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Effect of Organic Fertilizer and Plant Growth-Promoting Microbes on Growth, Flowering, and Oleanolic Acid Content in *Calendula officinalis* under Greenhouse Conditions

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THE CULTIVATION of *Calendula officinalis* has gained importance not only for its medicinal properties but also for its ornamental value. *C. officinalis* flowers contain carotenoids and oleanolic acid, valuable phytoconstituents with various medicinal properties. However, to meet the increasing demand for this plant, it is necessary to adopt sustainable cultivation practices that optimize growth, enhance flowering, and maximize the content of bioactive compounds. An experiment was conducted under greenhouse conditions during the 2021/2022 and 2022/2023 seasons on *C. officinalis* using chemical fertilizer (NPK-RD: recommended dose of NPK), organic fertilizer (poultry manure), and biofertilizers. The biofertilizers included different combinations of plant growth-promoting microbes: N-fixing bacteria (Az), P-solubilizing bacteria, K-solubilizing bacteria (Bc), and vesicular-arbuscular mycorrhizal (VAM) fungi. The results showed that the application of poultry manure was more pronounced compared to biofertilizers. The principal component analysis (PCA) revealed that NPK-RD, moderate and higher levels of organic fertilizer, and Az+Bc+VAM treatments achieved significant superiority in all tested parameters. A higher dosage of poultry manure (95.20 m^3 ha^{-1}) was significantly superior to chemical fertilizer in plant height, branch number, plant dry weight (DW), chlorophyll, carbohydrates, flower number, flower yield fresh weight (FW), and carotenoids content. However, the flowering start, flowering period, flower yield DW, and oleanolic acid content obtained by poultry manure at 95.20 m^3 ha^{-1} were significantly equal to NPK-RD treatment. The inoculation with Az+Bc+VAM microbes positively impacted carotenoids content in the *C. officinalis* flower. These results underline the potential of organic fertilizer and plant growth-promoting microbes in improving growth, flowering and phytochemical quality of the *C. officinalis* plant with the possibility of avoiding chemical fertilization.

Keywords: Marigold; VAM fungi; organic farming; carotenoids; flowering quality and secondary metabolites.

1. Introduction

Ornamental and medicinal plants are an essential source of affordable agricultural revenue and are among the oldest plant species. Additionally, there has been a recent boom in medicinal plants and floriculture cultivation. Among them is *Calendula officinalis* L., or marigold, a plant in the Asteraceae family. While *C. officinalis* is native to the Mediterranean region, it is grown worldwide, including in China, Europe, and the United States (Soliman et al., 2024). *C. officinalis* is a winter annual herb for its decorative and therapeutic blossoms. It has simple leaves, a lengthy root system with several secondary roots, a branching stem, and a maximum height of 70 to 90 cm. It blooms in winter and spring. Two rows of hairy bracts encircle the inflorescence of the flower head (Jan et al., 2017). *C. officinalis* is grown extensively

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in many countries across the world, including Poland, Germany, Egypt, China, Russia, Hungary, and Bulgaria (Ruzmetov et al., 2020).

C. officinalis has been used as an ornamental plant in landscape gardening and is planted in beds and borders. In addition, the vivid yellow-to-orange colors of the flowers make them appealing for use as cut flowers (Barut and Tansi, 2024). Conversely, *C. officinalis* is grown professionally as a medical herb. The bioactive substances found in flowers include flavonoids, coumarins, oleanolic acid, terpenoids, glycosides, carotenoids, and volatile oils (Kurkin and Sharova, 2007; Crabas et al., 2011; Ullah et al., 2023). The flower of the *C. officinalis* plant is frequently used to nourish the skin, cure wounds, and relieve gum disease. It also possesses anti-inflammatory, antioxidant, antihelminthic, antidiabetic, antimicrobial, anticancer, and hepatoprotective effects (Jan et al., 2017; Shahane et al., 2023). A recent study conducted by Zhang et al. (2024) revealed that bioactive compounds, including chlorogenic acid, 3, 4-dicaffeoylquinic acid, rutin, isorhamnetin 3-O-glucoside, and calenduloside E extracted from *C. officinalis* flowers act via PI3K and ERK signaling pathways to provide neuroprotective effects against Parkinson's disease.

Oleanolic acid and carotenoids have both been recognized for their medicinal value in numerous studies. Oleanolic acid, a natural triterpenoid compound, has shown promising therapeutic potential. It exhibits anti-inflammatory, antioxidant, hepatoprotective, and anticancer activities, among others (Wang and Liu, 2024). It has also shown promise in promoting wound healing and protecting against cardiovascular diseases (Luo et al., 2024). Carotene, a group of pigments responsible for the orange colour in many flowers, fruits, and vegetables, is a precursor of vitamin A. It possesses significant antioxidant properties and is known for its beneficial effects on vision, immune function, and overall health. Carotenoids, including beta-carotene, alpha-carotene, and lycopene, have been associated with a reduced risk of chronic diseases, including certain types of cancer, cardiovascular diseases, and age-related macular degeneration (González-Peña et al., 2023).

The 17 global Sustainable Development Goals are directly or indirectly intertwined with soils due to their connection to factors like crop productivity, environmental sustainability, and human health (El-Ramady et al., 2022). The effective management of soils in a sustainable manner poses a significant obstacle in ensuring future global food security (El-Ramady et al., 2022). One of the main goals of sustainable agriculture nowadays is to reduce the usage of chemical fertilizers to maintain sustainable crop productivity (Moradzadeh et al., 2021). Excessive chemical fertilization of plants causes several problems, such as those related to the environment, loss of soil fertility or quality, contamination of water sources, decreased plant response to fertilizers, decreased plant resistance to pests or diseases, and finally, an impact on the quality of crops and food (Mustafa et al., 2023). As a result, the use of organic or biofertilizers is a critical component of sustainable agriculture since it lessens the need for chemical fertilizers, encourages "greener" alternative production systems, and improves the use of sustainable and environmentally friendly techniques for growing plants (Anderson, 2013; Gamage et al., 2023; Mosaad et al., 2024).

Quality and production metrics are positively impacted by using organic and biofertilizers in cultivating crops of high economic value (Onofrei et al., 2017; Nada et al., 2022; El-Beltagi et al., 2023). Furthermore, improving nutrient status and lowering the rate of chemical fertilization applied are two significant goals of organic farming. The best method for enhancing the physical properties of soil and chelating nutrients in the soil is generally organic fertilization (Singh et al., 2024). Horticultural crops grown organically are of higher quality and yield a safe product that humans may use (Nada et al., 2022; Abou Elhassan et al., 2023; Awad et al., 2024). Previous research on *C. officinalis* showed that applying organic fertilization enhanced the plant's vegetative growth, flowering characteristics, and chemical composition (Kheiry et al., 2016; EL-Zawawy et al., 2021; Filipović et al., 2023). Microbial inoculants, such as bacteria, algae, and fungi, are applied to seeds or soil to act as biofertilizers. Because biofertilizers fix nitrogen and solubilize phosphorus and potassium, they can increase soil fertility and the availability of minerals in fertilizers and the soil, which in turn stimulates plant growth and flowering traits (Farid et al., 2023; Al-sayed et al., 2024; El-Naqma et al., 2024).

Growing plants in greenhouses reduces some of the environmental stresses that hinder growth. Furthermore, the crop quality is superior to open-field farming (Chávez-Servín et al., 2017). Greenhouse plants develop greater vigor because of higher carbon dioxide levels in the surrounding environment, providing ideal plant growing conditions (Bergstrand, 2017). To our knowledge, there are not sufficient previous studies on the production of *C. officinalis* flowers under the greenhouse system, especially organic production through the application of organic and biofertilizers, which could represent a more sustainable system and achieve high-quality production. Thus, this study aims to evaluate the use of organic and bio-fertilization on *C. officinalis* as a sustainable alternative to chemical fertilizer under a greenhouse system.

2. Materials and Methods

2.1. Experiment Site and Design

This investigation was carried out under greenhouse conditions in a private farm in El-Santa, Gharbia, Egypt (30°44′23.3″ N 31°09′02.1″ E) during the two successive seasons 2021/2022 and 2022/2023. The greenhouse was 40 m in length, 6 m in width, and 2.75 m in height. It was covered with 200 µ thick polyethylene sheets.

Before planting, the experimental soil was analyzed physically and chemically according to Page et al. (1982) and Sparks et al. (2020). The experimental soil was clay soil: 11.12% coarse sand, 8.24% fine sand, 18.23% silt, 62.41% clay, pH (1:2.5 soil water suspension) 7.54, EC_e (soil paste extract) 2.24 dS m^{-1}, 48.26 mg kg^{-1} available N, 9.22 mg kg^{-1} available P, 234.95 mg kg^{-1} available K, and 59.21% water holding capacity.

Calendula officinalis L. seeds were obtained from the Horticulture Research Institute, Giza, Egypt. The seeds were sown in a nursery bed on October 5th for both seasons. After 45 days, seedlings were transplanted to the plastic greenhouse. The experiment was carried out in plots (2 × 2 m) of 4 rows 50 cm apart and 40 cm between plants. A complete randomized block design was employed for the experiment design. Each treatment contained three replicates, and each replicate included 24 plants.

2.2. Treatments

The mineral sources of N, P and K fertilizers were ammonium sulphate (20.6% N) at 357 kg ha^{-1}, calcium superphosphate (15.5% P_2O_5) at 238 kg ha^{-1}, and potassium sulphate (48% K_2O) at 238 kg ha^{-1}, respectively, as the recommended dose (RD) (Nada, 2014). N and K fertilizers were added to the soil three times after transplanting, at 15-day intervals, while calcium superphosphate was counted as one dose during soil preparation.

Table 1. The average value of air temperature (°C), relative humidity (%), and possible sunshine duration (h) during the 2021/2022 and 2022/2023 seasons inside the experimental greenhouse.

Months	Mean value of air temperature (°C)		Relative humidity (%)		Possible sunshine duration (h)	
	1st season	2nd season	1st season	2nd season	1st season	2nd season
October	29.7	30.4	82	84	11.8	11.5
November	26.4	27.5	80	80	10.9	10.7
December	24.6	25.8	81	80	10.3	10.2
January	24.9	24.8	83	84	10.3	10.4
February	25.8	25.1	81	79	10.9	11.1
March	20.8	21.2	79	77	11.7	12.0
April	21.4	21.0	78	76	12.6	12.9
May	22.2	23.6	75	79	13.4	13.6
June	25.4	25.9	79	78	14.1	14.3

Organic fertilization as broiler poultry manure (M) was provided from a private farm in El-Santa, Gharbia, Egypt. The pH (1:5) value was 6.69, EC 3.29 dS m^{-1}, organic carbon 33.56%, organic matter 57.72%, 2.38% total N, 14.10:1 C/N ratio, 0.37% total P, 0.94% total K, 4.52 mg kg^{-1} available P, 957.11 mg kg^{-1} available K, 163% saturation percentage (SP). Three doses of poultry manure, 47.60, 71.40, and 95.20 m3 ha−1 (M1, M2, and M3, respectively), were added to the soil before planting.

For biofertilization, nitrogen-fixing bacteria (*Azospirillum brasilense*) (Az), phosphate-solubilizing bacteria (*Bacillus megaterium* var. *phosphaticum*), and potassium-solubilizing bacteria (*B. circulans*) (Bc) were obtained from Soil Microbiology Laboratory, National Research Center, Egypt (Fig. 1). These bacterial cultures were grown following the methods of Abd-el-Malek and Ishac (1968) and Dobereiner et al. (1976). Vesicular arbuscular mycorrhizal (VAM) fungi, which contained three effective strains (*Glomus etunicatum*, *G. intraradices*, and *G. fasciculatum*) were inoculated in the soil (200 VAM spores $plant^{-1}$) after transplanting. Each plant was inoculated with 5 mL of bacterial suspension. The microbes were inoculated ten days after transplanting. A plant residue-based compost (Nile Company, Cairo, Egypt) was applied at 12 ton ha^{-1} as a carrier material. The compost includes 14.1 g kg^{-1} of total N, 17.7 g kg^{-1} of total P, 6.5 g kg^{-1} of total K, 41% of organic matter, 21.08% of organic C, C/N ratio 14.95:1, EC 5.95 dS m^{-1}, and pH 7.24. The soil moisture was maintained at field capacity for all treatments. Table 1 shows the average value of air temperature (℃), relative humidity (%), and possible sunshine duration (h) during the two growing seasons inside the greenhouse.

2.3. Measurements

Vegetative and floral measurements

The recorded parameters were plant height (cm; as the main plant stem), branches number $plant^{-1}$, plant dry weight (DW; g $plant^{-1}$), flowering start (days; considered at the first opened flowering head), flowering period (days; recorded from the first flared flowering head until the end of the flowering), flowers number $plant^{-1}$ (7 cuts), flower yield fresh weight (FW) and DW (ton ha^{-1}).

Chemical measurements

The content of chlorophyll-a, chlorophyll-b, total chlorophyll in leaves, and total carotenoids in fresh petals (mg/g FW) was determined spectrophotometrically in the acetone extract according to the methods of Dere et al. (1998). The phenol-sulfuric acid method determined the dry leaves ' total carbohydrates (%) (Dubois et al., 1956). The percentages of nitrogen, phosphorus, and potassium were determined in dried leaves. N was estimated by following the protocol presented in AOAC (1995). P and K were determined according to Cottenie et al. (1982). Oleanolic acid content was estimated in the dried petals, according to El-Gaingihi et al. (1982).

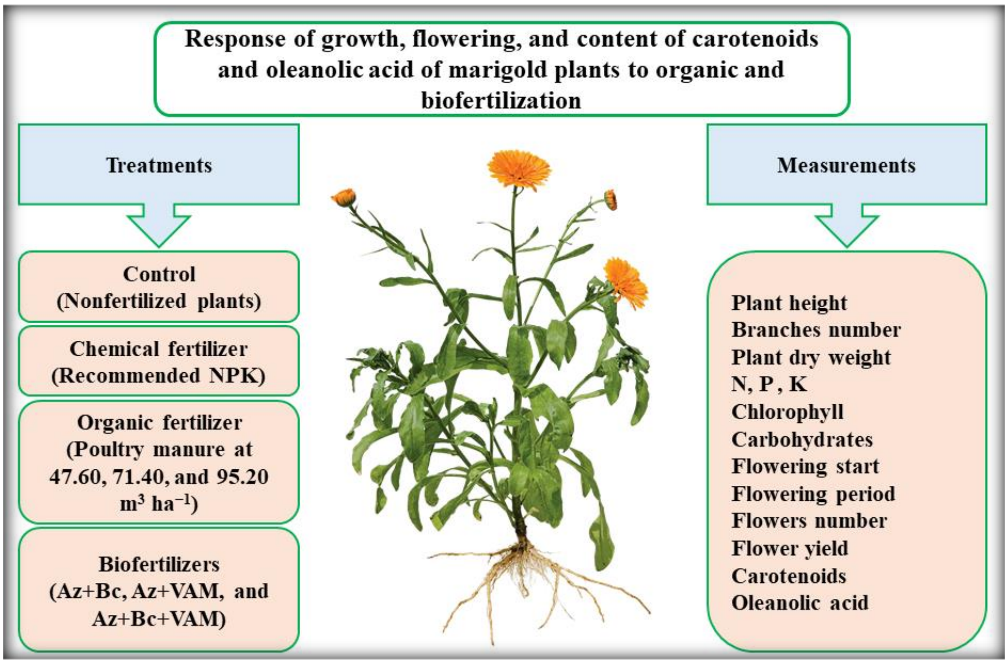


Fig. 1. General description of the study. Az: N-fixing bacteria (*Azospirillum brasilense*), Bc: P-solubilizing bacteria (*Bacillus megaterium* var. *phosphaticum*) and K-solubilizing bacteria (*B. circulans*), VAM: vesicular arbuscular mycorrhiza (*Glomus etunicatum*, *G. intraradices*, and *G. fasciculatum*).

Microbiological population screening

After 40 days of treatment with microbial inoculants, the numbers of microorganisms in the soil samples were counted immediately after collection according to the technique described by Louw and Webley (1959) and Bunt and Rovira (1955). The most probable numbers were used for counting *Azospirillum* spp. (Dobereiner et al., 1976; Abd-el-Malek and Ishac, 1968). The percentage colonization of roots with VAM fungi was assessed using the magnifying cross method (McGonigle et al., 1990).

2.4. Statistical analysis

All experiments were conducted in a randomized block complete design. The statistical analysis of data was subjected to analysis of variance (ANOVA) (Snedecor and Cochran, 1980) followed by Duncan's multiple range test (DMRT) at $P \leq 0.05$ using COSTAT package ver. 6.4 (CoHort software Monterey, USA). OriginPro 2021 (OriginLab Corporation, Northampton, MA, USA) was used to design the principal component analysis (PCA).

3. Results

3.1. Growth parameters

Data tabulated from the growing seasons (2021/2022 and 2022/2023) show obviously that there is a significant difference in the growth parameters (plant height, branch number, and plant DW) in response to NPK-RD, organic, and biofertilization (Table 2). The growth parameters were gradually enhanced due to increased poultry manure levels. Within the applied biofertilizers, the combination of Az+Bc+VAM (B3) was superior to Az+Bc (B1) and Az+VAM (B2).

The tallest plants in both seasons were obtained from M3 treatment, which recorded 103.50 and 118.68 cm in the 1st and 2nd seasons, respectively. No statistical differences were noticed among the results of NPK-RD and M2 in the 1st season or M1 and B1 in the 2nd season. The most significant increase in branch number was seen in the treatment of M3 at 95.20 m^3 ha^{-1}, which recorded 100.53 and 107.93 branches $plant^{-1}$ in both seasons, respectively, followed by M2 and NPK-RD treatments. The second season took the same line as the first season. However, treatment B3 was significantly equal to NPK-RD and M2 in the number of branches recorded. All treatments increased plant DW significantly compared to the control. The highest significant ($P \leq 0.05$) values of plant DW; 440.51 and 461.39 g $plant^{-1}$, were recorded for M3 treatment in the first and second seasons, respectively. No statistical differences in plant DW were observed between the plants that received NPK-RD, M2, or B3.

Table 2. Effect of organic and biofertilization on growth parameters (plant height, branches number, and plant dry weight) of *C. officinalis* during 2021/2022 and 2022/2023 seasons under greenhouse conditions.

Treatments	Plant height (cm)		Branches number $plant^{-1}$		Plant dry weight (g $plant^{-1}$)	
	1st season	2nd season	1st season	2nd season	1st season	2nd season
Control	44.86±1.22g	43.14±1.35f	34.67±1.53e	39.60±2.25e	162.28±9.96e	172.27±2.31f
NPK-RD	92.68±2.64b	92.56±2.28c	95.83±1.15b	95.33±2.03b	392.69±2.80b	396.08±3.95b
M1	80.74±1.04d	81.39±0.86d	81.07±1.86d	83.67±1.07d	353.36±24.25c	364.94±14.81c
M2	95.23±2.16b	98.76±1.98b	95.17±1.65b	95.77±0.65b	389.74±6.15b	392.10±9.98b
M3	103.50±3.05a	118.68±7.23a	100.53±2.19a	107.93±3.60a	440.51±39.46a	461.39±2.45a
B1	76.69±1.61e	79.74±2.47d	83.00±2.46d	83.10±1.54d	299.79±8.67d	312.36±3.67e
B2	72.78±1.36f	71.90±1.80e	86.27±1.70c	88.13±1.37c	311.67±16.90d	329.84±4.17d
B3	84.93±1.93c	88.23±3.56c	88.00±2.14c	93.23±3.19b	374.71±7.19bc	384.12±10.44b

Control: nonfertilized plants. NPK-RD: recommended dose of NPK, i.e., ammonium sulphate (20.6% N) 357 kg ha^{-1}, calcium super phosphate (15.5% P_2O_5) 238 kg ha^{-1}, and potassium sulphate (48% K_2O) 238 kg ha^{-1}. M1, M2, and M3 are poultry manure at 47.60, 71.40, and 95.20 m^3 ha^{-1}, respectively. B1, B2, and B3 are Az+Bc, Az+VAM, and Az+Bc+VAM, respectively. Az: N-fixing bacteria (*Azospirillum brasilense*), Bc: P-solubilizing bacteria (*Bacillus megaterium* var. *phosphaticum*) and K-solubilizing bacteria (*B. circulans*), VAM: vesicular arbuscular mycorrhiza (*Glomus etunicatum*, *G. intraradices*, and *G. fasciculatum*). Data are displayed as mean ±SD, (n=3). Values followed by different letters in the same column are significantly different according to DMRT ($P \leq 0.05$).

3.2. Microbiological population screening

Data on the effects of biofertilization on microbial diversity in the rhizosphere are shown in Table 3. Biofertilization treatments increased the microbiological population in the rhizosphere soil of marigold plants in comparison with control treatment. The number of *Bacillus* spp. in the inoculated soil was 5×100^3 and 6.5×100^3 compared to control soil (15×10^2 and 17×10^2) in the first and second seasons, respectively. Biofertilization treatments increased the Azospirillum spp. population to 6×100^3 and 8×100^3 compared to control soil (2×10^2 and 2.5×10^2) in both seasons, respectively. Furthermore, the data of microscopic examinations of root samples showed that the roots of marigold plants treated with VAM were characterized by higher percentage of mycorrhizal colonization (74% and 79%) in comparison with the roots of nonfertilized plants (13% and 15%) in both seasons respectively.

Table 3. Effect of biofertilization on microbial diverted in the rhizosphere.

Treatments	*Bacillus* spp.		*Azospirillum* spp.		Mycorrhizal colonization (%)	
	1st season	2nd season	1st season	2nd Season	1st season	2nd Season
Control	15×10^2	17×10^2	2×10^2	2.5×10^2	13%	15%
Biofertilization treatments	5×100^3	6.5×100^3	6×100^3	8×100^3	74%	79%

3.3. Nutrient content

The addition of chemical fertilizers (NPK-RD) and poultry manure to marigold plants was superior to the inoculation with the plant growth-promoting microbes in accumulating higher content of N, P, and K (Fig. 2). In general, nutrient contents increased by increasing levels of poultry manure**.** The plants inoculated with B3 treatment accumulated higher nutrient content than B1 and B2 inoculations. M3 treatment recorded the highest significant content of N in both seasons (4.26% and 4.31%, respectively). This was significantly followed by NPK-RD, B3, and M2, in the 1st season (Fig. 2a). NPK-RD and higher addition of poultry manure (M3) were superior to other treatments in increasing P content (Fig. 2b). The P % reached 0.46 and 0.49% in the first season and 0.51 and 0.52% in the second with NPK-RD and M3 treatments, respectively. Similarly, in both seasons, the most significant content of K (2.75 and 2.81%, respectively) was recorded for the M3 treatment followed by NPK-RD and M2 (Fig. 2c).

3.4. Chlorophyll and carbohydrate contents

Data illustrated in Fig. 3 show that the contents of chlorophyll and carbohydrates enhanced significantly in response to different fertilizers compared to nonfertilized plants. The content of chlorophyll and carbohydrates increased by increasing the dose of poultry manure. B3 of biofertilization recorded a higher content of chlorophyll and carbohydrates than B1 and B2. M3 treatment recorded the topmost significant content of chlorophyll-a (3.50 and 3.49 mg g^{-1} FW), chlorophyll-b (1.42 and 1.41 mg g^{-1} FW), total chlorophyll (4.91 and 4.90 mg g^{-1} FW), and carbohydrates (73.21 and 72.42%) in the first and second seasons, respectively. The chlorophyll and carbohydrate contents in plants of treatments NPK-R, M2, and B3 were significantly similar.

3.5. Flowering parameters

Fertilization treatments led to earlier flowering than nonfertilized plants (Table 4). A significant equivalence was observed between the flowering start of all plants treated with chemical, organic, and biofertilization; the earliest time the first flower opened occurred with M3 treatment, which recorded 39.00 and 36.00 days in the first and second seasons, respectively. This was significantly earlier than the control plants by about seven days. The flowering period of the fertilized plants increased significantly compared to the control. However, no significant differences were detected between different fertilization. M3 treatment resulted in the highest value of the flowering period in both growing seasons (160.67 and 164.00 days, respectively).

In contrast to the flowering period, there is significant differences between the fertilization treatments regarding the number of flowers per plant (Table 4). The number of flowers per plant of the fertilized plants ranged from 58.00 to 150.67, while 33.00 to 36.33 for nonfertilized plants. The M3 treatment tended to achieve the highest number of flowers, with 144.67 and 150.67 flowers per plant in the first and second seasons, followed by NPK-RD treatment (110.00 and 120.33 flowers, respectively). The treatments of B1, B2, B3, M1, and M2 increased the number of flowers compared to the control but remained lower than that recorded by NPK-RD. *C. officinalis* plants inoculated with B3 treatment produced more flowers than B1 and B2.

3.6. Flower yield

Significant differences in flowering yield were recorded between the treatments (Table 5). The same trend in the significant differences recorded between treatments in the first season was also observed in the second growing season. Increasing the rate of addition of organic fertilization was accompanied by a parallel increase in the yield of fresh and dry flowers. Application of higher dose of poultry manure (M3) registered the maximum significant yield of flower FW (18.98 and 19.35 ton ha^{-1}) and flower DW (3.85 and 3.90 ton ha^{-1}) followed by NPK-RD, which recorded 16.81 and 17.81 ton ha^{-1} of flower FW and 3.58 and 3.78 ton ha^{-1} of flower DW in the first and second seasons, respectively. Here, M3 and NPK-RD treatments caused an increase in fresh and dry flower yield estimated at 2.50-3.01 and 2.08-2.32 times that of nonfertilized plants, respectively. Treatment B3 of biological fertilization produced a significantly higher yield of flowers than treatments B1 and B2.

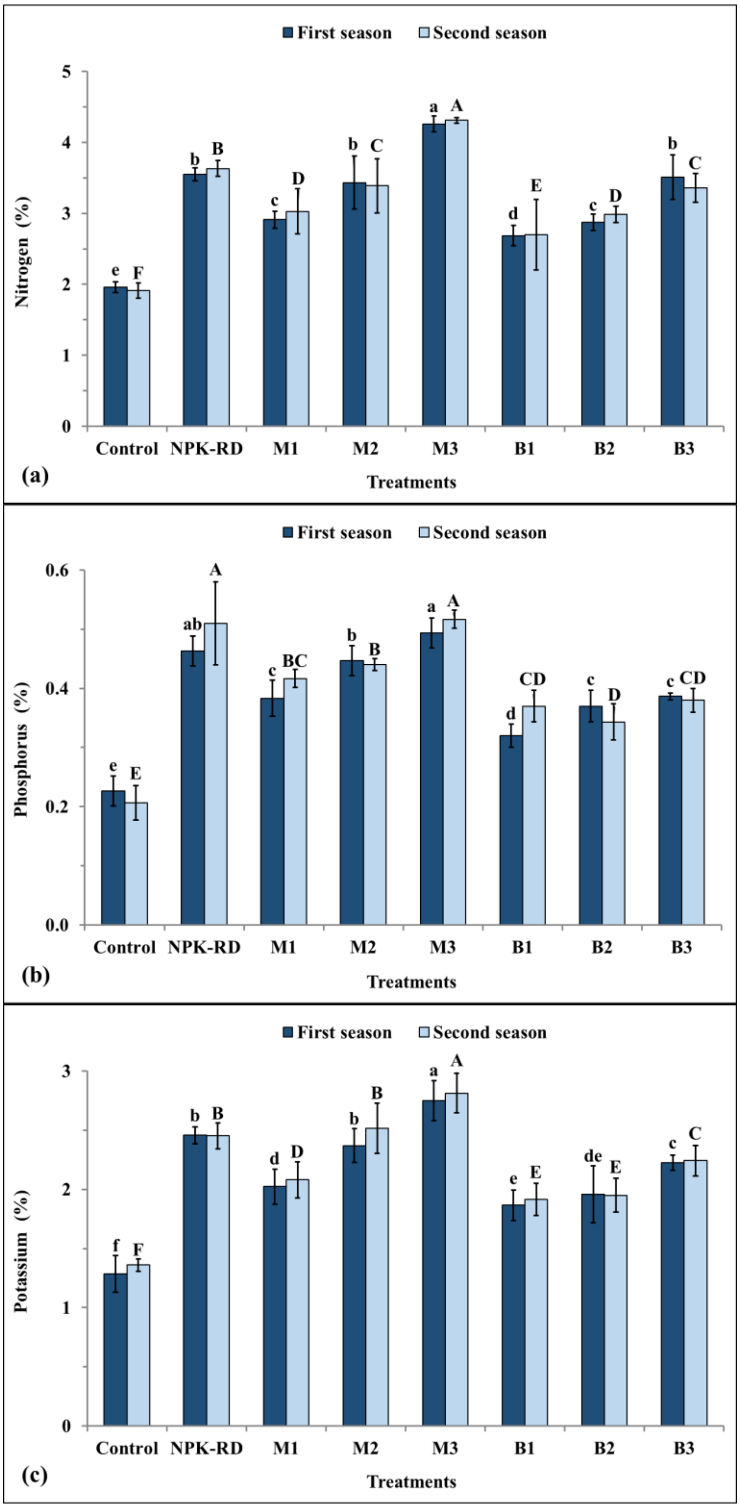


Fig. 2. Effect of organic and biofertilization on nitrogen (a), phosphorus (b), and potassium (c) percentage of *C. officinalis* during seasons 2021/2022 (first season) and 2022/2023 (second season). Bars represent ±SD, (n=3). Different letters showed statistical differences (*P*≤0.05) according to DMRT. Control: nonfertilized plants. NPK-RD: recommended dose of NPK, i.e., ammonium sulphate (20.6% N) 357 kg ha^{-1}, calcium super phosphate (15.5% P_2O_5) 238 kg ha^{-1}, and potassium sulphate (48% K_2O) 238 kg ha^{-1}. M1, M2, and M3 are poultry manure at 47.60, 71.40, and 95.20 m^3 ha^{-1}, respectively. B1, B2, and B3 are Az+Bc, Az+VAM, and Az+Bc+VAM, respectively. Az: N-fixing bacteria (*Azospirillum brasilense*), Bc: P-solubilizing bacteria (*Bacillus megaterium* var. *phosphaticum*) and K-solubilizing bacteria (*B. circulans*), VAM: vesicular arbuscular mycorrhiza (*Glomus etunicatum, G. intraradices,* and *G. fasciculatum*).

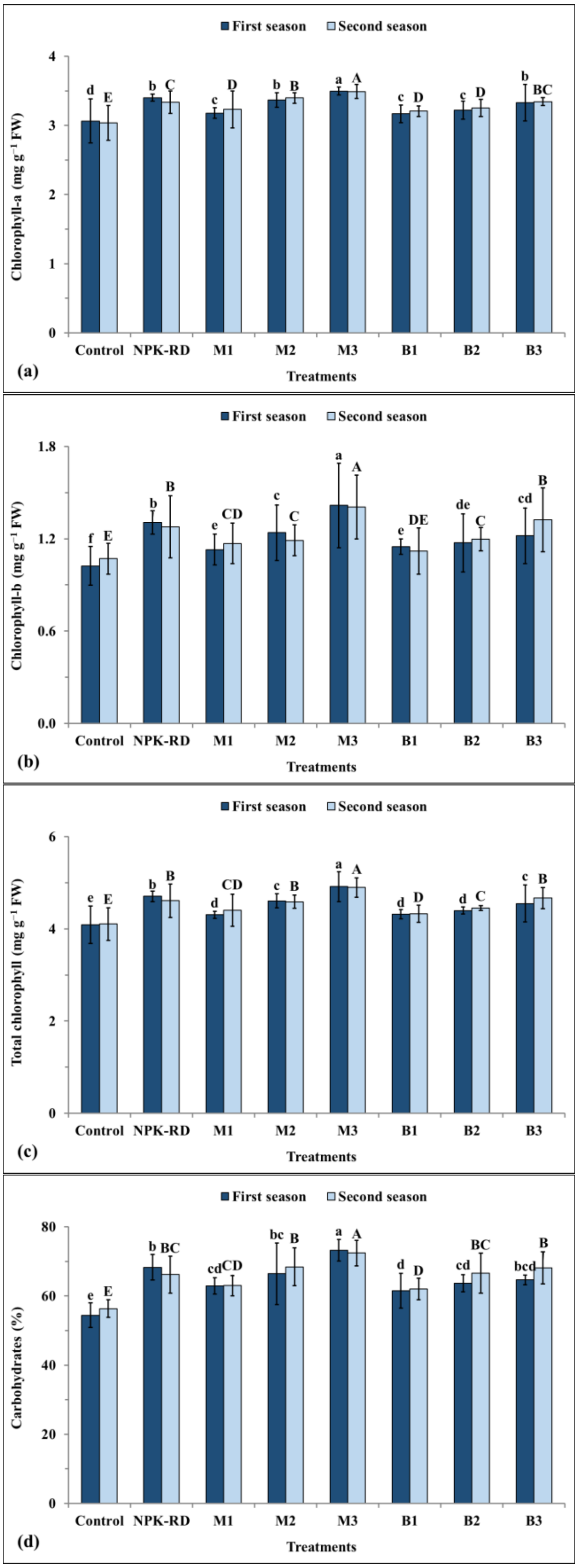


Fig. 3. Effect of organic and biofertilization on photosynthetic pigments [chlorophyll-a (a), chlorophyll-b (b), and total chlorophyll (c)] content (mg g^{-1} FW) and carbohydrates (d) of *C. officinalis* during seasons 2021/2022 (first season) and 2022/2023 (second season). Bars represent ±SD, (n=3). Different letters showed statistical differences (*P*≤0.05) according to DMRT.

Table 4. Effect of organic and biofertilization on flowering parameters (flowering start, flowering period, and flower number) of *C. officinalis* during 2021/2022 and 2022/2023 seasons under greenhouse conditions.

Treatments	Flowering start (days)		Flowering period (days)		Flowers number plant $^{-1}$	
	1st season	2nd season	1st season	2nd season	1st season	2nd season
Control	46.00±1.00a	43.33±1.53a	151.00±2.65c	153.00±3.61c	33.00±2.00e	36.33±5.51f
NPK-RD	39.67±2.08bc	38.00±1.73bc	159.00±3.61ab	157.67±3.06bc	110.00±7.21b	120.33±10.26b
M1	41.33±1.53bc	39.33±0.58b	156.33±3.21ab	156.67±2.89bc	62.33±3.51d	67.00±2.00e
M2	40.33±1.53bc	38.67±1.53bc	158.67±2.08ab	160.00±2.65ab	89.67±5.13c	91.67±2.52c
M3	39.00±2.00c	36.00±2.65c	160.67±3.06a	164.00±4.36a	144.67±8.33a	150.67±8.02a
B1	41.67±0.58b	39.33±1.53b	156.00±3.61ab	158.00±3.61abc	66.33±3.51d	64.33±3.21e
B2	41.33±0.58bc	39.67±1.53b	155.00±2.65bc	154.00±2.65bc	58.67±7.64d	58.00±2.65e
B3	40.33±0.58bc	39.00±1.00bc	156.67±2.08ab	160.00±2.65ab	81.67±3.21c	81.00±3.61d

Data are displayed as mean ±SD, (n=3). Values followed by different letters in the same column are significantly different according to DMRT ($P \leq 0.05$).

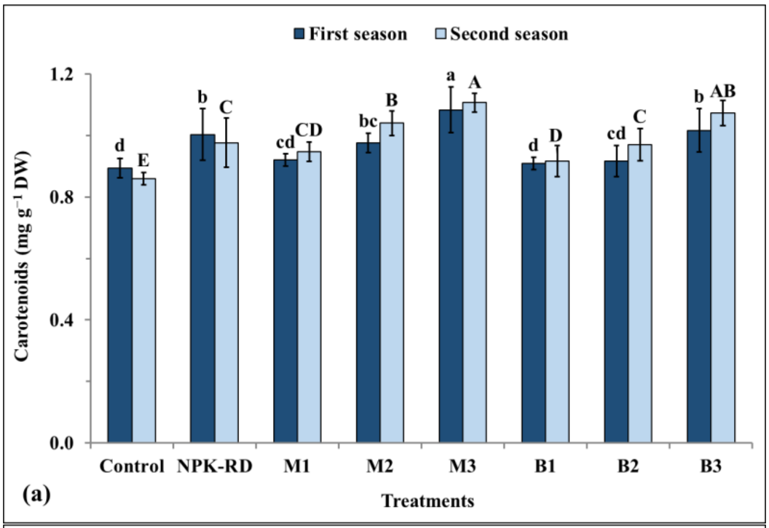
Table 5. Effect of organic and biofertilization on flower yield of *C. officinalis* during 2021/2022 and 2022/2023 seasons under greenhouse conditions.

Treatments	Flower yield FW (ton ha $^{-1}$)		Flower yield DW (ton ha $^{-1}$)	
	1st season	2nd season	1st season	2nd season
Control	6.30±0.85f	7.12±0.35g	1.66±0.16e	1.82±0.24f
NPK-RD	16.81±0.42b	17.81±0.17b	3.58±0.09ab	3.78±0.10ab
M1	11.95±0.17d	12.35±0.40e	3.04±0.10d	3.16±0.17d
M2	15.76±0.41c	16.30±0.21c	3.35±0.14bc	3.49±0.07bc
M3	18.98±0.25a	19.35±0.16a	3.85±0.36a	3.90±0.15a
B1	8.61±0.37e	8.54±0.23f	2.84±0.13d	2.82±0.27e
B2	9.02±0.40e	8.82±0.22f	2.79±0.06d	2.77±0.17e
B3	12.84±0.88d	13.07±0.32d	3.11±0.14cd	3.22±0.19cd

Data are displayed as mean ±SD, (n=3). Values followed by different letters in the same column are significantly different according to DMRT ($P \leq 0.05$).

3.7. Carotenoids and oleanolic acid contents

According to the data in Fig. 4, the carotenoids and oleanolic acid content in *C. officinalis* flower increased significantly in response to different fertilization treatments (chemical, organic, and biological fertilization) compared to the control in both seasons. The carotenoids and linolenic acid content in organically fertilized plants increased with increased levels of added organic fertilizer. The carotenoids content in the flowers of plants inoculated with the mixture of microbes B3 was significantly higher than those vaccinated with B1 and B2 (Fig. 4a). The carotenoids content reached its highest significant level in the first season in the M3 treatment (1.08 mg g^{-1} DW), followed by B3 (1.02 mg g^{-1} DW) and NPK-RD (1.00 mg g^{-1} DW). In comparison, the carotenoids content was significantly equal in the flowers of the M3 and B3 treatments during the second season (1.11 and 1.07 mg g^{-1} DW, respectively). Moreover, in the second growing year, the carotenoids content was significantly lower in the NPK-RD treatment than in the M2 treatment, while it was pretty equal in the B2 and NPK-RD treatments. The most significant accumulation of oleanolic acid was recorded in the flowers of plants that received M3 (0.49 and 0.52 mg g^{-1} DW) and plants of NPK-RD treatment (0.47 and 0.50 mg g^{-1} DW) in both seasons, respectively, without significant differences between them, followed by M2, B2, and B3 (Fig. 4b).



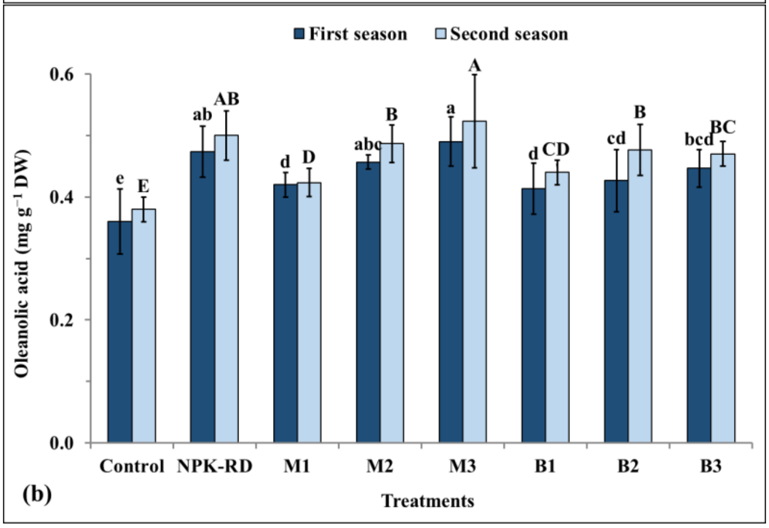


Fig. 4. Effect of organic and biofertilization on the carotenoids and oleanolic acid content (mg g^{-1} DW) of *C. officinalis* flower during seasons 2021/2022 (first season) and 2022/2023 (second season). Bars represent ±SD, (n=3). Different letters showed statistical differences (*P*≤0.05) according to DMRT.

3.8. Principal component analysis

The study employed principal component analysis (PCA) to assess the correlation between various parameters and treatments in *C. officinalis* plants. Under multiple treatments, including control, NPK-RD, M1, M2, M3, B1, B2, and B3, the PCA revealed considerable differences in growth, flowering, yield, and biochemical indicators (Fig. 5). The results for the various traits were shown in a two-dimensional PCA diagram. Two distinct principal component (PC) variability percentages, PC1 and PC2, which stand for 90.1% and 3.3%, respectively, were displayed in the data. Of the entire data variability, 93.4% could be explained by the first and second PCs. PC1 was positively associated with all studied parameters except flowering start. PC2 was best described by flowering beginning on the positive side of the Y-axis. PCA also revealed evident clustering differences between treatments, as control, M1, B1, and B2 treatments were located on the left side of PC1, whereas NPK-RD, M2, M3, and B3 treatments were found on the right side of PC1. The flowering start is the unique characteristic of the control treatment, as nonfertilized plants were late at the beginning of flowering compared to all chemical, organic and biofertilization treatments. M1, B1, and B2 treatments were not unique in the studied characteristics.

On the contrary, NPK-RD, M2, M3, and B3 treatments achieved significant superiority in all tested parameters. All growth, flowering (except flowering start), yield, and biochemical traits were positively correlated. A negative correlation was observed between flowering start and all other measurements.

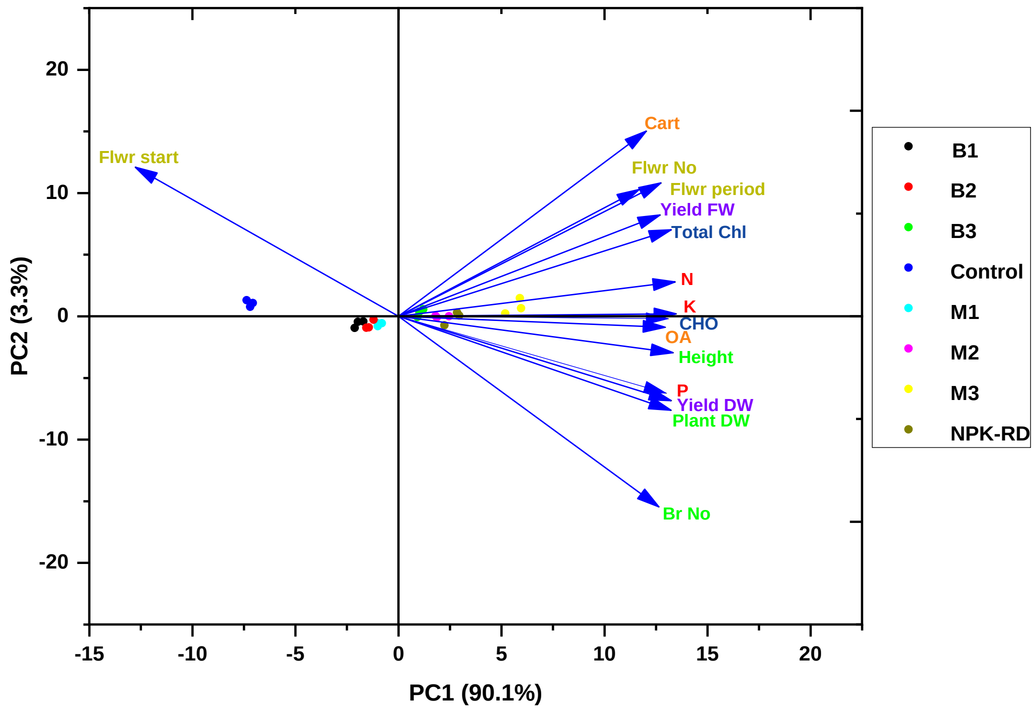


Fig. 5. Principal component analysis (PCA) based on the correlation matrix of growth parameters [plant height (Height), branches number (Br No), and plant dry weight (Plant DW)], nutrients content (N, P, and K), total chlorophyll (Total chl), carbohydrates (CHO), flowering parameters [flowering start (Flwr start), flowering period (Flwr period), and flowers number (Flwr No)], flower yield FW (Yield FW), flower yield DW (Yield DW), carotenoids content (Cart), and oleanolic acid content (OA) in response to different fertilizations. Colored dots represent treatments.

4. Discussion

The current study intends to determine the best fertilizer to increase *C. officinalis* growth, flowering, carotenoids and oleanolic acid content. There is currently a need to investigate the use of organic fertilization or plant growth-promoting microbes to improve the production of medicinal plants as an alternative to scientific chemical fertilizers. Chemical NPK fertilizers were applied to plants at the recommended dose (NPK-RD). Therefore, organic and biofertilizer treatments positively impact *C. officinalis* when the results are noticeably better than those achieved with NPK-RD. The current study shows that chemical, organic, and biological fertilization treatments enhanced several aspects of vegetative and flowering growth. The most significant increase was observed with a higher addition of poultry manure at 95.20 m^3 ha^{-1} (M3) and NPK fertilizers. However, overuse of synthetic NPK fertilizers will raise production costs, cut export prospects, contaminate the environment, and impact soil fertility (Abdrabbo et al., 2019). When applied to soil, organic sources enhance the physical characteristics of the soil, including aeration, aggregation, permeability, and water-holding capacity (Yadav et al., 2023), all of which support plant growth and development. The role of organic manure in holding moisture and maintaining sufficient pore spaces to permit good air circulation and drainage of the excess water produced may be responsible for the enhancing effects of organic manure on plant height, number of branches, and plant DW. Because organic fertilizer has little to no soluble salt, it can be applied at high concentrations without harming the roots. However, this can happen if the same amount of nutrition is used for the plants (Shaji et al., 2021). Moreover, poultry manure emerged as the most effective organic source of nutrients, the best source of organic manure which helped in improving the physico-chemical properties of soil (pH, EC, organic carbon, macro, and micronutrients) because of its higher analytical values (Idan et al., 2014). Many studies reported the significant effect of poultry manure on medicinal and ornamental plants, such as Helmy and Zarad (2003) on *Borago officinalis*, Kumar and Saravanan (2019) on *Gladiolus grandiflora*, and EL-Zawawy et al. (2021) on *C. officinalis.* Sultana et al. (2015) reported that applying vermicompost boosted the growth and flowering parameters of *Zinnia elegans* plants including shoot height, root length, leaf number, flower number, flower diameter, FW and DW of flower, when compared to NPK fertilizer.

The interaction of VAM fungi with bacteria significantly enhanced the effect of biofertilizer on the growth, flowering, flower yield, and carotenoids content of marigold plants. Previous reports on medicinal plants confirmed the stimulating effects of arbuscular mycorrhizal fungi on plant growth and productivity (Rydlová *et al.,* 2016; Amiri *et al.,* 2017; José de Almeida *et al.,* 2020; Merlin *et al.,* 2020; Taghizadeh *et al.*, 2023). Compared to the control in *Foeniculum vulgare* plant, applying biofertilizers, particularly bacterial mixture with VAM, also boosted the total plant fresh weight, dry weight, and number of branches (Nada *et al.*, 2022). According to Merlin et al. **(**2020**)**, introducing VAM to coarse mint increased the plant's P and N content. In a greenhouse study, Sinclair et al. (2014) studied the effects of colonization by VAM of strawberry cultivars. They found that mycorrhization of roots enhanced root length, diameter, and the number of lateral roots. In our study, the roots of marigold plants treated with VAM were characterized by higher percentage of mycorrhizal colonization compared to the roots of control plants.

In the current study, application of the biofertilizers increased the number of bacteria (*Bacillus* spp. and *Azospirillum* spp.) and mycorrhizal colonization (%) compared with nonfertilized plants. This confirms the findings of Ding et al. (2013) and Derkowska et al. (2015). They found that the application of biofertilizers increased the overall number of bacteria, actinomycetes, and VAM spores, as well as increased the degree of mycorrhizal colonization.

The highest dose of poultry manure (M3) outperformed all other treatments in terms of increasing the amount of N, P, and K. Similarly, higher amounts of poultry organic manure led to the highest N, P, and K content in *C. officinalis* plants under open field conditions (EL-Zawawy et al., 2021). This increase in mineral content could be attributed to several factors, including the higher degree of water retention, slow rate of nutrient release in forms that were sufficient for growing plants, and relatively high nutrient contents (Elsayed et al., 2020). Moreover, organic fertilizers are well-known for their capacity to enhance nutrient uptake, particularly of phosphorous, sulfur, and nitrogen, chelate soil nutrients, increase soil biological activity, and solubilize minerals (Frank and Roeth, 1996).

Plant health and nutrient availability can be inferred from the amount of chlorophyll in the plant **(**Bautista-Diaz et al., 2021). The increase of chlorophyll content under organic fertilization can be explained physiologically by the organic fertilizers' function as pyridines' constituent, forming chlorophyll and cytochrome (Joo et al., 1999). The carbohydrate percentage varied depending on the treatments applied compared to the control. Nutrients actively contribute to chlorophyll synthesis, so using NPK may enhance vegetative traits and raise the amount of carbohydrates in the plant. Potassium is necessary to increase the quantity of chlorophyll in plant tissues and in processes connected to photosynthesis, which improves the efficiency of photosynthesis and accelerates the accumulation of carbohydrates **(**Tränkner et al., 2018). Marigold plants microbially inoculated with Az, BC, and VAM individually or in combinations accumulated more carbohydrates than the control. Similar findings were reported by El-Beltagi et al. (2023) on fennel.

There is a variation in the flowering and yield parameters in response to NPK-RD, organic, and plant growth-promoting microbes. Consistent with our findings, it was observed that the application of organic and biofertilization resulted in a significant increase in the number of flowering heads, flowering head diameter, flowering start, and flowering period when compared to the nonfertilized marigold plants (EL-Zawawy et al., 2021). In the current study, a higher rate of organic fertilization (M3) resulted in the highest flower number value and yield. Similar results were found by El-Maadawy (2007) on *Tagetes erecta* and Vieira et al. (1999) on *C. officinalis*. Chang et al. (2010) reported that *Anthurium andreanum* plants receiving organic fertilizer (pea and rice hull compost) had the same growth, yield, and quality of cut flowers as those receiving chemical fertilizers. This suggests that organic fertilizer can replace chemical fertilizers as a source of nutrients for *A. andreanum* cut flower production.

A plant's ability to grow more vegetatively is reflected in its ability to produce more flowers with better quality. The improvement of flowering parameters might be attributed to the role of increased nitrogen in the commencement of new cells and the positive effects of mycorrhiza and bacteria on the characteristics of the soil. In addition to N-fixation and phosphate dissolution, the growth-promoting compounds produced by N-fixers and P- and K-dissolving bacteria are responsible for the positive effects on plant development (Saha et al., 2023). Furthermore, gibberellins can be produced by several soil microbes (Rademacher, 1994).

In the current study, as the level of organic manure was increased from 47.60 to 95.20 m^3 ha^{-1}, along with biofertilizer including VAM, the carotenoids content increased more than the control. Organic fertilizers, regarded as one of the least expensive forms of organic additions, enhance the soil's structure, capacity to retain water, and aeration and drainage, all of which contribute to healthy root development and improved nutrient absorption (Shaji et al., 2021). Elhindi (2012) found that applying composted green garbage to *C. officinalis* greatly enhanced the plant's FW, DW, plant height, number of flowers per plant, flower diameter, and content of chlorophyll and carotenoids.

The highest increase in oleanolic acid content was attributed to the application of NPK-RD, poultry manure at a high level (M3), and biofertilization at Az+Bc+VAM treatment. In a previous study, the application of humic acid increased the amount of oleanolic acid in *C. officinalis* flowers (Azzaz et al., 2007). Azzaz and Hassan (2008) also noted that applying varying organic fertilization at 20, 30, and 40 m³ fed^{-1} increased secondary metabolites and essential oil in the fennel plant. Furthermore, the accumulation of oleanolic acid in *C. officinalis* flower was maximum when poultry manure was given to plants at a rate of 60 m³ fed^{-1} under open field conditions (EL-Zawawy et al., 2021). El-Beltagi et al. (2023) recorded that total phenolics, flavonoids, and essential oil content increased in fennel fruits upon applied NPK and organic fertilization. Our findings can be explained by the fact that organic manure contains microorganisms like Azotobacter and Azosprillum, which are crucial for fixing nitrogen and releasing phytohormones like indole-3-acetic acid (IAA), GA_3, and cytokinins, which encourage growth, the content of dry matter, and the absorption of nutrients (Reynders and Volassak, 1982). According to Szakiel et al. (2003), oleanolic acid is produced in the same manner as gibberellic acid: starting with acetyl Co-enzyme A, moving via farnesyl pyrophosphate, squalene (C-30), B-amyrin, and finally oleanolic acid.

VAM have the potential to colonize plant roots by forming hyphal networks. In this mutualistic and symbiotic connection, the fungus plays an important role in enabling nutrient uptake, particularly phosphorus, by the plant roots via its mycelia (Kumar et al., 2021). Recent reports suggests that VAM and associated bacterial communities can influence the quantity and quality of metabolic products in the host plant. Furthermore, these investigations have identified numerous other plant features that are undergoing changes, such as increased biomass, greater stress tolerance, enhanced nutrient absorption and water regulation, and changes in the synthesis of plant hormones (Iakab et al., 2024). In our experiment, the oleanolic acid content was increased more effectively by combining bacterial inoculation with VAM (Az+VAM) than by Az+Bc alone. In this regard, plant secondary metabolite production may alter because of VAM's symbiotic relationships with plant roots (José de Almeida et al., 2020). Similarly, Iakab et al. (2024) found a difference in the compounds prevalent in the essential oil of *Echinacea purpurea* plants treated with VAM and untreated plants. Consistent with our results, El-Beltagi et al. (2023), Arango et al. (2012), Rydlová et al. (2016), Amiri et al. (2017), José de Almeida et al. (2020), Merlin et al. (2020) reported that VAM fungi improved plant growth and increased the secondary metabolites content of fennel, peppermint, dill, geranium, chamomile, and coarse mint plants, respectively. Rakbar et al. (2024) found that putrescine, combined with mycorrhizal fungus, had a beneficial influence on *Gerbera jamesonii* growth parameters, increased nutrient absorption, and improved post-harvest indicators.

In the study of El-Hindi et al. (2009), *C. officinalis* plant was exposed to three concentrations of nitrogen-fixing bacteria (*Azospirillum* sp. and *Azotobacter* sp.) and phosphate-dissolving bacteria (*Bacillus megaterium*). They demonstrated that many chemical constituents, including oleanolic acid, were improved by all treatments. Additionally, *C. officinalis* inoculated with biofertilizer-effective microorganisms increased carotene and oleanolic acid contents (Ali, 2013).

With a growing population, improving productivity through the use of greenhouses in agricultural production to control the crop production environment is thought to be a potential strategy (El-Ramady et al., 2022). Previous studies have compared the growth and productivity of ornamental and medicinal crops under open field and greenhouse conditions. These studies proved that there are significant differences in growth, flowering, phytochemicals, chlorophyll and nutrient contents, and thus yield, between cultivation in the open field and the greenhouse (Gantait and Pal, 2011; Lee et al., 2022). According to Lee et al. (2022), spinach grown in an open field has higher phytochemical quality than spinach grown in a greenhouse. On the contrary, the flower yield and content of carotenoids and oleanolic acid reported here are higher than those recorded in the study of EL-Zawawy et al. (2021) on *C. officinalis* under open filed conditions.

Conclusions

In conclusion, the study investigating the effect of organic and biofertilizers on the growth and flowering traits, carotenoids and oleanolic acid accumulation in *C. officinalis* plant under greenhouse conditions has provided valuable insights into the potential benefits of these interventions. The findings of this study suggest that the use of organic fertilizer and plant growth-promoting microbes positively influenced the growth and flowering of *C. officinalis* compared to the control treatment. The application of poultry manure was more pronounced compared to biofertilizers. A higher dosage of poultry manure (95.20 m^3 ha^{-1}) was significantly superior to chemical fertilizer in plant height, branch number, DW, chlorophyll, carbohydrates, flower number, flower yield FW, and carotenoids content. However, the flowering start, flowering period, flower yield DW, and oleanolic acid content obtained by higher addition of poultry manure was significantly equal to NPK fertilizer treatment. Oleanolic acid is a valuable compound with various medicinal properties, and its increased production in response to poultry manure and microbial inoculation indicates a potential strategy for enhancing the therapeutic value of this plant. The inoculation with a mixture of N-fixing, P-solubilizing, K-solubilizing bacteria, and VAM fungi (B3) positively impacted carotenoids content in *C. officinalis* flower. The results of this study have important implications for sustainable agriculture and the cultivation of ornamental and medicinal plants. Using organic fertilizers and plant growth-promoting microbes can offer an environmentally friendly approach to enhance plant growth and flowering. Furthermore, these interventions can reduce reliance on synthetic fertilizers and chemical inputs, promoting a more sustainable and ecologically balanced agricultural system. However, further research is needed to elucidate the specific mechanisms through which organic fertilizers and biofertilizers influence the growth, flowering, and secondary metabolite production in *C. officinalis*. Understanding these mechanisms can aid in optimizing the application of these interventions and maximizing their benefits. Future studies combining biological and organic fertilizers will enhance flowering and the content of carotenoids and oleanolic acid content in the *C. officinalis* plant.

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