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Research Article

Investigation of a Bacterial and Arbuscular Mycoorhizal Fungus Spore Inoculation in Cultivation of Saffron

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ABSTRACT

Saffron (*Crocus sativus* L.) is prized for its stigma, a valuable spice rich in bioactive compounds crucial for various industries and traditional medicine. Enriched with bioactive compounds crucial for numerous industries and traditional medicinal practices, saffron cultivation expansion hinges on assessing the vegetative process in soils with low organic content. Optimal fertilization, considering plant and soil conditions, is crucial. Leaves indicate vegetative status and serve as an agricultural byproduct. In this study, saffron was grown in clay loam soil (1.88% organic matter) with supplementation of the bacterium *Rhodobacter sphaeroides*, spores of an arbuscular mycorrhizal fungus *Glomus iranicum* var. *tenuihypharum*, and both. Sole bacterium application increased leaf fresh and dry weight by 25.5% and 36.8%, respectively, demonstrating growth promotion. *Rhodobacter sphaeroides* and AMF combination elevated leaf P, Mg, and Cu concentrations, while AMF alone increased Zn, Mn, and B accumulation. *Rhodobacter sphaeroides* reduced Fe, Zn, Mn, and B soil concentrations, with no corresponding increase in their accumulation in the plant, as observed with mycorrhiza and the combination of microorganisms, hinting at its applicability for saffron cultivation in environments contaminated with heavy metals. In summary, the findings underscore the importance of microorganism supplementation in saffron cultivation, offering insights into optimizing growth conditions.

1. Introduction

Saffron (*Crocus sativus* L.) is an angiosperm in Iridaceae family. It is distributed in Mediterranean-Europe and Western Asia. The dried stigma which is the upper part of the female reproductive system of the saffron flowers serves as a highly esteemed spice, recognized as the most expensive spice globally [1]. In traditional medicine it is widely used for treatment of constipation, depression, cough, inflammation, and menstruation difficulty [2]. The stigmas of the saffron flowers are rich in many bioactive chemicals: more than 300 volatile and non-volatile metabolites making the plant medicinally invaluable. That is why most researchers focused on the stigma and its valuable ingredients [1, 3–5].

After the harvest of flowers and the stigma, the daughter corms are left in the land for the leaves to dry and fall, then the corms are removed and kept in a dry place for 1-2 months and replanted. The size of corms influences the development of the plants, especially the number of flowers obtained, the stigma size and weight. The larger the circumference the higher the yield, and the suggested corm size was 7-10 cm circumference length [6]. The primary objectives of saffron cultivation encompass the production of its prized stigma and corms.

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Meanwhile, the leaves are often regarded as an agricultural byproduct. The green leaves of *Crocus sativus* L. were found to be rich in bioactive compounds and research demonstrated the antibacterial activity of the leaves against *Listeria* species and anti-radical activity well comparable to alpha tocopherol [7]. Moreover, the leaves are the indicator of the situation of the plant in terms of plant nutrition, which can well be used to determine the nutrition status and the effectiveness of the fertilizer regime. It is also very important for the determination of plant status of flowering plants having a short flowering period, such as saffron which has a flowering period of only 3-4 weeks [8]. Flowers are one of the most important quality parameters of plant development, however, to understand the effects of fertilizers and nutrient elements on growth and development of plants, vegetative parts should be investigated.

Saffron has a significant market size in the world. Spain was the leading country in terms of saffron export volume in 2022 with 51.8 million USD followed by United Arab Emirates with 13.8 million USD [9]. The cultivation of this plant is restricted to one city in Türkiye right now, but proliferation of saffron cultivation is desired to expand the market size. This brings about the need for fertilizer use depending on the soil's properties. Microbial fertilization is of great interest to increase both the yield and the fertility of the soil, which has decreased dramatically due to unconscious agriculture applications such as immense use of chemical fertilizers. Such applications have damaged the microbial populations in the soil which decreased the fertility of soil [10].

To support soil fertility, organic fertilizers as well as microbial fertilizers have been suggested to use. Mycorrhizae are fungi that form symbiotic relationship with plant roots and enhance the plant growth, as well as protect the plants from diseases [11]. *Glomus iranicum* var. *tenuihypharum* is an arbuscular mycorrhizal fungus (AMF) which has been used in recent studies to enhance root system of plants and productivity [12–14]. Besides fungi, some bacteria have plant growth promoter abilities. Among them, nitrogen fixing bacteria can be used as fertilizers to enhance the plant growth and soil [15–17]. Different bacteria and fungi species are of research focus for their potential to be used as microbial fertilizers for different agricultural crops. *Rhodobacter sphaeroides* is a purple non sulfur bacterium which is a plant growth promoter because it can fix nitrogen and contribute to the enrichment of soil besides the ability of producing plant hormones such as indole-3-acetic acid and 5-aminolevulinic acid [16, 18, 19].

In this context, this study aimed to determine the nutritional status of saffron leaves upon the use of three different microbial inoculation regimes: a nitrogen fixing bacterium, AMF spore, and the combination of these two.

2. Materials and Methods

Crocus sativus L. corms, which are the seeds of Saffron plant, were obtained from a local provider. Saffron multiplies by cormlets which form corms. Each corm contains 1-4 buds which form flowers and leaves [20] The corms with fibrous and thin roots were planted into the pots of 6L volume, which were filled with topsoil obtained from the experimental field of Tekirdağ Namık Kemal University Faculty of Agriculture, Tekirdağ, Türkiye. There were 5 corms in each pot. After the germination and formation of 3-4 leaves, applications of bacteria and mycorrhiza solutions were carried out to the root area. The experiment was carried out due to randomized block design with 3 pots of each treatment (control, bacteria, AMF, bacteria+AMF) The plants were watered with tap water when needed and plants were harvested after 2 months. The experiment was carried out in lab conditions with controlled temperature and humidity, 22 °C and 80%, respectively, during October and November (Figure 1).

Figure 1. Photos from the experiment. Left: pot experiment showing the leaves of saffron. Right: Saffron flowers

The bacterium applied to the bacteria and bacteria+AMF groups was *Rhodobacter sphaeroides* DSM O.U.001 which was obtained from Hydrogen Research Lab of Middle East Technical University, Ankara, Turkey. The bacteria were grown in Modified Peptone Yeast Extract (MPYE) medium which contains peptone, yeast extract, Ca and Mg sources [21]. When the bacterial concentration reached 1.5 x 10^8 cells/mL, the bacteria were collected, centrifuged, washed with sterile distilled water and resolved in distilled water. 10mL of bacterial suspension (1.5 x 10^9 bacterial cells in total) was applied around the root area in bacteria and bacteria+AMF groups at the time of planting the corms to the pots using pipettes. Equal amount of distilled water was applied to the other pots. Similarly, mycorrhiza solution containing 4.8 x 10^3 propagule of *Glomus iranicum* var. *tenuihypharum* (Symborg) was applied to the root area according to the mycorrhiza manufacturer's recommendations (after dissolving in water applying slowly to the root area) and according to a previous study [22].

2.1.Soil analysis

Soil samples were air-dried and sifted with 2 mm sieve. The pH and electrical conductivity values of the soil were established using 1:2.5 soil:water mixture [23, 24]. Lime ($CaCO_3$) content was measured using Scheibler calcimeter [24]. Bouyoucos Hydrometer method was adopted for the determination of the soil texture [25]. Organic matter content was measured using modified Walkey-Black method [26]. to determine the concentrations of available phosphorus, extractable potassium, magnesium, calcium, available iron, copper, zinc, manganese and extractable nickel, DTPA method with the buffer 0.005 M DTPA+0.01M $CaCl_2$+0.1 M TEA (pH 7.3) was used in ICP-OES [27]. The available boron content was determined according to Wolf (1971) [28]. The determined soil characteristics are given in Table 1.

2.2.Elemental analysis

The micro and macro nutrient element contents of the dried plant material (the aboveground parts without flowers) and the soil obtained in each treatment were determined with ICP-OES (Agilent 700, Agilent) in the Central Lab of Namık Kemal University (NABILTEM). The nitrogen contents in soil and plant samples were determined with Kjeldhal method in the same lab [21, 22].

Table 1. Some physical and chemical characteristics of the trial soil

Parameters	**Measurements**	**Evaluation**
pH	7.10	Neutral
Lime (%)	15.70	High
Salt (µs/cm)	1630	Low
Texture	Clay %45, sand %35, silt %20	Clay loam
Organic matter (%)	1.88	Low
Available phosphorus (P) ($mgkg^{-1}$)	17.04	Sufficient
Extractable boron (B) ($mgkg^{-1}$)	0.17	Insufficient
Extractable potassium (K) ($mgkg^{-1}$)	204.7	Sufficient
Extractable magnesium (Mg) ($mgkg^{-1}$)	208.44	Sufficient
Extractable calcium (Ca) ($mgkg^{-1}$)	4822.07	High
Available iron (Fe) ($mgkg^{-1}$)	1.13	Sufficient
Available copper (Cu) ($mgkg^{-1}$)	0.77	High
Available zinc (Zn) ($mgkg^{-1}$)	0.71	Sufficient
Available manganese (Mn) ($mgkg^{-1}$)	4.32	Sufficient
Extractable nickel (Ni) ($mgkg^{-1}$)	5.02	Non-toxic

2.3.Statistical analysis

The biological parameters of the harvested plants, macro and micronutrient element concentrations of plant and soil samples were analysed for normality with Rjan Joiner test [23]. Followed by the comparison of groups either by ANOVA or Kruskal Wallis test depending on the normality test results with post hoc tests Duncan or Dunn's test, respectively, using SPSS version 22 [24, 25].

3. Results and Discussion

Saffron corm cultivation under controlled laboratory conditions was undertaken, accompanied by some morphological and

biological measurements conducted both throughout the experimental phase and post-harvest. The measurements are given in Table 2. The average leaf length of the control and treatments did not differ significantly, however, the root length of saffron upon mycorrhiza addition alone was almost twice that of control (Table 2). The root enhancement of mycorrhizae (root volume, root length, area etc.) were shown in previous studies carried out with different plant species [29, 30]. On the other hand, the root length of the plants treated with both mycorrhiza and bacteria was almost the same with the control, and lower than that of the bacterial treatment only. This result might suggest competition between fungi and bacteria. If the experiment was carried out for a longer time, a synergistic relationship might have probably been observed [31].

Table 2. Some morphological parameters of *Crocus sativus* L. grown by bacteria and AMF applications

	Leaf length (cm)	**Fibrous root length (cm)**	**Leaf fresh weight (g)**	**Leaf dry weight (g)**
Control	25.6±0.69	11.27±1.74	3.02±0.10	0.57±0.02
Bacteria	26.33±0.1	14.11±1.20	3.79±0.40	0.78±0.04
AMF	25.1±3.51	22.93±2.42	2.77±0.09	0.56±0.06
Bacteria + AMF	24.4±2.01	11.48±1.02	2.75±0.45	0.52±0.09
P value	0.712	0.000	0.01	0.003

The data are given as mean±standard deviation of triplicates.

The application of bacteria alone resulted in higher fresh and dry weight of leaves, 25.5% and 36.8% compared to the control condition (p=0.01 and p=0.003, respectively), while the other two treatments were not significantly different than the control condition. *R. sphaeroides*, but not AMF, exhibited some plant growth promoting effect on saffron. This experiment was carried out for one flowering period. If it proceeded for another flowering period, i.e., another year, the effect on the number of flowers could have been well observed. During the period of this experiment, the number of flowers formed was 8 for the control, 4 for bacteria, 3 for AMF and 5 for bacteria + AMF applications.

It is only safe to say that AMF application alone lowered the formation of flowers. Although the plant length (leaf length) was not significantly affected by the bacterial application, the fresh and dry weight of the leaves increased. Since this study aimed to investigate the development of above ground parts of the plant, it is safe to say *R. sphaeroides* positively affected the growth of saffron when applied alone. *R. sphaeroides* was tested for the growth of cucumber and sesame seedling. The results revealed that this bacterium enhanced the amino acid content and plant growth hormones such as IAA (indole acetic acid) and gibberellin and hence enhanced the growth of plants [32, 33]. Purple non sulfur bacteria were reported to produce 5-aminolevulinic acid (5-ALA) which helps the plants struggle with stress conditions and enhance the plant growth [34].

The effects of different microbial inoculations on some macro and micronutrient elements are tabulated in Table 3. The soil used in this study was low in organic content (1.88%). Therefore, the utilization of microbial inoculations holds promise for augmenting saffron cultivation, as these microorganisms facilitate the solubilization of essential elements making them more readily available to plants [35].

Macro and micro-nutrient elements' analyses of vegetative parts of the plants revealed how the plant benefit from the use of these microbial fertilizers. Among the macro nutrient elements, the nitrogen content of plants grown upon different applications was not significantly different from each other (Table 3). However, there were significant differences of all the other macro and micronutrient elements among the applications. The macro nutrient phosphorus (P) was observed to be the highest in saffron grown with the addition of bacteria and mycorrhiza combination, followed by the application of mycorrhiza alone (11.1% and 4.5% higher than the control) (p=0.008). *Rhodopseudomonas palustris*, a purple non sulfur bacteria were

documented to be a phosphate solubilizer as it increased the uptake of P of peanut from the soil [36].

In addition, two strains of *R. sphaeroides* were shown to increase the soil P by about 30% and these bacteria enhanced the plant height of pineapple by about 4% [37]. Phosphate solubilizer bacteria are therefore accepted as promising substitution for expensive chemical phosphate fertilizers [38]. This is achieved by the decrease of pH upon organic acid production by bacteria which results in solubilization of inorganic phosphate bound to the soil colloids [39].

Potassium solubilization in soil occurs via a similar mechanism with phosphate solubilization. The K content of saffron was found to be highest in the leaves of control condition and the lowest in mycorrhiza application alone. The solubilized K might have been used by the bacteria and fungi in the metabolism to replicate and increase population in this study. However, the combination of bacteria (2.36 mg kg^{-1}) and AMF resulted higher K content in leaves than the separate applications (2.30 and 2.01 mg kg^{-1} upon bacteria alone and AMF alone). In contrast to K, the highest Ca content was observed in saffron grown by the application of AMF only (p=0.000). It is known that mycorrhiza enhances the absorption of nutrients by plants [39]. On the other hand, the lowest was obtained by the application of only bacteria, and it was even lower than the control. This might suggest that bacteria can uptake Ca from the soil and compete with the plant roots, although the Ca in the soil was found to be high (Table 1).

Magnesium is another crucial macro nutrient element in plants as it has a central role in chlorophyll synthesis, besides being vital for protein synthesis [40]. The Mg content of saffron was found to be the highest in the plants grown upon the use of combination of bacteria and AMF, but the lowest in the application of bacteria alone. The Mg content of saffron upon application of AMF was 11.8% higher than the control and the combination of these microorganisms seemed to exert a synergistic interaction which served the plant to enrich in terms of Mg. Therefore, this combination can be suggested to be applied in saffron cultivation under the soil conditions used in this study.

Among the micronutrient elements, the application of AMF alone or in combination with *R. sphaeroides* led the plant to accumulate more Fe, Cu, Zn, Mn and B, where bacteria only treatment lowered the accumulation of Fe, Zn and Mn elements in saffron compared to the control condition (Table 3). The availability of Fe in the soil used in the trial was sufficient (Table 1). Bacteria can produce siderophores which are small organic molecules that act as iron chelator and keep most of the available Fe in rhizosphere [41]. In this study, the Fe concentration in soil was the highest in the pot where only bacteria were applied (Table 4). This might be the result of bacterial chelation of Fe in the soil. In terms of the micronutrients Fe, Zn, Mn and B application of the mycorrhiza can be suggested to enrich the soil.

Table 3. Macro and micronutrient element concentrations in leaves of *Crocus sativus* L.

	Control	**Bacteria**	**AMF**	**Bacteria + AMF**	**P value**
N (%)	4.19 ± 0.07	4.34 ± 0.05	4.21 ± 0.04	4.21 ± 0.04	0.178
P (%)	0.44 ± 0.01b	0.44 ± 0.02b	0.46 ± 0.00b	0.49 ± 0.01a	0.008
K (%)	2.88 ± 0.22a	2.30 ± 0.11b	2.01 ± 0.13b	2.36 ± 0.03b	0.002
Ca (%)	0.53 ± 0.01 c	0.48 ± 0.01 b	0.59 ± 0.01 a	0.57 ± 0.01 a	0.000
Mg (%)	0.17 ± 0.00 bc	0.16 ± 11.03 c	0.18 ± 0.00 ab	0.19 ± 0.00 a	0.001
Fe ($mgkg^{-1}$)	81.71 ± 7.71b	61.67 ± 0.53b	170.34 ± 12.67a	98.08 ± 22.04a	0.004
Cu ($mgkg^{-1}$)	6.61 ± 0.20b	7.58 ± 0.80ab	7.87 ± 0.62ab	8.77 ± 0.20a	0.010
Zn ($mgkg^{-1}$)	25.79 ± 0.58b	22.27 ± 0.20ab	30.14 ± 0.67a	28.04 ± 0.67ab	0.000
Mn ($mgkg^{-1}$)	15.6 ± 0.52 b	14.85 ± 0.59 b	18.13 ± 0.59 a	17.36 ± 0.22 a	0.000
B ($mgkg^{-1}$)	21.27 ± 1.01c	27.89 ± 0.80b	54.1 ± 0.30a	26.38 ± 0.52b	0.003
Ni ($mgkg^{-1}$)	0.51 ± 0.04b	1.29 ± 0.26a	1.06 ± 0.09a	0.86 ± 0.09a	0.001

The data are given as mean ±standard deviation of triplicates. Different letters indicate difference among groups according to Duncan's test following one way ANOVA or Dunn's test following Kruskal-Wallis test. No letters mean non-significant difference.

Table 4. Macro and micronutrient elements in soil with different applications

	Control	Bacteria	AMF	Bacteria + AMF	P value
N (%)	0.126 ± 0.002	0.124 ± 0.003	0.128 ± 0.003	0.125 ± 0.002	0.782
P (%)	0.002 ± 0.000	0.002 ± 0.000	0.002 ± 0.000	0.002 ± 0.000	0.786
K (%)	0.014 ±0.000	0.016 ± 0.001	0.015 ± 0.000	0.014 ± 0.000	0.562
Ca (%)	0.376 ± 0.004a	0.331 ± 0.002b	0.333 ± 0.003b	0.338 ± 0.004b	0.000
Mg (%)	0.027 ± 0.000a	0.025 ± 0.001b	0.024 ± 0.000b	0.024 ± 0.000b	0.009
Fe ($mgkg^{-1}$)	1.543 ± 0.036ab	1.681 ± 0.005a	1.444 ± 0.038bc	1.310 ± 0.083c	0.000
Cu ($mgkg^{-1}$)	1.012 ± 0.009a	0.927± 0.034b	0.986 ± 0.010a	1.032 ± 0.012a	0.007
Zn ($mgkg^{-1}$)	6.526 ± 0.108	5.838 ± 0.236	6.663 ± 0.131	6.457 ± 0.179	0.098
Mn ($mgkg^{-1}$)	3.262 ± 0.054	2.918 ± 0.118	3.332 ± 0.066	3.228 ± 0.090	0.091
B ($mgkg^{-1}$)	0.153 ± 0.003a	0.141 ± 0.010b	0.157 ± 0.004a	0.157 ± 0.007a	0.006
Ni ($mgkg^{-1}$)	0.777 ± 0.002a	0.721 ± 0.010b	0.763 ± 0.004a	0.772 ± 0.006a	0.000

The data are given as mean ±standard deviation of triplicates. Different letters indicate difference among groups according to Duncan's test following one way ANOVA or Dunn's test following Kruskal-Wallis test. No letters mean non-significant difference.

Sole mycorrhiza application also increased the concentration of these elements in plant (Table 3). However, for Cu the combination of microorganisms resulted in higher content in the plant (32.7% higher than the control). Micronutrient elements are vital for activation of several enzymes such as those playing role in redox balance in the cells, antioxidant enzymes such as catalase, peroxidase, superoxide dismutase, etc. [42]. Therefore, in the germination phase, the application of *R. sphaeroides* cannot be suggested alone, while AMF application or the combination of this bacterium with AMF can be suggested for healthy Saffron cultivation

The Ni content in the bacteria only condition after the harvest was found to be the highest, followed by mycorrhiza, the combination, and the control condition (Table 3). The increased solubility of the heavy metal Ni by the microorganisms applied resulted in the uptake of the metal by the plant. The content of Ni in soil after the harvest was the lowest by the application of the bacteria only (Table 4). This suggests that *R. sphaeroides* might be used in the enhancement of phytoremediation of this heavy metal from the soil. It solubilizes the heavy metal and helps the plant to uptake it more by the plant. The augmentation of phytoremediation of hexavalent chromium by purple non sulfur bacterium *Rhodobacter capsulatus* was shown in our previous study [43].

The contents of macro nutrients N, P and K were not significantly different from each other in the soil after the harvest of saffron. However, the Ca and Mg content in soil decreased upon applications of *R. sphaeroides,* AMF and in combination (Table 4).

Among the micronutrient elements, there is no significant difference between the treatments in terms of Zn and Mn. However, the application of *R. sphaeroides* increased the Fe in soil, but not in plant (Table 3). But the combination of microorganisms lowered the Fe content compared to the control condition. The results suggest that the combination of mycorrhiza and bacterium both solubilize the elements and make them available for the plant. However, the contents of Cu, Zn, Mn, and Ni were all the lowest in the bacterial only treatment. This suggest that *R. sphaeroides* might use the elements and might compete with the plant for uptake. Therefore, this bacterium cannot be suggested as a microbial fertilizer for Saffron cultivation alone [44, 45].

4. Conclusion

Saffron is a highly prized spice derived from the stigmas of saffron plant (*Crocus sativus* L.). After the harvest of the flowers, the corms of the plant are kept for the next season while the leaves are considered as agricultural byproduct. This investigation focused on assessing the macro and micronutrient composition of saffron leaves following inoculation with *R. sphaeroides*, *Glomus iranicum* var. *tenuihypharum*, and their combination, particularly in a soil characterized by low organic content.

When the microbial fertilizers were applied, the morphological results revealed that the root length of saffron upon AMF spore inoculation alone was almost twice that of control. The application of bacteria alone resulted in higher fresh and dry weight of leaves. *R. sphaeroides* exhibited plant growth promoter effect saffron. The macro nutrient phosphorus (P) was observed the highest in saffron grown with the addition of bacteria and AMF combination, followed by the application of AMF alone. The macro nutrients Ca and Mg were found to be the lowest by bacterial application alone, however, the Mg content of saffron was found the highest in the plants grown upon the use of combination of bacteria and mycorrhiza. Therefore, in the germination phase, the application of *R. sphaeroides* cannot be suggested for saffron alone, while AMF application or the combination of these microorganisms can be suggested. The nutrient contents of the plant are important for the phytochemicals, too.

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MG: Design of the study, data collection, data analysis, writing, critical review of content, literature review
SY: Design of the study, data collection, data analysis, writing, critical review of content, literature review
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