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Integrated Effect of Organic, Biological, and Chemical Fertilizers on Roselle's (*Hibiscus sabdariffa* L.) Growth and Quality Characteristics

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ABSTRACT

This study investigates the effects of integrated nutrient management on Roselle's quantitative and qualitative traits (*Hibiscus sabdariffa*). The experiment assessed various treatments in a randomized complete block design with three replications over the 2021-22 and 2022-23 cropping seasons in the Jiroft region of Iran. These included 100% recommended poultry manure (PM), 100% recommended chemical fertilizer (CF), inoculation with arbuscular mycorrhizal fungi (AMF), application of *Azospirillum* (*Az*), their combined application (AMF + *Az*), and various combinations with reduced organic and chemical fertilization. Results showed that significant impacts of fertilizer treatments on Roselle's growth and quality traits. Specifically, the treatments of 50% PM + *Az* and 50% PM + 50% CF yielded the highest plant height, total number of bolls per plant, bolls fresh weight per plant, sepal dry weight, biomass yield, and sepal harvest index. Moreover, the highest quality traits were observed in the 50% PM + *Az* and 50% PM + AMF + *Az* treatments. These integrated treatments are recommended for reducing CF usage while enhancing Roselle's production and quality.

Keywords: *Azospirillum*, Mycorrhiza, Poultry manure, Sepal yield

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INTRODUCTION

Roselle (*Hibiscus sabdariffa* L.), an annual or perennial plant from the Malvaceae family, due to its versatility and multiple uses, has a long history of food and medicinal uses [1]. The plant's most crucial economic organ is the fleshy sepal surrounding the fruit, which contains organic acids, vitamin C, protein, minerals, and anthocyanin [2].

The content of effective compounds in Roselle sepals is influenced by agricultural management practices, such as field fertilization [3]. In the arid and semi-arid regions, soil's low nutrient and organic matter content and reliance on conventional agricultural methods and CFs have led to unstable production systems [4]. Therefore, organic inputs such as organic and biological fertilizers containing beneficial microorganisms are suggested. These compounds affect plant growth and performance, maintain soil fertility, and promote sustainable production [5]. The results of researchers' studies have shown the beneficial effect of using organic and environmentally friendly fertilizers in improving the traits of the medicinal plant [6]. One such organic fertilizer is poultry manure (PM), which, in addition to having a suitable decomposition speed, contains higher nitrogen levels than other organic fertilizers [7].

Bio-fertilizers contain beneficial microorganisms that stimulate plant growth by colonizing the rhizosphere or internal plant tissues. These microorganisms bioavtive compounds that enhance the plant's ability to absorb mineral nutrients, thereby improving plant growth [8]. *Azospirillum*, a beneficial bacterium and plant growth stimulant, facilitates the plant's absorption of essential elements, increases the solubility of insoluble phosphates, and creates synergistic relationships with other beneficial soil bacteria [5].

Among fungi, mycorrhizal fungi are significant biological resources with high efficiency in producing growth-stimulating factors and providing nutrients in an absorbable form [9]. Researchers are increasingly investigating the beneficial effects of the coexistence of these microorganisms with various plants. Studies have reported improved growth rates and enhanced effective substances in basil (*Ocimum basilicum* L.) [10] and peppermint (*Mentha piperita* L.) [11] when inoculated with mycorrhizal fungi. In studies on applying organic and inorganic fertilizers to Roselle, researchers observed increased quantitative yields with the combined application of PM and ostrich manure [12]. Al-Sayed *et al*. [13] positively reviewed the effects of *Azospirillum* on many characteristics of Roselle. Conversely, Ghanbari *et al*. [14] reported a negative effect of mycorrhizal inoculation and CFs on the phenolic content of saffron, while compost application had a positive impact. Other studies also observed increased sepal anthocyanin content with bio-fertilizer application on Roselle [15].

Despite the positive effects reported in many studies, the combined application of PM and chemicals under simultaneous inoculation with *Azospirillum* and mycorrhizal fungi, particularly in Roselle, has not been accurately investigated. Therefore, this research aims to examine the effects of organic, chemical, and biological fertilizers on some quantitative and qualitative traits of

Roselle and to identify a suitable integrated fertilizer treatment to reduce CF use and increase the fertilizer use efficiency.

MATERIALS AND METHODS

Location of Experiment

This study was conducted over two cropping seasons (2021 and 2022) in the Jiroft region of Iran (latitude: 28°40′ N, longitude: 57°45′ E, elevation: 680 m). According to the UNESCO classification, the region has a dry climate with extremely hot summers and mild winters [16]. Meteorological data from the study period and the soil's physical and chemical properties (0-30 cm depth) are detailed in Tables 1 and 2, respectively.

The Fertilizers Used in the Experiment

The experiment followed a randomized complete block design with three replications. Treatments included inoculation with arbuscular mycorrhizal fungi (AMF), application of *Azospirillum* (*Az*), simultaneous application of AMF and *Az* (AMF+ *Az*), 100% recommended poultry manure (100%PM), 100% recommended chemical fertilizer (100% CF), combinations of 50% PM with 50% CF, AMF, or *Az*, 50% PM+ AMF+ *Az*, combinations of 50% CF with AMF or *Az*, 50% CF+ AMF+ *Az*, and a mixed treatment of 25% PM+ 25% CF+ AMF+ *Az*. These were compared against control (no fertilizer and microorganism application).

Table 1 Meteorological statistics of the studied area.

Year	Month	Average temperature (^{0}C)	Average maximum relative humidity (%)	Average minimum relative humidity (%)	Average total evaporation (mm)	Average total sunshine hours per month (h)
2021-2022	May	37.73	43.90	16.70	445.6	267.1
	June	37.20	44.45	16.29	403.8	199.6
	July	36.83	34.25	12.41	338.8	207.9
	Aug.	34.66	36.23	11.26	249.7	231.8
	Sept.	28.14	37.16	16.80	182.1	225.2
	Oct.	21.11	41.06	22.03	138.0	189.6
2022-2023	May	36.92	29.60	10.80	443.3	288.9
	June	35.40	57.35	20.58	289.5	225.0
	July	36.20	20.70	10.29	402.4	202.5
	Aug.	33.81	38.76	10.56	341.7	243.5
	Sept.	31.00	24.74	8.51	326.7	260.8
	Oct.	24.30	53.53	20.90	139.6	203.8

Table 2 The physical and chemical characteristics of the used soil in this experiment (depth of 0-30cm).

Year	EC (dS/m)	pH	Total N (%)	Organic matter (%)	Available phosphorous (ppm)	Available potassium (ppm)	Clay (%)	Silt (%)	Sand (%)
2021-2022	1.5	7.1	0.027	0.34	21	100	10	12	78
2022-2023	1.8	7.5	0.075	0.52	22.4	145	10	12	78

Table 3 Some chemical properties of poultry manure used.

EC (dS/m)	pH	Total N (%)	Organic carbon (%)	Phosphorous (ppm)	Potassium (ppm)
4.5	7.5	3.58	34.02	2.43	4.4

Fertilizer requirements were estimated prior to the experiment, and the application rates of animal and chemical fertilizers were determined based on regional guidelines and previous studies. Relevant details have been added to the Materials and Methods section. Each experimental plot measured 1.8 × 3 meters, with a spacing of 60 cm between rows, 30 cm between plants within rows, and 1 meter and 2 meters between plots and replications, respectively [17]. For CFs, triple superphosphate ($Ca(H_2PO_4)_2$) (160 kg/ha), potassium sulfate (K_2SO_4) (264 kg/ha), and one-third of urea (CH_4N_2O) (234 kg/ha) were applied before seed sowing. Additionally, one-third of the urea was added during the vegetative stage and another third before flowering. For the PM treatment, 20 days before planting, the required amount (based on 3 ton/ha) was mixed into the soil. AMF (*Funneliformis mosseae*) (spores mixed with associated material), sourced from the Organic Plant Protection Clinic in Asadabad, Hamadan, were applied under the seedbed during planting at a rate of 5 grams per planted seed (based on the amount recommended by the relevant company). For growth-promoting bacteria treatments, seeds were inoculated with an *Azospirillum* solution, strain *Azospirillum lipoferum* (10^7-10^8 CFU/g) before planting and applied at the seed site. The Research and Development Department of Green Biotech Incorporation, Tehran, Iran, provided the *Azospirillum* bacteria. The seeds used were from the local population (Jiroft population) that, in both years, were cultivated on the 12th of June. Manual weeding and other farming practices were performed as per regional customs. Plants were harvested on November 22 in both years, eliminating marginal effects. Plants from the middle of each plot (excluding the first and last row margins) were harvested over one square meter to determine the biological yield and harvest index.

Measuring Qualitative Traits

The total acidity of Roselle sepal, anthocyanin content, and total phenolic content were measured using the sodium hydroxide titration method [18], the Wagner method [19], and the Folin-Ciocalteu reagent method, respectively [20].

Vitamin C content was determined using the iodometric method [21], while protein concentration was measured using the Bradford method with slight modifications [22].

Data Analysis

The effects of treatments on traits were analyzed using a combined analysis of variance (ANOVA) in SAS software (Ver 9.2). The Duncan test ($p< 0.05$) determined significant differences between means. The results of Bartlett's test are presented in Table 4.

Table 4 Bartlett's test (Chi-Square values) results for homogeneity of examined traits variance in Roselle

	Chi-Square										
	Plant height	Number of total bolls per plant	Boll's fresh weight	Sepal dry weight	Biomass yield	Harvest index sepal	Sepal's total acidity content	Sepal's phenol content	Sepal's anthocyanin content	Sepal's vitamin C content	Percentage of sepal's protein
Year	0.333 ^{ns}	0.095 ^{ns}	0.018 ^{ns}	0.028 ^{ns}	0.144 ^{ns}	0.158 ^{ns}	0.597 ^{ns}	0.026 ^{ns}	3.283 ^{ns}	0.449 ^{ns}	0.024 ^{ns}

* and ^{ns}, Significant at 5% probability levels and not significant, respectively

RESULTS

Plant Height, Number of Total Bolls, Boll Fresh Weight, and Sepal Dry Weight

Combined analysis of variance showed (Table 5) that different nutrient sources significantly affected plant height of Roselle, the total number of bolls per plant, fresh weight of bolls per plant, and dry weight of sepals per plant in both growing seasons ($P \leq 0.01$). The tallest plants were observed in the 50% PM + *Azospirillum* (*Az*) treatment, while the shortest plants were in the control group. The highest number of bolls per plant was observed in the 50% PM + *Az* treatment. The 50% PM + 50% CF treatment was not significantly different from 50% PM + *Az* treatment. The highest fresh boll weight per plant was recorded in the 50% PM + 50% CF, 50% PM + *Az*, and 50% PM + AMF + *Az* treatments, with increases of 83.75%, 75.90%, and 71.93% compared to the control, respectively (Table 6). For sepal dry weight, the highest values were recorded in the 50% PM + 50% CF treatment. The lowest fresh boll weight and sepal dry weight were found in the control and AMF inoculation (Table 6).

Table 5 Analysis of variance (mean squares) for Roselle's vegetative growth characteristics, yield, and yield components affected by different nutritional treatments during the 2021-2022 and 2022-2023 seasons.

Source of variance S. O. V	df	Mean of Squares (MS)					
		Plant height	Number of total bolls per plant	Boll's fresh weight per plant	Sepal dry weight per plant	Biomass yield	Harvest index sepal
Year	1	3.81 ^{ns}	12.04 ^{ns}	62.26 ^{ns}	1.37 ^{ns}	0.03 ^{ns}	0.26 ^{ns}
Block (Year)	4	62.92	10.94	535.88	0.28	0.92	4.09
Treatment	13	1398 **	742.43 **	40553 **	178.54 **	6.55 **	20.50 **
Year × Treatment	13	40.96 ^{ns}	2.37 ^{ns}	503.27 ^{ns}	0.58 ^{ns}	0.03 ^{ns}	0.41 ^{ns}
Error	52	95.59	13.14	636.80	0.75	0.36	1.30
C. V (%)	-	6.21	5.39	7.11	3.80	7.22	7.64

$^{*, **,}$ and ^{ns}, Significant at 5% and 1% probability levels and not significant, respectively.

Table 6 Mean comparison of vegetative growth characteristics, yield, and yield components of sepals Roselle affected by different nutritional treatments during the 2021- 2022 and 2022- 2023 seasons.

Treatment	Plant height (cm)	Number of total bolls per plant	Boll's fresh weight per plant (gr/plant)	Sepal dry weight per plant (gr/plant)	Biomass yield (ton/ha)	Harvest index sepal (%)
Control	118.96 f	52.75 g	278.81 f	15.90 g	6.94 c	12.62 e
AMF	142.31 e	58.60 efg	284.30 f	16.16 g	7.01 c	12.67 e
AZ	153.24 bcde	61.79 de	297.99 def	18.71 f	7.85 bc	13.18 de
AMF+ *AZ*	148.75 cde	61.79 d	297.97 def	18.69 f	7.46 bc	13.79 cde
100%PM	168.00 ab	74.43 c	396.92 b	26.66 c	9.64 a	15.25 bcd
100%CF	160.62 bcde	66.56 d	342.28 cd	22.28 e	7.97 bc	15.41 bc
50%PM+50%CF	168.31 ab	83.79 ab	512.32 a	31.45 a	9.85 a	17.60 a
50%PM+AMF	164.08 bcd	63.58 de	304.98 def	18.31 f	7.87 bc	12.82 e
50%PM+ *AZ*	183.68 a	88.27 a	490.44 a	30.11 ab	9.61 a	17.30 ab
50%PM+AMF+ *AZ*	166.55 ab	80.28 b	479.38 a	28.92 b	9.31 a	17.26 ab
50%CF+AMF	146.80 de	54.66 fg	290.86 ef	18.25 f	7.23 bc	13.94 cde
50%CF+ *AZ*	157.71 bcde	60.10 def	300.51 def	24.00 d	8.20 b	16.14 ab
50%CF+AMF+ *AZ*	159.37 bcd^{e}	60.47 def	333.42 cde	21.10 e	8.25 b	14.08 cde
25%PM+25%CF+AMF+ *AZ*	164.62 bcd	74.84 c	360.29 bc	28.75 b	9.47 a	16.75 ab

Means followed by similar letters in each column are not significantly according to Duncan test ($p \leq 0.05$). Control: no fertilizer and microorganism application, AMF: Arbuscular Mycorrhizal Fungi, *AZ*: *Azospirillum*, PM: poultry manure, CF: chemical fertilizer.

Biomass Yield and Harvest Index

Analysis of variance indicated that different nutrient sources significantly affected the biomass yield and harvest index of dried sepals of Roselle ($P \leq 0.01$) (Table 5). The highest biomass yields were observed in the 50% PM + 50% CF, 100% PM, 50% PM + *Az*, 25% PM + 25% CF + AMF + *Az*, and 50% PM + AMF + *Az* treatments. The lowest biomass yields were in the control and AMF inoculation treatments (Table 6).

The highest harvest index of dried sepals was in the 50% PM + 50% CF treatment, showing a 39.46% increase compared to the control. The lowest harvest index was in the control, AMF inoculation, and 50%PM+AMF treatments (Table 6).

Total Acidity of Roselle Sepal

Different nutritional sources in both growing seasons significantly affected the total acidity of Roselle sepals ($P \leq 0.01$) (Table 7). The highest total acidity was found in the 50% PM + *Az* treatment, which did not significantly differ from the 50% PM + AMF + *Az* treatment. The lowest total acidity was observed in the control treatment, with no significant difference from the 100% CF treatment (Table 8).

Total Phenol Content of Roselle Sepal

Different nutrient sources significantly affected the total phenol content of Roselle sepals in both growing seasons ($P \leq 0.01$) (Table 7). The highest phenol content was observed in the 25% PM + 25% CF + AMF + *Az* treatment, which did not significantly differ from the 50% PM + AMF + *Az* and 50% PM + *Az* treatments. The lowest phenol content was in the control treatment. Phenolic content increased significantly in the second year compared to the first year (Table 8).

Anthocyanin Content of Roselle Sepal

Analysis of variance showed a significant effect of different nutrient sources on the anthocyanin content of Roselle sepals in both cropping seasons (*P*≤0.01) (Table 7). The highest anthocyanin content was found in the 50% PM + AMF + *Az*, 50% PM + 50% CF treatments, while the lowest was in the control group. Anthocyanin content significantly increased in the second year compared to the first year (Table 8).

Table 7 Analysis of variance (mean squares) for Roselle's sepal qualitative attributes affected by different nutritional treatments during the 2021- 2022 and 2022- 2023 seasons.

Source of variance S. O. V	df	Mean of Squares (MS)				
		Sepal's total acidity content	Sepal's phenol content	Sepal's anthocyanin content	Sepal's vitamin C content	Percentage of sepal's protein
Year	1	0.014 ^{ns}	7.808 *	0.010 *	1.469 ^{ns}	0.829 ^{ns}
Block (Year)	4	0.022	4.060	0.001	4.062	0.740
Treatment	13	0.124 **	15.731 **	0.031 **	38.281 **	31.950 **
Year × Treatment	13	0.003 ^{ns}	0.320 ^{ns}	0.002 ^{ns}	2.654 ^{ns}	1.041 ^{ns}
Error	52	0.014	1.653	0.002	2.307	0.690
C. V (%)	-	8.72	8.74	8.10	7.04	9.04

*, **, and ^{ns}, Significant at 5% and 1% probability levels and not significant respectively.

Vitamin C Content of Roselle Sepal

Different nutritional sources significantly affected the vitamin C content of Roselle sepals (*P*≤0.01) (Table 7). The highest vitamin C content was in the 50% PM + *Az* treatment, which did not significantly differ from the 50% PM + AMF + *Az* and 50% PM + 50% CF treatments. The lowest vitamin C content was in the control treatment (Table 8).

Table 8 Mean comparison of qualitative attributes of sepals of Roselle affected by different nutritional treatments during the 2021-2022 and 2022-2023 seasons.

	Sepal's total acidity content (gr/100 gr dry matter)	Sepal's phenol content (mgr/gr dry matter)	Sepal's anthocyanin content (µmol/gr dry matter)	Sepal's vitamin C content (mgr acid ascorbic /100cc extract)	Percentage of sepal's protein (%)
Year					
2021- 2022	1.326 a	14.405 b	0.484 b	21.433 a	9.097 a
2022- 2023	1.351 a	15.014 a	0.506 a	21.698 a	9.295 a
Different nutritional sources					
Control	0.956 e	11.707 e	0.385 g	16.027 f	6.736 f
AMF	1.243 cd	13.448 de	0.415f g	17.680 ef	7.178 ef
AZ	1.274 bcd	13.560 de	0.430 efg	20.696 cd	8.373 de
AMF+ *AZ*	1.315 abcd	14.495 bcd	0.465 def	21.806 bcd	7.602 def
100%PM	1.462 abc	15.384 abcd	0.577 ab	23.463 abc	12.502 a
100%CF	1.183 de	13.329 de	0.412 fg	19.360 de	7.434 ef
50%PM+50%CF	1.417 abc	16.008 abc	0.544 abc	24.073 ab	10.517 b
50%PM+AMF	1.379 abcd	13.650 cde	0.518 bcd	21.711 bcd	7.533 ef
50%PM+ *AZ*	1.523 a	16.327 ab	0.592 a	25.130 a	13.652 a
50%PM+AMF+ *AZ*	1.474 ab	16.631 ab	0.604 a	24.130 ab	12.752 a
50%CF+AMF	1.365 abcd	13.576 de	0.465 def	21.120 cd	7.310 ef
50%CF+ *AZ*	1.394 abcd	15.227 bcd	0.469 def	22.127 bcd	8.261 def
50%CF+AMF+ *AZ*	1.346 abcd	14.847 bcd	0.491 cde	21.703 bcd	9.067 cd
25%PM+25%CF+AMF+ *AZ*	1.408 abc	17.719 a	0.561 abc	22.893abc	9.832 bc

Means followed by similar letters in each column are not significant according to Duncan test (*p≤ 0.05*). Control: no fertilizer and microorganism application, AMF: Arbuscular Mycorrhizal Fungi, *AZ*: *Azospirillum*, PM: poultry manure, CF: chemical fertilizer.

Protein Content of Roselle Sepal

Analysis of variance indicated that different nutritional sources significantly affected the protein content of Roselle sepals (*P*≤0.01) (Table 7). The highest protein content was observed in the 50% PM + *Az*, 50%PM+ AMF+ *Az*, and 100% PM treatments. The lowest protein content was in control (Table 8).

DISCUSSION

Quantitative and Growth Characteristics of Roselle Sepals

The results indicated that the maximum values for all quantitative traits of Roselle (*H. sabdariffa*) were achieved through combined treatments. Like other vegetative or reproductive organs, plant height, the fresh and dry weight of vegetative parts are significantly influenced by nutrient elements and water availability [23]. Using eco-friendly inputs in agriculture can lead to increased soil cation exchange capacity, improved nutrient transfer to plants, increased soil nitrogen content and aeration, and higher soil moisture levels [24]. In addition to the report by Naderi *et al*. [25] that the highest Hyssop (*Hyssopus officinalis* L.) height was observed from the cow manure nutritional treatment, Dahmardeh (12) reported the highest plant height in Roselle from a combined treatment of poultry and ostrich manure.

The positive impact of growth-promoting microorganisms around plant roots in stabilizing certain nutrients, improving soil structure, reducing pollution from CFs, and consequently increasing yield has also been documented [26]. Al-Sayed *et al*. [13] attributed the increased plant height under bio-compound applications to optimal nutrient absorption, enhanced synthesis, and secretion of growth-stimulating substances, leading to increased cell division, internode elongation, and overall plant height. In this study, the positive role of combined PM and *Azospirillum* bacteria in improving moisture and nutrient absorption resulted in increased plant height, total number of bolls per plant, boll weight per plant, and sepal dry weight.

According to the results, nitrogen fixation by *Azospirillum* and PM presence increased vegetative growth under the combined treatment. Existing reports suggest that bio-fertilizers, by enhancing soil microbial activity, secreting growth-promoting phytohormones, and consequently increasing the availability of nutrients from stimulatory substances, lead to enhanced photosynthesis rate, vegetative growth, and thus improved dry matter production [27].

Many researchers have reported that integrating fertilizer sources is better than using these compounds separately due to better soil nutrient balance, resulting in more efficient plant utilization [28]. In this study, improving the plant's nutritional status and increasing its access to water by enhancing the physical properties of soils with livestock manure (especially in light-textured soils) leads to increased plant growth, more flowers, and, subsequently, more fruits per plant. Additionally, using bio-fertilizers to increase nutrient availability for the roots and stimulate plant growth will increase yield components and dry sepal weight. Gomaa *et al*. [29] also reported the highest number of total bolls per plant and maximum fresh and dry boll weight of Roselle from the combined treatment of 30 tons of compost and bio-fertilizers containing nitrogen-, phosphorus-, and potassium-fixing bacteria, compared to chemical, organic, and biological fertilizers. Some researchers have attributed the increased number of fruits to increased nitrogen and phosphorus absorption when using *Azotobacter* and *Azospirillum* bacteria. These researchers noted the accumulation of dry matter and minerals in the leaves and stems of the plants following the application of these resources, which ultimately move toward the fruits during their growth period [30].

In this study, achieving the highest biomass yield from the combined treatment of 50% PM + 50% CF indicates that using 50% of the recommended CFs with PM improves plant growth. In other words, the applied PM provided the essential nutrients for plant growth and was effective even when using half the amount of CF. Pandey *et al*. [31] also reported that PM, by increasing the organic matter and humus in the soil, thus increasing the soil's cation exchange capacity and phosphorus availability, and being rich in other elements, led to increased biomass in treated plants. Other researchers also evaluated the positive effect of organic compounds on chicory plant growth [32]. The harvest index is a critical metric for evaluating the investment of crops in economic organs. The highest harvest index for Roselle sepal was observed in treatments containing 50% PM. Additionally, by providing nutrients to the plants, bio-fertilizers create an optimal condition for plant growth and improve the allocation of photosynthates to the reproductive parts in combination with organic fertilizers, resulting in the highest harvest index. Rezvani Moghadam *et al*. [28] reported the highest harvest index of dried Roselle sepal from the combined treatment of CFs and cow manure. The observations from this study align with the findings of researchers who stated that the combined use of manure and biological fertilizers is a significant factor in increasing growth sepal yield [27].

Qualitative Characteristics of Roselle Sepals

Based on Han's [33] research, adding organic compounds to soil enhances plant growth by increasing the availability of both macronutrients and micronutrients. Specifically, using PM reduces soil pH, enhancing plant nutrient availability [34]. Kianimanesh *et al*.'s [35] observations also showed that the use of organic compounds, in addition to improving the quantitative growth of saffron, increased the quality of the product.

In this study, the highest titratable acidity of Roselle sepals was observed in the combined treatment of PM and *Azospirillum*. Weaam *et al*. [36] reported improved acidity and anthocyanin content in Roselle sepals with combined bio-fertilizer treatments over two years of organic cultivation using natural minerals, organic, and biological fertilizers.

Phenolic compounds are the most common phytoconstituents and crucial components of medicinal and aromatic plants that are effective antioxidants [37]. They play roles in many physiological activities, including cell growth [38]. While both environmental and genetic factors influence the production of these secondary metabolites, genetic factors have a more substantial impact [39]. Organic fertilizers can raise secondary metabolite levels by increasing plant sugars and carbon [40]. The significant differences in phenolic content over the two studied years may be due to variations in environmental conditions [41], such as light intensity [42], cumulative average evaporation and relative humidity, which affect phenolic content in Roselle sepals. Therefore, the inability to control these factors explains the variation in phenol content observed in Roselle sepal between the two years.

Anthocyanin, a glucoside compound in plant tissues, is a broad group of secondary plant metabolites influenced by various factors such as nutrients, temperature, water availability, and light [43]. Kahil *et al*. [15] observed the highest anthocyanin content in Roselle sepals and the highest vegetative yield in treatments combining nitrogen- and phosphorus-fixing bacteria. Livestock manure enhances soil physical properties, increases root nutrient absorption, and boosts vegetative biomass and photosynthetic surface area, leading to higher production of anthocyanin [44]. Gomaa *et al*. [29] reported the highest anthocyanin levels with the highest amounts of organic and bio-fertilizer applications, while Abou-Sreea *et al*. [45] noted the highest anthocyanin content with combined treatments of cow manure, CFs, and royal jelly. The findings of this study align with those of other researchers, indicating that organic fertilizer positively impacts anthocyanin content and that *Azospirillum* bacteria also play a crucial role. The statistical difference in anthocyanin content within Roselle sepals between the first and second years, as shown in Table 1's meteorological data, could be attributed to stress factors such as higher average temperatures, increased total sunlight hours, and increased overall evaporation experienced during the plant's growth stages in the second year.

According to reports, by balancing nutrient uptake, bacteria enhance plant growth and improve the nutritional value of fruits by maintaining internal plant compound balance [46, 47]. Adequate availability of potassium and phosphorus is crucial for improving the vitamin C quality index in various crops. The enhancement of these elements' uptake through growth-promoting bacteria is well-documented [48]. The presence of growth-promoting bacteria, due to their significant role in rapid root system development and increasing the green surface area of the field, improves the quality of target crops, including increased vitamin C content. Although many factors, such as crop type, genotypic differences, pre-harvest climatic conditions, agricultural practices, and harvest conditions, influence this trait, the positive effect of nitrogen-fixing bacteria on vitamin C content in tomatoes has also been reported [49]. Recent studies show that biofertilizers enhance nutrient uptake by improving both soil nutrient availability and plant physiological responses.

Microbes such as PGPR and mycorrhizal fungi mobilize nitrogen, phosphorus, and micronutrients, while also stimulating root growth, branching, and nutrient transporter activity. These interactions increase root surface area, enhance photosynthesis through improved N and P supply, and strengthen plant metabolism, resulting in higher nutrient-use efficiency and better stress tolerance [50, 51]. The positive impact of PM application in the plant growth environment to increase nitrogen, along with the nitrogen-fixing effect of *Azospirillum* bacteria, were among the main reasons for the positive effect of the combined treatment of PM + *Azospirillum* bacteria on protein content in this study.

CONCLUSION

In this study, the positive effect of PM combined with *Azospirillum* bacteria was observed for many traits. For some traits, the combination with CFs also showed positive effects. Additionally, for the evaluated qualitative traits, the combined use of PM and *Azospirillum* bacteria had a more positive impact than the combined use of PM and CFs. Therefore, the application of organic fertilizers, in combination with *Azospirillum* bacteria, can be recommended as a suitable solution for achieving maximum sustainable yield in arid and semi-arid regions. These comprehensive findings highlight the significant benefits of combining organic and bio-fertilizers to enhance the qualitative characteristics of Roselle sepals, including increased acidity, phenolic compounds, anthocyanin, vitamin C, and protein content. Integrating these practices can improve plant growth, yield, and quality, providing a sustainable and eco-friendly approach to agriculture.

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