, which is one of several architectural and biochemical changes in the root system induced by humic substance (Nardi et al., 2009; Canellas and Olivares, 2014). Inoculation of humic acids with plant growth promoting rhizobacteria may be used for both plant growth stimulation (Dobbss et al., 2010; Busato et al., 2012; Schoebitz et al., 2016; Silva et al., 2017; Shah and Wu, 2019) and plant disease protection (Cakmakci et al., 2001). The studies with co-application of humates and different PGPR strains previously reported remarkable growth and yield increases in safflower (Sharifi et al., 2017; Nosheen et al., 2018).

Conclusions

Recently, interest in safflower production has been renewed as a result of drought tolerance and the suitability of its oil for nutritional, industrial or biofuel purposes. This study shows that safflower variety Dincer adapted well to semi-arid conditions of Turkey and is a viable alternative to other oilseed crops. In the study, alone application of safflower seeds with both humic acid and plant growth-promoting rhizobacteria (*Bacillus megatorium* and *Bacillus subtilis*) produced highly increases in terms of seed

and oil yields of safflower in semi-arid area. However combined application of plant growth promoting rhizobacteria and humic acid significantly improved growth and yield more than alone application of each partner under rainfed field condition. This increase was also supported by the enhancement of nutrient contents in the safflower seeds. Finally, the *Bacillus* inoculants should be assessed for their potential to increase crop yields and food production under real field environment and this integrated approach could become an effective method for safflower production under semi-arid conditions in sustainable agriculture systems.

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