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Foliar Application of Iron Fortified Bacterio-siderophore And Rhizobium Seed Inoculation Promote Growth and Grain Fe Contents in Soybean and Chickpea

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ABSTRACT

Iron (Fe) is a vital micronutrient essential for plant development and grain quality. Chickpea (*Cicer aeritum*) and soybean (*Glycine max*), known for their high protein content, often suffer reduced grain quality due to low bioavailable Fe levels. To address this, a study was conducted to evaluate the combined effect of rhizobium seed inoculation and bacterio-siderophore foliar spray on legume crop growth and development. *Rhizobium sp*., isolated from chickpea nodules, was used for seed inoculation and *Bacillus megaterium* selected for its high siderophore production, was applied as foliar spray. Field experiments on chickpea and soybean crops included treatment with bacterio-siderophore alone or combined with inorganic Fe during the flowering stage. Parameters such as plant height, number of nodules, pods per plant, grain yield, nutrient (Fe, N and P) contents were measured. The synergistic application significantly enhanced grain Fe content, with increment of 1.44-fold in soybean and 4.07- fold in chickpea compared to control (water). Maximum plant height, number of nodules and pods were observed in combined application of bacterio-siderophore enriched with Fe and rhizobium. Grain yield improved by 2.16- fold in chickpea and 1.6-fold in soybean with rhizobium seed inoculation and bacterio-siderophore foliar spray enriched with Fe. This study demonstrates that foliar application of siderophore-producing bacteria enriched with Fe is an economical strategy for Fe fortification in leguminous crops, particularly in alkaline calcareous soils.

Key words: Bacterio-siderophore, iron, *Bacillus megaterium*, chickpea, soybean

INTRODUCTION

Almost all kinds of life depend on iron as one of their fundamental elements. Limitation of iron bio-availability in alkaline calcareous soil to plant roots is due to its fixation, despite its abundance in the Earth's crust (Merry et al., 2022). Fe chlorosis is a common agricultural disease that lowers crop productivity and nutritional value. Ideal growth and yield depend on a steady supply of iron coming from the roots and being remobilized from older to younger leaves. Iron availability in soil depends largely on pH, and it becomes immobile within plants, especially in high pH conditions. The quick transformation of iron added to the soil into solid Fe (III), which is inaccessible, highlights the need for an alternate approach to treating iron insufficiency (Molnár, Solomon, Mutum, & Janda, 2023). Significant progress has been made in understanding the potential of many beneficial organisms to improve agricultural productivity and guarantee food security (Daniel et al., 2022). Rhizobium seed inoculation has been employed in a variety of crops in the past. One of the most cost-effective and focused methods for treating Fe deficiency in crops is foliar fertilization combined with seed inoculation (R. Zhang et al., 2022).

Numerous organic and inorganic Fe compounds have been applied topically, yet the outcomes on Fe chlorotic plants were inconsistent (Mutlu, 2021). Fe chelates have been proposed as a more effective treatment for Fe chlorosis than inorganic salts (Sachin Sharma, Singh, Rai, Yadav, & Singh, 2022). When exposed to air, Divalent Fe salts quickly oxidize on exposure to air, but trivalent Fe salts form a hydrous gelatinous polymer as soon as the pH rises above 2.0 (H. Zhang et al., 2024). Therefore, chelated forms of Fe are better for foliar application than inorganic Fe salts because they are more readily translocated within plants when applied to foliage than other Fe-containing compounds (Sandeep Sharma et al., 2019). However, since synthetic chelates like EDTA and EDDHA are either non-biodegradable or weakly biodegradable, their overuse could pose a threat to the environment (Checa-Fernandez, Santos, Romero, & Dominguez, 2021). To stop iron chlorosis, it is necessary to find and create organic Fe chelators that are safe for the environment.

Within the rhizosphere exist new microorganisms known as plant growth promoting rhizobacteria (PGPRs) (Santoyo, Urtis-Flores, Loeza-Lara, Orozco-Mosqueda, & Glick, 2021). PGPRs exhibit versatility in their functions, exerting a considerable direct and indirect influence on plant growth through a variety of pathways (Vocciante, Grifoni, Fusini, Petruzzelli, & Franchi, 2022). When there is a lack of iron, a number of soil microorganisms create low molecular weight Fe-chelating compound with a strong affinity for ferric ions called "bacterio-siderophore," (Timofeeva, Galyamova, & Sedykh, 2022). Greek term "siderophore" means "iron barrier." Bacterial products known as siderophores use certain receptors to bind iron and speed up its bacterial transport from the environment into the cell. In general, the siderophore functions as a transporter of Fe into plant cells by forming a thermodynamically stable complex with trivalent Fe cation (Drechsel & Winkelmann, 2022). At present, five hundred siderophore compounds have been extracted from various microorganisms and classified according to their functional group: hydroxamate, which is generated by fungi and bacteria and gives blue color, catecholates, which are produced by bacteria and give red color, and carboxylates, which are produced by rhizobectin and give green color (Butler, Harder, Ostrowski, & Carrano, 2021). Identification of these bacteria was substantially aided by the accurate, dependable, and reasonably priced genome sequencing (Church et al., 2020). For example, the carboxylate siderophore that binds iron ions to the hydroxyl and carboxyl groups is generated by Salmonella typhimurium and E. Coli (Ammendola et al., 2021). By means of many methods, the siderophore enters the microbial cell and forms a combination with the ferric form of iron. The ferric ion enters the cell, converts to ferrous, and loses its siderophore (Khasheii, Mahmoodi, & Mohammadzadeh, 2021). It has been observed that inoculating seeds or roots with microbial inoculants that produce siderophores improves plant development and Fe uptake (Shahwar et al., 2023). Different mechanisms allow plants to absorb iron-siderophore complex, including direct absorption, chelation and release of Fe, and ligand exchange processes (Ghosh, Bera, & Chakrabarty, 2020). The quantity of divalent or trivalent cations in the soil influences the formation of the Fe-siderophore complex because they compete with Fe for binding sites.

Due to the aqueous nature of bacterio-siderophore, it becomes very appropriate for foliar treatment, hence circumventing the constraints associated with siderophore application in soil (Malhotra, Pandey, Sharma, & Bindraban, 2020). Previous research examined the effects of foliar application of bacterio-siderophore on several growth parameters and Fe concentrations in grains and seeds of wheat and chickpea crops, either with or without the addition of Fe (Ehsan et al., 2022). Thus, the goal of the current study was to determine the effects of foliar application of bacterio-siderophore with and without Fe addition, as well as rhizobium seed inoculation on the growth, yield, and iron concentration of legumes such as chickpeas and soybeans.

Legumes are among the food crops that are a key source of iron and are therefore important for human iron intake. Given that iron is a component of cytochrome and myoglobin, which is the hemoglobin heme group transporter of oxygen, legumes can be very helpful in enhancing iron nutrition to help people overcome hidden hunger. Legumes that fix nitrogen are highly desirable for more sustainable agriculture, especially in organic farming systems (Barbieri, Starck, Voisin, & Nesme, 2023). Given this, smallholder farmers in sub-Saharan Africa stand to benefit greatly from biological nitrogen fixation (BNF), a sustainable source of N fertilizer. Legumes rank second in the country after cereals, accounting for 12% of total food production. Grain legumes such as soybean (*Glycine max L.*) and chickpea (*Cicer aeritium*) are cultivated for their edible beans, which are a major global source of low-cost, high-quality protein and carbohydrates. They partially meet their own demands for nitrogen in this symbiosis and also give some of the leftover nutrients to crops that come after them through the breakdown of their biomass, roots, and nodules (Abd-Alla, Al-Amri, & El-Enany, 2023). In Punjab's highly alkaline calcareous soils, there is not enough soluble iron present for plants and microbes to grow healthily. In soils, Fe^{2+} is easily oxidized to generate iron hydroxides or oxides, the solubility of which reduces as pH rises to 7.5–8.5. The World Health Organization (WHO) has developed a strategy called biofortification to enhance the nutritional properties of cereals and legume crops. This strategy makes use of a variety of microorganisms, crop genetics and breeding technology, and agronomic methodologies (Prasad & Shivay, 2020).

Considering the aforementioned information, the current study's objectives were as follow

- To assess the potential of PGPRs for siderophore production
- To observe the synergistic effects of seed-inoculation and foliar application on soybean and chickpea crops biological yield
- To find the improvement in grain quality through iron enrichment.

MATERIALS AND METHODS

Isolation of Rhizobium Sp for Seed Inoculation: Root samples collected from chickpea at flowering stage were given washing with tap water to remove soil particles. Then nodules were plucked from roots with tweezer and kept in petri-plates for further surface sterilization with 95% C_2H_5OH and 0.2% $HgCl_2$ for 3-5 min and the rinsed with sterilized water to remove all types of contamination (Tariq et al., 2024). The crushing of sterilized nodules in a small quantity of distilled water with the help of glass rod done to get a milky suspension containing rhizobium. Petri-plates were prepared with sterilized yeast extract mannitol agar medium enriched with Congo-red indicator. Then a loop-full of milky suspension containing rhizobium spp. were streaked on agar medium in plates and incubated at 28±1^{O}C for 3-4 days. Purification of isolated colonies was done by re-streaking on fresh YEM containing agar plates to get a pure rhizobium strain. The rhizobium strain was then preserved in the refrigerator at 4±1 degree in cryo-vials for further testing and experimentation. They were used later for inoculum preparation using liquid broth of yeast extract mannitol medium (YMB). Fresh culture/inoculum were prepared 72 hours before use by culturing 1mL of cryo-preserved strains of *Rhizobium spp*. in 250mL of sterilized broth of yeast mannitol medium with continuous shaking get a maximum optical density of 0.5.

Isolation & Selection of Rhizobacteria for Foliar Spray: Rhizobacterial strains for foliar were selected on the basis of quantity of siderophore units produced and are called bacterio-siderophore Approximately 10.0 g wet soil was suspended in 95mL sterilized water to prepare 10^{-1} dilution with continuous shaking for 10 min. 1.0 mL of this suspension was taken with tip and transferred into 9mL sterilized water (10^{-2}) in test tube, similarly serial dilution of this suspension was continued up-to 10^{-10}, followed by pouring of 0.1mL suspension on different types of agar medium viz, Nutrient Agar (NA), King B (KB) containing basal nutrients i.e. glucose, yeast, potassium and magnesium sources. The suspension was spread homogeneously on agar medium plates and incubated for 2-3 days at 28ºC. The distinct separate colonies were then selected and sub-cultured again on fresh nutrient agar plates to get individual isolates. A total of 20 bacterial isolates thus obtained were cryo-preserved at -80ºC in 50% glycerol for future experiments. Fresh culture/inoculum were prepared 72 hours before use by culturing 1mL of cryo-preserved strains of rhizobium spp in 250mL of sterilized broth of yeast mannitol medium with continuous shaking get a maximum growth of 10^{7}cfu/mL. The DNA sequencing done through Macrogen Lab, Korea. BLAST analysis was done for the sequence received and comparison with already registered sequences in the Gen-bank database was done using NCBI Blast server.

Quantitative Production of Siderophore: Quantitative detection of siderophore was assayed spectrophotometrically as described by (Senthilkumar et al., 2021). Succinate medium was used for siderophore production (Meyer & Abdallah, 1978) at pH 7.0. The medium was inoculated with 24-hour old fresh culture/inoculum of rhizobacteria and rhizobium separately @ 1 % (v/v), and kept under incubation at 30-degree temperature for two days with constant shaking at 120 rpm. The siderophore secreted by microbes will come to liquid broth was then harvested by centrifuge and collected supernatant. To determine the presence of siderophore, the 0.5 mL of supernatant (24 h) was poured into the 0.5 mL dark blue Chrome Azurol Sulfonate (CAS) in 2mL of Eppendorf. Later on, 10 µL sulfosalicylic acid was added to this shuttle solution to develop color that was measured using the spectrophotometer at 630 nm wavelength after 20 min of dark place incubation. Necessary blank (uninoculated medium) called reference solution were used as a negative control.

In CAS Liquid Assay method, the strains depict sharp pigmentation and change of CAS liquid both from dark blue color to yellowish orange compared with the reference solution was cleared. The reference sample showed greatest absorbance percentage (blue color) as all the blue color is retained due to no siderophore production (Ar). Test samples showed lower absorbance as siderophore removes the iron from the dye complex (As). The values of the siderophore excreted determined using the formula:

The percentage (%) siderophore unit (SU) was calculated by using a formula given below:

$$\%\ siderophore\ unit = \frac{\mathrm{Ar} - \mathrm{As}}{\mathrm{As}} \times\ 100$$

As= Sample absorbance at the wavelength of (600 nm); Ar= Reference/blank absorbance at the wavelength of (600 nm).

Nutrient Solubilization and Hormone Production*:* Qualitative test for mineral solubilization of P and Fe was done by using Nautiyal (1999) and Nishio and Ishida (1989) agar medium. Specific media have the insoluble source of Fe as $FePO_4$ and P as $Ca_3(PO_4)_2$. One loopful of the 24-hour fresh culture was spot inoculated on the respective medium and incubated at 28^{0}C for 4 days. The P solubilization was observed by zones of clearance around colonies. The bacteria showed a change in color after 3-4 weeks due to reducing insoluble iron form to a soluble form. In the end, it was marked as positive for iron solubilization.

Characterization of selected strains was done on the basis of plant growth promoting traits. Brick *et al.*, (1995) method for hormone Indole acetic acid (IAA) production was used. For this purpose, 10mL of sterile peptone broth containing L-tryptophan {0.204 g}, peptone/tryptone {10g}, NaCl {5g}, beef extract {3g}, deionized water {1L} at pH 7 was inoculated with 100 microliter of cell suspension into culture tubes and kept under incubation for 3 days at 28ºC. Later on, after centrifugation, 1mL of Salkawaski color developing reagent (50mL of 35% of perchloric acid, 1mL of 0.5M FeCl3 solution) added to 1mL of the supernatant solution for pink color development, the color intensity was measured on spectrophotometer at 540 nm λ. The IAA concentration was measured by using standard curve prepared through working standards of IAA.

Field Experiment: Based on the characteristics, two bacterial strains, *Bacillus megaterium* and *Rhizobium sp.*, were chosen to validate the impact of foliar application of iron fortified bacterio-siderophore and seed inoculation, respectively on soybean and chickpea in field. Yeast mannitol broth was prepared for seed inoculation with rhizobium. Sowing of soybean crop was done on 25th July 2022 while wheat was sown on12th November 2022. Seeds of soybean were dibbled with a plant -to-plant distance of 3 inches on both sides of ridges (2-1/2 feet) and three ridges per plot. Sowing of chickpea was done manually with 4 rows per plot (22 cm row spacing) and 3 inches plant to plant distance. The plot size of 27 $m^2 \times 48$ m^2 for soybean and 1.2 $m^2 \times 4$ m^2 chickpea were used for field experiment.

Preparation of Bacterio-siderophore for Foliar Spray: Siderophore produced by rhizobacteria extracted from medium are called bacterio-siderophore. Foliar sprays solution was prepared by inoculating 5.0 mL of 24-hour old culture of bacterial strain in 5.0 L of Fe deficient Succinate medium (SM) with continuous shaking under incubation temperature of 32 degree for one day to get maximum growth 10^7cfu/mL. After making sure the bacterial growth through OD_{600nm}, culture solution was centrifuged at 10,000 rpm for 15 min to get bacterio-siderophore. The siderophore containing supernatant thus obtained from bacterial strain was divided into two portions. To prepare iron fortified bacterio-siderophore, one portion was incubated with $FeCl_3$ (2.0 mM) while the other portion was maintained as such without Fe. A total of eight treatments; namely T1. water as control (C), T2. Rhizobium Sp Inoculation (Sp), T3. Foliar 2.0mM Fe Spray (Fe), T4. Foliar bacterio-siderophore without Fe (Bs), T5. Foliar bacterio-siderophore with Fe (Fe + Bs), T6. Rhizobium Sp. + Foliar 2.0mM Fe spray (Sp+ Fe), T7. Rhizobium Sp. + foliar bacterio-siderophore without Fe (Sp+Bs), T8. Rhizobium Sp. + foliar Fe bacterio-siderophore with Fe (Sp+ Fe+ Bs). The chickpea and soybean seeds were surface sterilized for seed inoculation, and dipped in rhizobium inoculum for half an hour (Naveed, Mitter, Reichenauer, Wieczorek, & Sessitsch, 2014). Field experiment was launched in the farm area of Pulses Research Institute, Ayub Agricultural research Institute, Faisalabad, during 2022-2023 in loam textured soil having pH 8.1 with adequate potassium and phosphorus contents. Randomized complete block design/RCBD with three replications was used (Koirala et al., 2020). The foliar application of bacterio-siderophore was carried out at flowering stage in both crops. During spraying time, polythene was used to cover the soil surface to avoid dripping of excess solution from foliage into the soil and it was removed the next day. A set of treated and non-treated plants were harvested at physiological stage for plant height and nodules per plant while at maturity other set was sun dried and threshed to record yield attributes data. The Fe concentration in grains was estimated by wet digestion with di-acid mixture (HNO_3:$HClO_4$) and using atomic absorption spectrometer (ECIL,India).The iron contents were calculated multiplying the grain Fe concentration with their dry weight and expressed as mg per plant. Similarly, after wet digestion of grains, nutrient uptake was calculated as: Nutrient uptake = Nutrient content × Dry shoot weight

Statistical Analysis: The collected data will be analyzed with randomized complete block design (RCBD) and difference/significance between treatments will be checked through Turkey HSD at 95% level of confidence of interval.

RESULTS

Identification and Characterization of Strains: The two selected strains were *Bradyrhizbium Spp.* and *Bacillus megaterium* with accession number (KY426385.1) and (KJ476721.1) respectively according to DNA analysis from Macrogen Lab Korea (table 1). These bacteria were both gram positive and gram negative. The characterization of PGPR and rhizobium on the basis of siderophore production was done. All strains produced siderophore qualitatively and quantitatively with maximum siderophore units produced by *Bacillus megaterium*. All strains found positive for P, Fe solubilization along-with Indole acetic acid production (Table 1).

Table 1. Microbial characteristics of isolated strains

Isolate	IAA	Siderophore Unit (SU)	Gram staining	Qualitative Solubilization		Accession #
	(µg mL-)	(%)		Fe	P	
Bacillus megaterium	2.15	41.67	++	++	+	KY426385.1
Bradyrhizobium	2.09	26.66	--	++	+	KJ476721.1

Synergistic Effect of Bacterio-siderophore and Rhizobium on Physiological Parameters of Plant: A significant difference in number of nodules per plant was observed in seed and foliar applied bacteria in both crops (Table 2). The maximum number of nodules per plants (53.3 in soybean & 32.2 in chickpea) were observed in foliar applied bacterio-siderophore along with iron and rhizobium seed inoculation in soybean and chickpea respectively. Seed inoculation with *Bradyrhizobium* showed maximum response when applied along-with foliar bacterio-siderophore complex as compared to alone application. Alone application of iron foliar spray showed a smaller number of nodules (21.3) in soybean and 16.7 in chickpea but more as compared to control where no treatment was applied. No significant difference in No. of nodules was recorded between seed inoculation along-with bacterio-siderophore foliar spray with and without iron, however, their response was more as compared to alone foliar application with bacterio-siderophore or iron. Similarly seed inoculated chickpea (21.3) and soybean (33) plants showed high number of nodules as compared to foliar application of iron or bacterio-siderophore. Application of siderophore producing *B. megaterium* on leaves and rhizobium inoculation increases number of nodules per plant in both crops. Plant height of crop plants was statistically at par with the combined application of PGPRs as seed and foliar spray (Table 2). Foliar bacterio-siderophore invigorated with Fe and rhizobium seed inoculation (Sp+Fe+Bs) was found most effective in enhancing plant height (95.6cm) of the soybean plants. Sp+Bs was the second most effective treatment to increase the plant height (92.3cm) in chickpea. No statistically significant difference in plant height of chickpea plant was recorded where Sp, Sp+Fe and Bs+Fe were applied. However, their efficacy to enhance plant height was more as compared to alone application of Fe and bacterio-siderophore (Bs) foliar spray. In case of chickpea crop, nonsignificant effect on plant height was observed among seed and foliar applied treatments with maximum (54.4 cm) effect in Sp+Fe+Bs treatment and minimum in case of control (45.9 cm). While combined application of seed and foliar based bacterio-siderophore showed statistically significant impact on plant height as compared to seed inoculation or foliar alone application of treatments.

Synergistic Effect of Bacterio-siderophore and Rhizobium on Yield Parameters*:* A significant difference in pod production was recorded among rhizobium and bacterio-siderophore applied plants (Table 3). The maximum number of pods (46) were counted where iron invigorated bacterio-siderophore was sprayed along-with rhizobium seed inoculation in soybean. The iron foliar spray alone application plants produced lower number of pods (34) as compared to bacterio-siderophore and foliar iron (Fe+Bs) applied plants, however, were more as compared to control in case of soybean. Similarly, in case of chickpea, co-inoculation of bacterio-siderophore as foliar spray and bradyrhizobium (Sp+Bs) was statistically at par in producing maximum number of pods per plant with bradyrhizobium and iron fortified bacterio-siderophore complex (Sp+Bs+Fe) as shown in (Table. 3). The seed inoculation with bradyrhizobium produced lower number of pods as compared to seed inoculation and foliar iron spray treatment but showed more response as compare to foliar iron or bacterio-siderophore alone application. In case of grain yield iron fortified bacterio-siderophore complex as foliar along with seed inoculation showed significant impact to increase grain yield of chickpea and soybean crops Table 3). In case of chickpea maximum yield 5047 kg/ha was found in SP+Fe+Bs complex followed by Sp+Bs (4801 kg/ha) and minimum yield (3020 kg/ha) found in control, where no treatment was applied. All foliar applied treatment applications along-with rhizobium seed inoculation were statistically at par in case of grain yield of chickpea. The maximum yield of soybean was found in T8 (936 kg/ha) followed by T7 (Sp+ Bs) and T6 (Sp+ Fe) showing positive impact of seed and foliar co-inoculation of siderophore producing bacteria. Seed inoculation with bradyrhizobium showed less impact on increasing grain yield (863 kg/ha) of soybean but more as compare to foliar applied iron (508 kg/ha) and bacterio-siderophore spray (611 kg/ha) at flowering stage (Table 3)

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Table 2. Synergistic effect of seed inoculation and foliar spray of PGPR on physiological parameters of legume crops with means± standard error, n=3

Treatments	Number of nodules		Plant height (cm)	
	Soybean	Chickpea	Soybean	Chickpea
T1. Control	13.7±0.67^{c}	13.3±0.33^{e}	81.6±4.3^{c}	45.9±0.2^{c}
T2. Seed Inoculation (Sp)	33.0±6.51^{b}	21.3±0.73^{c}	87.3±4.1^{abc}	47.5±0.8^{bc}
T3. Foliar Fe (Fe)	21.3±1.86^{c}	16.7±2.03^{d}	83.0±3.1^{bc}	48.7±4.1^{bc}
T4. Foliar Bacteriosiderophore (Bs)	16.67±2.03^{c}	18.6±0.30^{d}	83.3±2.7^{bc}	47.8±1.7^{bc}
T5. Folira Fe+Bs	24.3±7.54^{bc}	23.6± 0.30^{c}	91.0±1.5^{abc}	52.0±2.0^{ab}
T6. Sp+Fe	47.3±2.67^{a}	26.9±0.48^{b}	88.3±3.5^{abc}	53.6±1.5^{a}
T7. Sp+Bs	49.67±3.93^{a}	30.1±0.33^{a}	92.3±4.4^{ab}	54.4±1.8^{a}
T8. Sp+Fe+Bs	53.3±6.97^{a}	32.3±1.63^{a}	95.7±7.4^{a}	55.0±1.7^{a}
LSD	**11.65**	**2.46**	**10.3**	**105.8**

Mean values of the same letter do not differ α=0.05

Table 3. Synergistic effect of seed inoculation and foliar spray of PGPR on yield parameters of legume crops with means± standard error, n=3

Treatments	Number of Pods		Grain yield (kg/ha)	
	Soybean	Chickpea	Soybean	Chickpea
T1. Control	28.0±0.58^{f}	8.20±0.42^{f}	432.7±24.7^{f}	3020±91.6^{f}
T2. Seed Inoculation (Sp)	38.2±0.54^{c}	14.3±1.25^{c}	863.3±31.8^{b}	4620±14.7^{c}
T3. Foliar Fe (Fe)	34.0±1.53^{e}	10.0±0.58^{e}	508.3±16.9^{e}	3583±63.7e
T4. Foliar Bacteriosiderophore (Bs)	36.0±0.58^{de}	12.1±1.03^{d}	611.3±27.2^{d}	4215±90.1^{d}
T5. Folira Fe+Bs	37.0±1.0^{cd}	11.1±0.10^{de}	769.3±43.0^{c}	4108±34.2^{d}

T6. Sp+Fe	39.3±2.41[ab]	16.8±0.32[b]	893.3±12.0[ab]	4759±59.3[bc]
T7. Sp+Bs	41.3±2.03[a]	17.9±0.09[ab]	920.0±26.5[ab]	4801±21.9[b]
T8. Sp+Fe+Bs	46.1±0.58[a]	19.4±0.67[a]	936.6±35.3[a]	5047±62.7[a]
LSD	**3.32**	**1.67**	**71.19**	**150.8**

Mean values of the same letter do not differ α=0.05

Effect of Synergism on Nutrients Uptake: Nitrogen and phosphorus uptake in grain contents of chickpea and soybean crops were determined to see the impact of foliar v/s soil application of siderophore producing bacteria (Table 4). Phosphorus and nitrogen uptake were maximum in combined effect of seed inoculation and iron enriched foliar application of bacterio-siderophore (Sp+ Fe+ Bs) in soybean crop followed by T7 (Sp+ Bs) and T6 (Sp+ Fe). Combined effect of rhizobium seed inoculation and foliar application of bacterio-siderophore showed significant impact on nitrogen and phosphorus uptake in grains. Rhizobium seed inoculation showed less impact on nutrient uptake but more as compared to sole application of iron and bacterio-siderophore foliar spray.

Similarly, in case of chickpea, phosphorus uptake was more in T8 (Sp+ Fe+ Bs) and less in T1 (control) where no treatment application was done. While in case of other combined application of seed v/s foliar, the treatments were statistically at par among each other. Similar is with case of nitrogen, bacterio-siderophore spray showed significant effect in increasing uptake of nitrogen in chickpea grain. Maximum nitrogen uptake (55 g/kg) obtained in case of T7 (Sp+ Bs) followed by T8 and T6 (Table 4).

Effect of Synergism on Protein and Iron Contents of Grain: Effect of co-inoculation of siderophore producing bacteria as seed and foliar application was checked on quality parameters of crops (Table 5). Maximum protein contents (40%) found in iron fortified bacterio-siderophore foliar application along-with rhizobium seed inoculation and minimum (23.9%) in case of control in soybean crop. Statistical analysis of data showed that treatments were statistically at par among each other in increasing protein contents in grain. But in case of iron contents in grain, significant impact of treatment was seen with maximum in T8, followed by T7 and T6. Sole rhizobium seed inoculation also showed increased iron content in grain (22.6 mg/100g DW) but this impact was less then combined foliar and seed inoculation. Sole application of iron and bacterio-siderophore showed less effect on iron contents but more as compared to control in soybean crop.

Similarly, in case of chickpea crop, protein and iron contents significantly improved in grain with foliar + seed application of siderophore producing bacteria (Table 5). Maximum protein and iron contents found in T8 (Sp+Fe+Bs) followed by T7 (Sp+Bs). Seed inoculation along with bacterio-siderophore application alone and with iron enrichment depicted significant impact on improving quality of grain crop. T8, T7 and T6 were statistically at par in increasing iron contents in chickpea grain followed by T2 (Sp) where seed inoculation with rhizobium was done

Table 4. Synergistic effect of seed inoculation and foliar spray of PGPR on nutrient uptake in legumes with means± standard error, n=3

Treatments	**Nitrogen uptake (g/kg)**		**Phosphorus uptake (g/kg)**	
	Soybean	Chickpea	Soybean	Chickpea
T1. Control	52.6±0.8[e]	24.7±1.6[f]	8.67±0.3[d]	3.04±0.4[e]
T2. Seed Inoculation (Sp)	126.6±4.4[bc]	50.8±1.1[c]	19.7±0.5[bc]	4.92±1.5[cd]
T3. Foliar Fe (Fe)	104.6±2.9[d]	34.9±1.7[e]	17.1±0.9[c]	3.90±0.1[de]
T4. Foliar Bacteriosiderophore (Bs)	108.3±4.4[d]	40.2±0.8[d]	20.9±0.7[b]	5.59±0.1[bc]
T5. Folira Fe+Bs	115.0±2.9[d]	40.2±0.9[d]	21.0±0.9[ab]	5.82±0.2[abc]
T6. Sp+Fe	140.3±8.7[b]	51.3±1.1[bc]	23.0±0.3[a]	6.03±0.2[abc]
T7. Sp+Bs	133.6±4.7[b]	55.0±1.7[a]	23.9±2.6[a]	6.76±0.1[ab]
T8. Sp+Fe+Bs	204.6±14.4[a]	54.4±1.8[ab]	24.2±1.4 [a]	7.16±0.1[a]
LSD	**16.02**	**3.45**	**1.74**	**1.35**

Mean values of the same letter do not differ α=0.05

Table 5: Synergistic effect of seed inoculation and foliar spray of PGPR on protein and iron contents in legumes with means± standard error, n=3

Treatments	**Protein %**		**Fe contents (mg/100g DW)**	
	Soybean	**Chickpea**	**Soybean**	**Chickpea**
T1. Control	23.9±1.0[e]	16.6±1.1[d]	16.6±1.1[d]	3.04±0.4[d]
T2. Seed Inoculation (Sp)	32.9±0.9[bc]	23.0±0.4[a]	22.6±0.4[ab]	8.19±1.7[b]
T3. Foliar Fe (Fe)	28.2±0.4[de]	19.7±1.0[c]	19.7±1.0[c]	6.13±0.7[bc]
T4. Foliar Bacteriosiderophore (Bs)	31.3±2.2[cd]	20.9±0.5[c]	20.8±0.5[c]	5.75±0.1[c]
T5. Folira Fe+Bs	34.3±0.4[bc]	21.2±0.1[bc]	21.0±0.4[bc]	7.43±1.3[bc]

T6. Sp+Fe	35.5±0.5[abc]	22.6±0.4[ab]	23.0±0.4[a]	11.3±0.6[a]
T7. Sp+Bs	37.3±0.5[ab]	24.2±0.4[a]	24.2±0.4[a]	11.5±0.5[a]
T8. Sp+Fe+Bs	40.0±4.4[a]	23.9±1.0[a]	23.9±1.0[a]	12.4±0.8[a]
LSD	**4.48**	**1.71**	**1.74**	**2.22**

Mean values of the same letter do not differ α=0.05

DISCUSSION

Plants like corn, chickpea, wheat and soybean crops need their extra boost in form of foliar fertilization during boom and seed development. Beans require a proper nutrition for optimal productivity; this involves both basal and foliar fertilizers which provide macro and micro elements. In high pH soils in arid regions where some elements such as Fe & Zn cannot be adequately absorbed and accordingly we hypothesized that using chelating agents like bacterial siderophore as foliar spray could alleviate nutrient deficiency under such conditions. Also, among different priming methods, bio-compounds including Plant growth promoting bacteria (PGPB) are one of the effective methods which is called bio-priming. Therefore, the findings of present study clearly indicated that biopriming of seed and later on foliar application with bacterial siderophore can improve crop physiological and yield attributes under arid conditions. Plant growth promoting rhizobacteria has the potential to fortify iron content in the edible portion of crop plants through iron solubilization and siderophore production. Rhizobia by their ability to convert nitrogen to ammonia, which can be used by plants also belong to PGPR. Biofortification of wheat and potato with such siderophore producing PGPR based inoculants is an alternative way of providing micronutrients to the human diet especially in rural areas of less developed countries (Mushtaq, Asghar, & Zahir, 2021).

PGPB that can simultaneously produce siderophores and possess nitrogen fixation have been described along-with other activities like indole acetic acid production, phosphate solubilization under biotic and abiotic stress; Inoculation with these strains was shown to positively improve plant growth. The significant variation in the quantity of siderophore produced by bacterial strains also called "Bacterio-siderophore" found under Fe deficient conditions as shown in Table 1. The effect of foliar application of bacterio-siderophore fortified with Fe on physiology, yield and Fe content depends upon the quantity of siderophore secreted by bacteria. The bacterium *Bacillus megaterium* is known to produce two hydroxamate siderophores (shizokinen and N-deoxyshizokinen) under Fe-limited conditions (Byers, Powell, & Lankford, 1967). The *Rhizobium leguminosarum* is also known to produce the schizokinen siderophore (Storey, Boghozian, Little, Lowman, & Chakraborty, 2006). Some strains of *Rhizobium radiobacter* are capable of producing hydroxamate-type siderophores (M. Smith & Neilands, 1984); for example, *Rhizobium meliloti* produces a siderophore called rhizobactin (M. J. Smith, Shoolery, Schwyn, Holden, & Neilands, 1985).

The plant experiment results also exemplify that seed bacterized with rhizobia and foliar iron fortification through *Bacillus megaterium* results in increase in various biological parameters viz. No. of nodules/pods, plant height, biomass accumulation and seed yield mainly be associated with enhancement in Plant growth promoting attributes viz IAA, siderophore production and phosphate solubilization leading to enhanced amino acid and protein contents as described previously (Sandeep Sharma et al., 2019). Very few literatures are available on the use of foliar applied PGPR for growth enhancement but the effect of foliar applied bacterio-siderophore in combination with Fe is lacking. Previous research findings (Esitken, Karlidag, Ercisli, & Sahin, 2002) showed that when Bacillus strain (OSU142) was applied as foliar spray in apricot during two successive years, yield increment of 30% and 90% respectively found. Further, experimentation on foliar implanted PGPR resulted to improved photosynthesis and boost metabolism in Sesame by *Bacillus methylotrophicus* strain KE2 (Radhakrishnan & Lee, 2017); increase height along-with nitrogen status of canola plants (Valizadeh-Rad *et al*., 2023). Moreover, previous studies also concluded that more nodulation on roots provides means more niches available for rhizobia, which ultimately leads to enhance nitrogen fixation and growth of leguminous crops (Zahir et al., 2018).

Study suggests that bacteria associated with root and stem nodules can be promising resource to enhance nodulation, plant growth promotion and crop yield in chickpea (Mir, Kumar, Gopalakrishnan, Vadlamudi, & Hameeda, 2021). In the field, both soybean and chickpea crops showed significant increase in No. of nodules, pods, plant height and grain yield due to inoculation of seeds with root associated rhizobia and foliar application of iron along-with bacillus bacterio-siderophore. PGP bacteria colonize roots efficiently, increase availability of nutrients (Meena et al., 2017), regulate nitrogenase activity, synthesize plant growth regulators like phytohormones (Chen et al., 2023) and siderophore production. In soybean, the foliar application of bacterio-siderophore + Fe along-with seed inoculation resulted in increased in number of nodules (20%), plant height (9.5%), pod number per plant (61.5%), grain yield (8.49%) as compared to alone seed inoculation. Similar effect on crop parameters was shown by *Bacillus megaterium* as foliar spray along-with Fe and rhizobium seed inoculation application in chickpea crop. Our results demonstrate that the foliar application of Fe alone or in combination with siderophore producing *Bacillus Sp*. had similar effect on plant growth and yield but combination of Fe with siderophore producing *Bacillus* bacterium along with *Rhizobium sp.* significantly improved yield and yield parameters. Our findings are in accordance with (Hafezi Ghehestani, Azari, Rahimi, Maddah-Hosseini, & Ahmadi-Lahijani, 2021) that bacterial siderophore could be usefully applied to improve physiological traits, nutrient absorption and grain yield under arid regions. Similarly, high efficiency and profitability of biopriming with bacterial siderophore have been reported in fenugreek seed (Solouki et al., 2023) that resulted in producing more vigorous seedlings & improving seedlings establishment.

Phosphorus is most limiting nutrient after nitrogen in most of agricultural soils due to low level of soluble phosphate, the potential of rhizobacteria to solubilize insoluble phosphate improved plant development and yield by enhancing its availability to plants. Rhizobium inoculation along-with foliar bacterio-siderophore improved nutrient solubilization and uptake in legume crops. Significant improvement in nitrogen uptake (63%) and phosphorus uptake (21%) in soybean and 54% and 45% in chickpea found in combined application of foliar and seed treated application compared to seed inoculation alone (Table 5). Moreover, increase in nutrient uptake due to increase solubilization of minerals by rhizobacteria found. Our results are in accordance with the previous findings of researches that combined application of seed and foliar treatment help plant growth development and improvement (Meena et al., 2017).

The quality parameters of legume crops are very important factor responsible for its being proteinaceous diet (Banti & Bajo, 2020) The concentration of protein and partitioning of Fe in grain contents of soybean and chickpea were significantly influenced by foliar treatments (Table 5). In soybean crop, foliar spray of bacterio-siderophore and Fe at flowering stage, significant increase in protein concentration (32-40%) and iron contents (19-23%) when compared to control (water). Although all foliar treatments improved seed Fe contents in soybean but higher retention in foliage at harvest was observed in combined seed and foliar application of plant growth promoting rhizobacteria. Our results are in accordance with the findings of (Sandeep Sharma et al., 2019) that foliar spray of high siderophore rhizobacteria resulted in improved iron contents and plant growth in chickpea crop. Moreover, it was observed that seed soaking of legume crops with rhizobium and later foliar fertilization with bacterio-siderophore + Fe provoked yield and yield contributing parameters due to continuous supply of iron through siderophore. They believe that the microorganisms can either increase the Fe availability or cellular use or regulate Fe homeostasis in the plant (Vélez-Bermúdez & Schmidt, 2022).

CONCLUTION

The study confirmed that siderophore produced by indigenous plant growth promoting rhizobacteria called bacterio-siderophore have the potential to improve quality of crops through iron enrichment It is also obvious that seed inoculation with rhizobium and foliar application of *Bacillus megaterium* has promising effects in promoting growth and yield of leguminous crops. Besides these promising isolates possess desirable plant growth promoting traits, foliar application of bacterio-siderophores enriched with iron lead to increase in plant height, number of nodules, pods per plant and yield under field condition. The synergistic effect of seed and foliar application of bacterio-siderophore enhanced P and K uptake in chickpea and soybean plants and boost their protein and iron contents. The overall result of this study proved that "*B. megaterium* and *rhizobium*" can be used for plant growth promotion through combined use as seed and foliar application under field conditions.

AUTHORS CONTRIBUTION

Shabana Ehsan: Writing original draft, Conceptualization, Swebba Waheed: Writing – review & editing, Data curation, Formal analysis. Aleem Sarwar and Neelam Chaudhary– review & editing. Waqas Ashraf and Quais M. Affan– review & editing. Fraz Anwar: Field Resources, Software, Supervision, Validation, Visualization, Hafsa Zafar and Amar Iqbal Saqib: Investigation, Methodology, Project administration.

CONFLICT OF INTEREST

The authors declare that the research was conducted without any conflict of interest.

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