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Effect of Fluorescent-Producing Rhizobacteria on Cereal Growth Through Siderophore Exertion

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Abstract

Despite soil having an abundance of iron (Fe), it is unavailable for proper plant growth and development. One of the mechanisms plants use to deal with iron deficiency is the uptake of iron by chelating phytosiderophores. *Pseudomonas fluorescence* can produce pyoverdine-type siderophore and has potential application in agriculture as an iron chelator. Therefore, bacterial isolates collected from different areas of district Faisalabad were screened for their fluorescent, siderophore production and indole acetic acid equivalents. After selecting efficient strains from a screening test, they were evaluated for improving wheat and maize production under field conditions. The results showed that out of 15 isolates, 7 were found to have significant plant-beneficial microbial traits. Efficient strains promoted grain yield by 24.2% and 20.2%, plant height by 30.9% and 23.7%, total grain weight by 25.3% and 13.4% over control in wheat and maize, respectively. Similarly, significant improvements in the number of grains per cob/spike were also observed. Analyses of grain iron contents depicted 67% increase as compared to control in for maize. Therefore, based on the results, it is concluded that bio-fortification of cereal crops through fluorescent producing siderophoric microbes is an effective strategy favorable for plant growth and development through nutrient solubilization/mobilization.

Keywords*. Siderophore, Fluorescence, Wheat, Maize, Iron solubilization, Rhizobacteria*

Introduction

Micronutrients play a crucial role in the growth and development of living organisms. Intensive cereals production to feed the rising population and increasing demand for synthetic agrochemicals (chemical fertilizers & pesticides) is the present-day concern in the agriculture sector (Please give a reference). Soil inhabiting plant growth promoting rhizobacteria (PGPR) has a vital role in sustainable cereals production. Utilizing beneficial bacteria (PGPR) is an eco-friendly strategy for enhancing cereals production by modulating the root system architecture, imposing systemic resistance, and producing certain allelochemicals. Moreover, the production of primary and secondary metabolites improved plant growth, nutrient uptake, quorum sensing, and defence against phytopathogens (Jha & Saraf, 2015); (Alori, Babalola, & Prigent-Combaret, 2019). The PGPR potential to improve the growth of plants like maize, rice, wheat, etc., has been reported previously (Adjanohoun et al., 2011; Gopalakrishnan et al., 2013; Islam et al., 2014). Many strains of *Pseudomonas fluorescence* belonging to PGPR are known to enhance plant growth by secreting a soluble greenish fluorescent pigment under UV light called fluorescence (Vacheron *et al*., 2016). *Pseudomonas fluorescence* is known to produce siderophore "pyoverdine", a biotechnologically significant iron chelator with great potential for application in the agricultural and medicinal sectors (Joshi *et al*., 2018). Low molecular weight (<1500 Da) siderophores are Fe^{3+} chelating agents which deliver free iron to the cells by interrelating with membrane receptors (Johnstone and Nolan, 2015; Saha *et al*., 2016). Siderophore-producing bacteria are classified into three main classes: Hydroxamates, Catecholate, and Carboxylates, depending on the Fe-chelating category (Schalk & Mislin, 2017). By binding with the iron tightly, siderophores reduce the bioavailability of iron for plant pathogens and facilitate the killing of phytopathogens (Beneduzi *et al*., 2012; Ahmed and Holmström, 2014; Herlihy *et al*., 2020). Moreover, the development of the Fe-siderophore complex is affected by the concentration of divalent or trivalent cations such as Cd^{2+}, Ni^{2+}, and Al^{3+} in soil, which competes

with Fe for binding sites in siderophores, thereby reducing the chances of Fe-binding (Herlihy *et al.*, 2020; Gorshkov and Tsers, 2022). Iron, the second most prevailing metal on earth, is the imperative element for the production of all living microbes because it catalyzes the enzymatic mechanism, electron relocation, oxygen metabolism, and DNA and RNA synthesis ((Aguado-Santacruz, Moreno-Gómez, Jiménez-Francisco, García-Moya, & Preciado-Ortiz, 2012). Iron exists in an aqueous solution in two interconvertible states between divalent (Fe^{2+}) and trivalent (Fe^{3+}) ionic forms (Buziashvili & Yemets, 2022). The stability of these two states in the soil is regulated by pH, aerification, salinity, and biotic matter (material that originates from living things) content. The existence of oxygen and pH results in speedy oxidation, and the Fe^{2+} ion is oxidized to Fe^{3+} (Colombo, Palumbo, He, Pinton, & Cesco, 2014). Ferrous (Fe) ions translocation in plants occurs in two ways. As H^+ is released, Fe chelate reductases are activated, reducing Fe^{3+} to Fe^{2+} ions, which are subsequently translocated into the intracellular cavity of the epidermis cells. Alternately, Fe^{3+} ions are first bound to phytosiderophores and ejected into the rhizosphere (Kim & Guerinot, 2007); Kobayashi and Nishizawa, 2012; (Tsai & Schmidt, 2017). However, the siderophore's leading role is to ensure the bioavailability of iron-by-iron mobilization (Schwabe et al., 2020). Phytosiderophores function as high-affinity chelators, and the Fe^{3+} phytosiderophore complex is transferred into the cells by the Fe-phytosiderophore Yellow stripe 1 (in maize) or Yellow stripe-like proteins (in other grasses) (Nozoye *et al.*, 2011). Arthrobacter is the most abundant bacterial genera present in rhizosphere of gramineous crops (Cavaglieri, Orlando, & Etcheverry, 2009). The concentration of Fe is low in cereals, and approximately 2 billion people globally have iron deficiency (Please put a reference here). Iron is deficient in the soils of Pakistan because of its calcareous nature with alkaline pH and higher contents of carbonates (Zulfiqar et al., 2020). The nutritional value of Fe in food grains should be enough not only to meet the adults' dietary iron requirements but also to fulfil the Fe deficiency (Please give reference). Iron (Fe) deficiency can be compensated by the diverse option available such as genetically modified crops, food fortification, chemical fertilizers, nutritional diversification, and agronomic biofortification (García-Bañuelos, Sida-Arreola, & Sánchez, 2014); (Zunjare *et al.*, 2018); (Velu & Singh, 2019); (Khan, Singh, Upadhayay, Singh, & Shah, 2019). Agronomic biofortification is a new way to mitigate micronutrient malnutrition (Bouis et al., 2011; Benkeblia, 2020; Roriz et al., 2020). Likewise, the biofortification of plants with free-living microbes is a promising strategy for improving the production of food crops (Glick, 2012). Cereals with iron biofortification improve iron's bioavailability, reducing iron malnutrition (Rana, Joshi, Prasanna, Shivay, & Nain, 2012). Wheat and maize are rich sources of proteins and micronutrients (Zhao *et al.*, 2020). Therefore, this study was planned to observe *Pseudomonas fluorescence* and then biofortification of cereal crops with these microbes to identify their role in crop growth and grain enrichment of iron.

Materials And Methods

Collection of Rhizobacteria: Fifty rhizobacterial isolates from wheat, maize, millet and sorghum were collected from the Faisalabad district. The isolates were preserved in a cold storage box to reduce the microbial action and taken to the laboratory for further characterization and analysis.

Isolation and purification of collected rhizobacteria: To isolate bacteria dilution plate technique was used. Briefly, around 10 g soil sample was dissolved in 99 mL of deionized water and shaken for 5-10 minutes to immerse Rhizosphere bacteria. Then 1 mL from this soil solution was taken and poured into 250 mL conical flask containing 99 ml of autoclaved deionized water to make 10^{-2} dilutions. The procedure was repeated to get a 10^{-6} dilution. A 100 microliter from each dilution of soil bacterial solution was taken using a sterilized nozzle and dropped on LB medium (Bertani, 1951). Afterwards, the proper dispersal of bacteria on the agar plate was made with a spreader. These plates were incubated at 28 ± 2^0C for 24-48 hours. After proper growth, further purification and repeated streaking of colonies were done on LB agar medium to get pure growth. The procedure was repeated twice or thrice to get purified strains. Finally, 15 pure strains were preserved in broth at -20^{o}C in eppendorf tubes in the freezer for further characterization (Kapoore et al., 2019).

Detection of rhizobacteria for the fluorescent pigment: King's B medium was used for the detection of fluorescent pigment by microbes (King *et al*., 1954). The efficacy of this medium was examined by supplementing it with extra iron. The optimal composition of the medium per litre contained 20 g of Bacto peptone (Difco), 1.5 g of dipotassium hydrogen phosphate, 1.5 g of magnesium sulphate heptahydrate, 15 mL of glycerol and 1.5 g of agar with 5 µmol and 50 µmol iron supplementation. After sterilization of medium, the purified bacterial colonies were inoculated on petri-plates containing solidified media, and after 48 hours, production of fluorescent pigment was observed under UV light.

Estimation of siderophore production-assay: ***Qualitative estimation:*** Chrome Azurol S (CAS) plates containing medium with lower iron contents were used for screening isolates. For spot inoculation of various

bacterial isolates, 5 places were marked at an equal distance on the plates (CAS-agar medium). The plates were kept in the incubator for proper growth of bacteria at 28 ± 2^0C for 48 hrs. The presence of halo zones around the colonies was used as an indicator of siderophore production. The whole process of estimation followed the method described by Milagres *et al*. (1999).

Quantitative estimation: CAS-shuttle assay was employed to quantify siderophore (SP) production (Kotasthane *et al*., 2017). An aliquot of culture filtrate measuring 0.5 mL was put into a test tube along with 0.5 mL of CAS reagent for the quantification assay. Afterwards, 0.5 mL of CAS reagent was added in an uninoculated blank. Ultimately, the colour was changed, and the intensity of this change was measured colourimetrically. The percentage (%) of siderophore units was calculated using the following equation Kotasthane *et al*. (2017).

% Siderophore unit (SU) = Ar – As/Ar × 100
As = Sample absorbance at a wavelength of 600 nm
Ar = Reference/blank absorbance at a wavelength of 600 nm

Growth promoting trait: Auxin biosynthesis was measured through IAA equivalents using L-Tryptophan (Trp) and L-Tryptamine (Trt) as hormone precursors. Basic medium ingredients used for general-purpose media included glucose (0.375 g), di-potassium hydrogen phosphate (0.125 g), magnesium sulphate heptahydrate (0.025 g), iron sulphate heptahydrate (traces) and ammonium sulphate (0.125 g) in 250mL of distilled water. Around 10 mL of broth was taken in test tubes and sterilized. The isolates were inoculated along with 1% precursors at 28 degrees for 24 hours in an incubator. The concentration of IAA equivalents was measured on a spectrophotometer at 540 nm after centrifuge and colour development with the Salkowski reagent (Brick *et al*., 1991).

Assessing the efficiency of fluorescent-producing microbes under controlled conditions: For screening, a germination experiment was carried out in a growth chamber. Bacterial isolates were selected based on the growth efficiency character under axenic conditions in the growth chamber. For the lab screening experiment, 7 isolates (top-performing isolates) were chosen on the basis of microbial characteristics. An inoculum of particular bacterial strains was prepared for the germination test in volumetric flasks of (250 mL). Then, the flask was kept in an incubator for 3 days with continued shaking. Surface sterilization of seeds was done using 3% hypochlorite solution (for 2-3 minutes). Before dipping in selective bacterial inoculums, the seeds were washed three times with deionized water and then spread on moist filter paper sheets. Sheets were kept moist and covered with polythene in a growth chamber under proper light and temperature. Growth parameters were recorded after (10) days of seed sowing. Agronomic traits were also measured, including root length, shoot length, and shoot fresh weight.

Assessing the efficiency of fluorescent-producing microbes under Field conditions: The field study was conducted to analyze the effectiveness of siderophore-producing bacteria to chelate insoluble iron and their ultimate impact on cereals (wheat and maize) growth and yield during 2021-22. Wheat variety Galaxy 2013 and maize variety Malka 2016 was used in the field experiment. In control (un-inoculated) treatment, seed coating was carried out using a mixture containing 10% sugar solution + sterilized broth + sterilized peat. Seed inoculation of experimental units was done with peat containing siderophore (SP) producing bacteria plus sterilized 10% sugar (as sucrose solution) at a 10:1 ratio. The recommended dose of NPK fertilizers (110-46-25 kg/ha and 120-90-60 kg/ha for maize and wheat, respectively) was applied at sowing. Wheat was sown manually with seeds dibbled at 6 inches depth in six rows having 9 cm plant-to-plant distance and 30 cm spacing between rows. On the other hand, maize seeds were dibbled on 5 ridges (7 feet*13 feet) at a plant-to-plant distance of 12 inches. The soil used for the experiment was free from salinity and sodicity hazards and deficient in organic matter, while phosphorus, potassium, and iron contents were sufficient. Sex treatments (T_1=control, T_2=SB1, T_3=SB2, T_4=SB3, T_5=SB4, T_6=SB5, T_7=SB9 and T_6=SB10) were applied using Randomized Complete Block Design (RCBD) with three replicates... The parameters (grain weight, plant height and grain yield) were recorded at harvesting yield. The grain analysis for iron was done through a wet digestion process using a di-acid mixture, and contents were determined using atomic absorption spectrophotometer. Statistical analysis was performed using Statistix v. 8.1 (Steel *et al.,* 1997).

Results And Discussion

Siderophore-producing microbial-mediated biofortification is an emerging approach to overcoming malnutrition. The PGPR can fortify Fe in the soil rhizosphere by SP production and Fe solubilization. The present study was conducted to isolate and purify siderophore-producing bacteria that can produce fluorescent pigment and improve the growth and yield of (wheat and maize).

Biochemical Characterization: Results of fluorescent pigment production under UV light showed that the ability of rhizobacteria to excrete fluorescent pigment depends on the synthetic media used for the growth of microbes (Table 1). The microbial characteristics determination showed that out of 50 collected strains,

15 produced a higher amount of siderophore and fluorescent pigment when examined under UV light. All the strains showed a positive response to pigment production except control, indicating a resemblance to typical characteristics of *Pseudomonas fluorescence*. The maximum fluorescent pigment was observed in SB10, followed by SB9 when supplemented with 50 µmol of iron chloride (Table 1). Siderophores improve iron nutrition through iron chelation in the rhizosphere. These iron-chelating siderophores also reduce the availability of iron to pathogens. During the qualitative test, siderophore detection was marked by transparent halo zones around colonies. 7 isolates out of 15 were found capable of producing siderophores. In quantitative measurement through a spectrophotometer, the maximum colour change was observed in SB10 (72.0%) followed by SB5 (64.5%). Likewise, the maximum siderophore unit percentage ranged from 33.8% to 72.0% (Table 1). Similar results were reported by Kumari *et al*. (2021) with a maximum production of siderophores (46.2 (SU %) with SB10. Moreover, results regarding IAA equivalents indicated that precursors induced the biosynthesis of auxin in all strains (Table 1). Maximum auxin biosynthesis with L-tryptophan was observed in SB3 (6.38 µg/mL), and the same strain produces more auxin biosynthesis when augmented with L-Tryptamine (7.35 µg/mL).

Table 1. Biochemical test of different isolates

Isolate	CAS-assay	Siderophore (%)	IAA + L-Trp (µg mL^{-1})	IAA +L-Trm (µg mL^{-1})	Fluorescent pigment
SB1	+	**33.8**	**6.37**	**2.16**	+
SB2	+	**36.6**	**3.13**	**3.70**	+
SB3	++	**47.5**	**6.38**	**7.35**	+
SB4	+	**45.4**	**4.06**	**4.90**	+
SB5	+	**64.5**	**3.95**	**4.77**	+
SB9	++	**51.2**	**3.74**	**3.88**	++
SB10	++	**72.0**	**3.97**	**4.58**	++

Efficiency character essay: Inoculation with siderophore and fluorescent pigment-producing bacteria manifested a positive response on maize seeds germination when the essay was conducted under controlled axenic conditions (Fig. 1). Data describes that all the strains are statistically on par with each other in increasing shoot length but statistically significant as compared to control. The maximum shoot length of maize was observed in strain SB10 (29.5 cm) and SB9 (28.6 cm). The maize plant has an embryonic root system consisting of primary, seminal and post-embryonic. Similarly, inoculation with selected bacterial isolates improved root length in SB9 and SB10 compared to other treatments. SB3, SB 4, and SB 5 were on par with each other for improvement in root length.

Germination test assay showed increased shoot/root length and weight in inoculated seeds over un-inoculated (control) seeds. Satish *et al*. (2020) also reported improvement in the growth of plants; after having contact with soil microbiota with the roots of the plant. Similarly, Kaur *et al*. (2020) reported that the inadequacy of essential micronutrients (vit. A, Zn, and Fe) can be improved by inoculating seeds with specific microbes. He *et al*. (2020) observed that some wheat-linked microbes, primarily the microbes of rhizospheric soil, produce SP and other metabolites, which increase the solubility of Fe in the soil. The current scenario may imply that an increase in physiological traits might be due to increased phosphorus solubilization, solubilization/uptake/translocation of iron, auxin and phytohormone production (Yavarian *et al*., 2021; Etesami, 2020; Mushtaq *et al*., 2021; Delaporte-Quintana *et al*., 2020). The addition of plant growth regulators (PGRs) to plant growth-promoting rhizosphere bacteria (PGPRs) showed improvements in chlorophyll content, leaf area, sugar content, oxidative stress, and reduction in peroxidation of lipids (Khan *et al*., 2019). The findings of the current study are also in line with the observations of Ekin (2019).

Findings of the field experiment: Biofortification of wheat (*Triticum aestivum* L.) through seed inoculation with siderophore-producing bacteria is an alternative approach to fulfilling desired micronutrients deficiency in the human diet in rural areas (Ehsan *et al*., 2022; Riaz *et al*., 2020). Radzki *et al*. (2013) reported that SP with low molecular weight binds the Fe and transports it into root cells via protein membrane. Field experiment results revealed that inoculation with iron-complexed siderophore-producing bacteria improved yield and yield attributes. Data in figure 2 showed the effect of siderophore-producing bacteria on the plant height of both crops. Maximum height in maize (255 cm) was observed where T6 was used as inoculum. In the case of wheat, the maximum height (110 cm) was found with the T7 strain. A significant increase in grain yield of cereals has been shown in figure 3 by siderophore-producing microbes. The maximum maize yield (5.93 t/ha) was found in T7 (SB9), while in wheat, T8 boosted the yield, i.e. 3.48 t/ha. The application of microbial inoculants, along with a

recommended dose of mineral fertilizers improved grain weight in both crops. The increase in grain weight of maize was in the range of 32.6-37.1 g and for wheat (26.7-33.9 g), which is statistically significant over control (Figure 4). These outcomes are in harmony with the results of Yadav *et al*. (2020) and Singh *et al*. (2020). Like the current study, Khalid *et al*. (2015) found a 13-18% increase in wheat grain yield, 12-16% plant shoot, 6-11% root length, and 34-60% chlorophyll contents with the inoculation of siderophore producing *Pseudomonas* in wheat. The interaction of plant-microbe is the basic factor contributing to the improvement in productivity, plant health, and soil fertility, as already observed in the case of potato (*Solanum tuberosum* L.) by Mushtaq *et al*. (2020). Kabiraj *et al*. (2020) found that bacterial inoculants can significantly increase agronomic parameters, which helps alleviate the cost of production and environmental pollution. Microbes are proven to be cost-effective, efficient, more promising, and sustainable approaches that can contribute to plant development

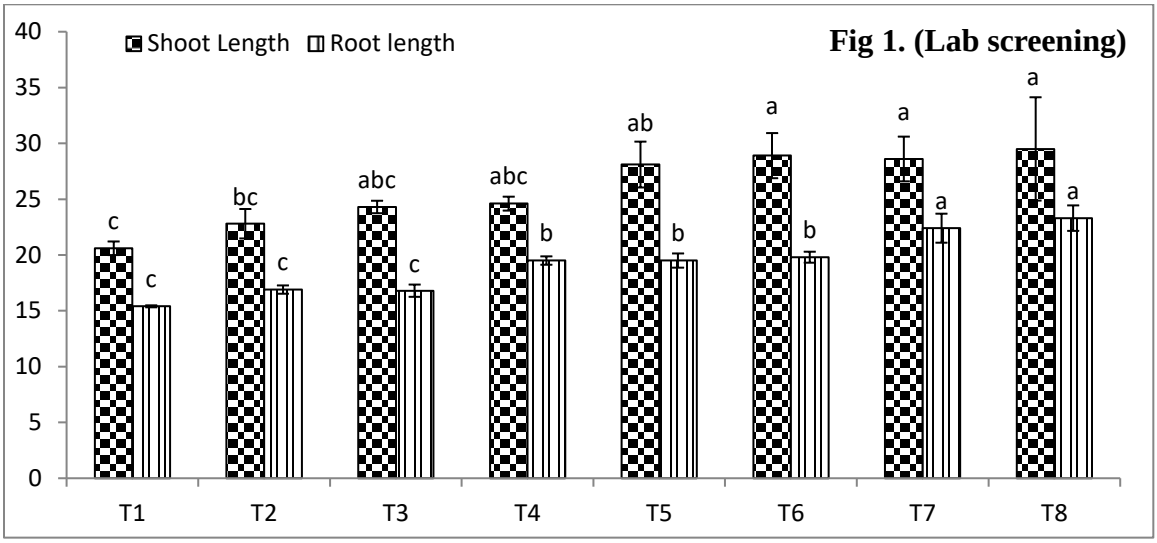


Figure 1. Germination bioassay of selected isolates on root and shoot length of maize grain under axenic conditions.

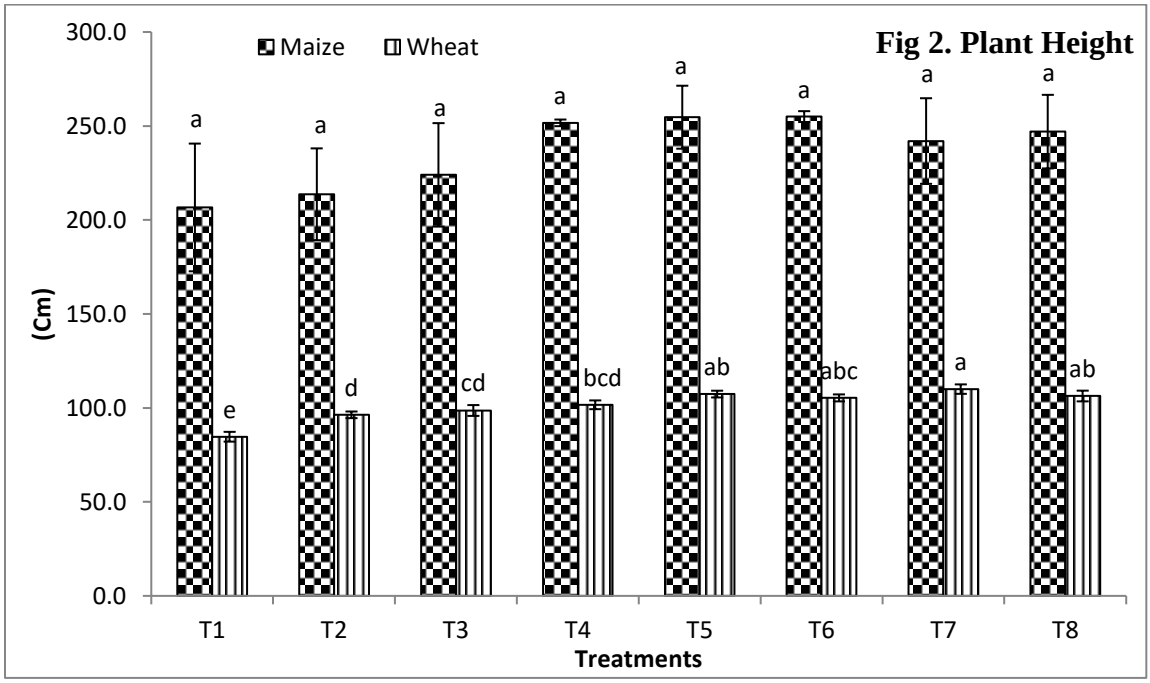


Figure 2. Effect of fluorescent producing Pseudomonas bacteria on plant height of wheat and maize at $p<0.05$ level of significance.

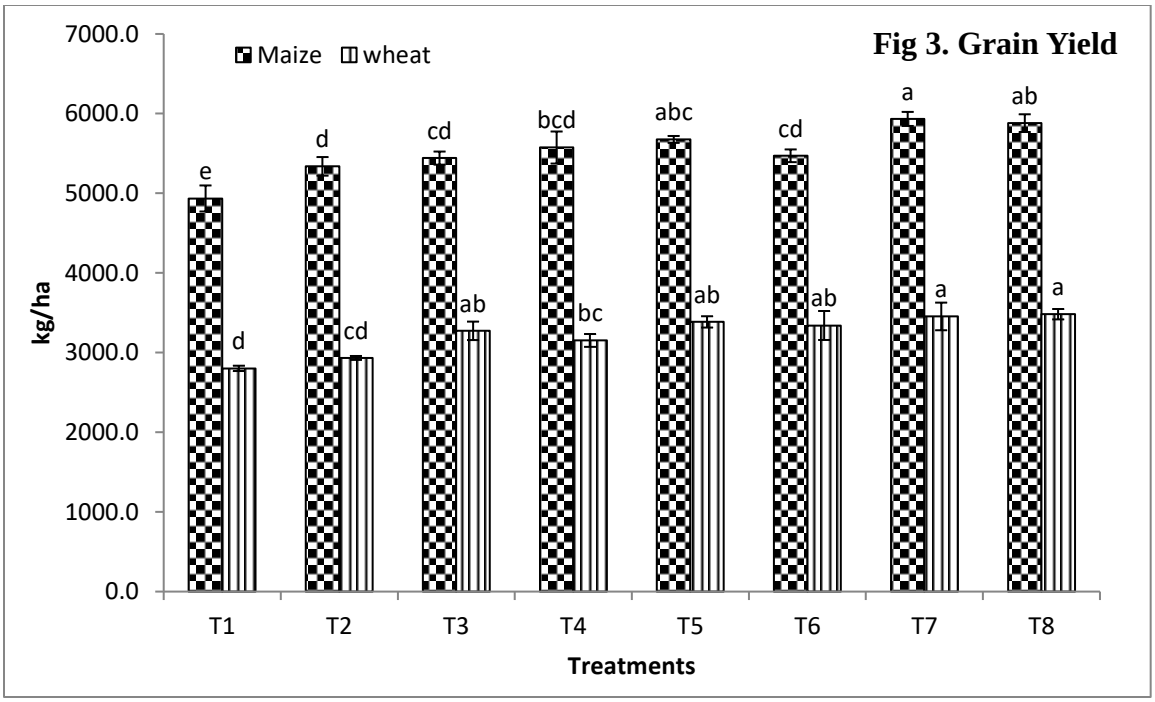
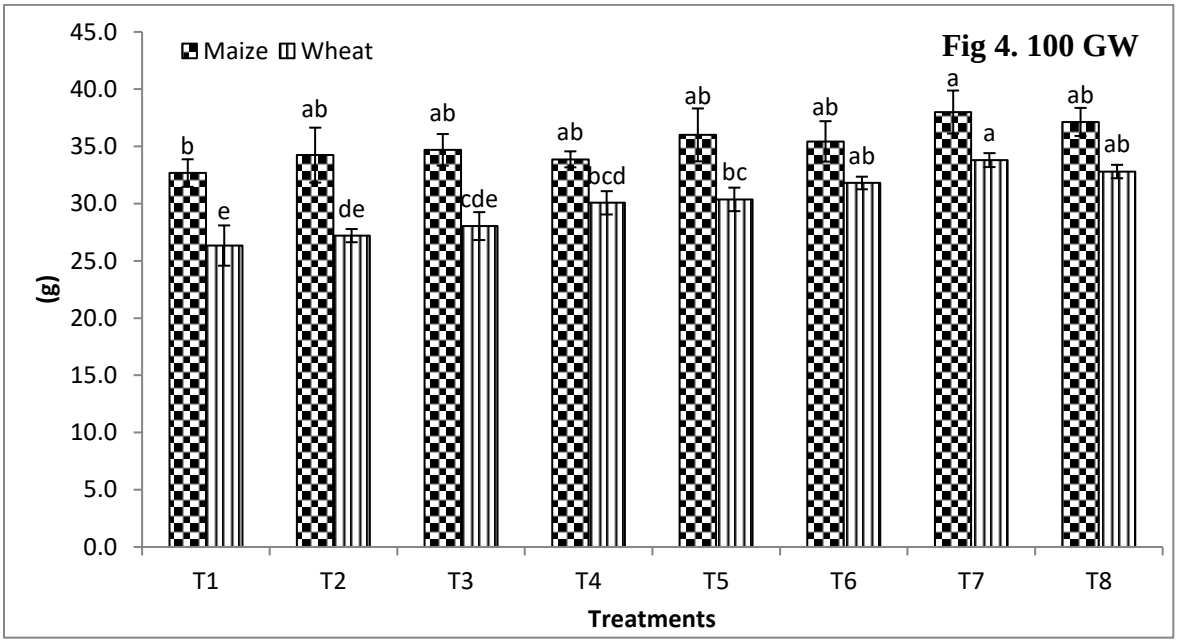


Figure 3. Effect of fluorescent producing Pseudomonas bacteria on grain yield of wheat and maize at $p<0.05$ level of significance.



Results regarding iron contents in grain are shown in figure 5. Both crops showed positive responses to iron biofortification, with iron contents in the range of 12.35%-28% (wheat) and 40.3%-67.7% (maize). Yield contributing parameters like spike length (wheat) and cob length (maize) are given in figure (6). Results indicated that SB8 promoted the development of the productive part of cereal crops with a 25% increment in spike length of wheat and a 33% increase in cob length of maize as compared to control. All the strains significantly increased the cob length of maize compared to the check treatment. But in the case of spike length, SB8, SB6 and SB2 showed remarkable impact compared to other strains in developing the productive part of wheat crop. Similarly, number of grains per cob/spike was recorded (Fig 7). Data revealed a significant effect of fluorescent-producing PGPR having siderophore-producing ability in increasing the number of grains per spike/cob.

Maximum grain count/spike was found in SB8 (75.6), followed by SB6 (74.6) and SB7 (74.5). Mushtaq *et al*. (2021) reported that microbes promoted nutrients concentration, plant physiological processes, plant development, yield and growth using various (direct or indirect) methods such as hormonal production, including (cytokinin, gibberellins, and auxin IAA). Similar findings were reported by (Ehsan *et al*., 2022).

Field studies demonstrated around 24 % increase in wheat grain yield, 30% increase in plant height, 25% increase in grains/spike and iron contents after inoculation with siderophore-producing bacteria. Such an increase in germination and yield attributes provides a baseline to test these siderophore and iron solubilizing PGPR for other cereal crops. Furthermore, around 60% increase in grain Fe contents and 13% increase in thousand-grain weight (TGW), 33% increase in cob length and 20% increase in grain yield of maize were observed in inoculated plants compared with uninoculated control. Likewise, a combination of six strains, including *Bacillus subtiles* & *Pseudomonas fluorescence*, showed high siderophore production and increased antioxidants activity that reduced fungal infection in maize and improved its yield (Lopez-Reyes *et al*., 2017; Ghazy and El-Nahrawy, 2021). Zarei *et al*. (2022) reported that the combination of four strains of *Pseudomonas fluorescence* significantly increased yield traits of sweet corn and canned seed yield by reducing harmful effects and improving crop productivity.

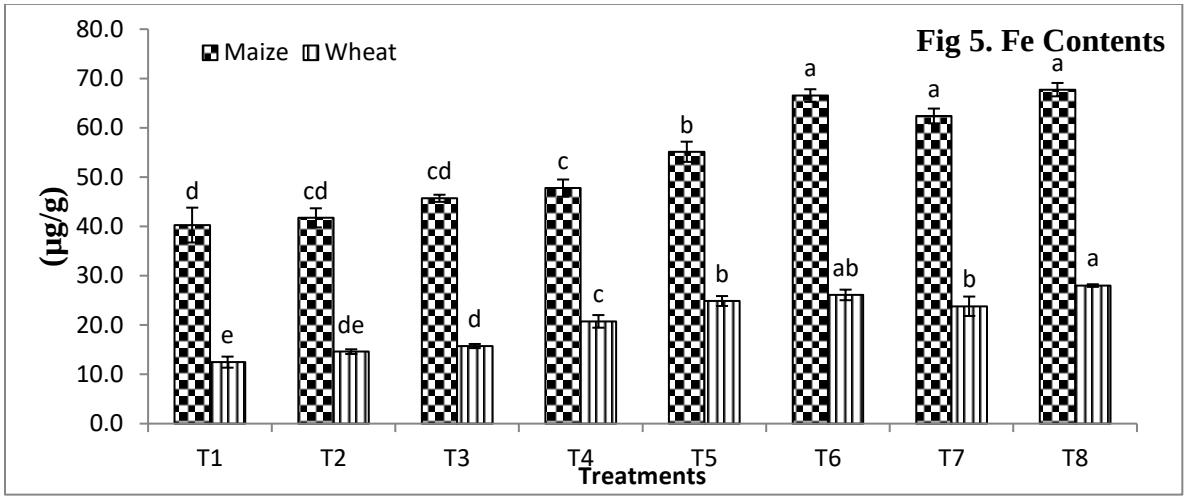


Figure 5. Effect of fluorescent producing Pseudomonas bacteria on Iron contents of wheat and maize grain at p<0.05 level of significance.

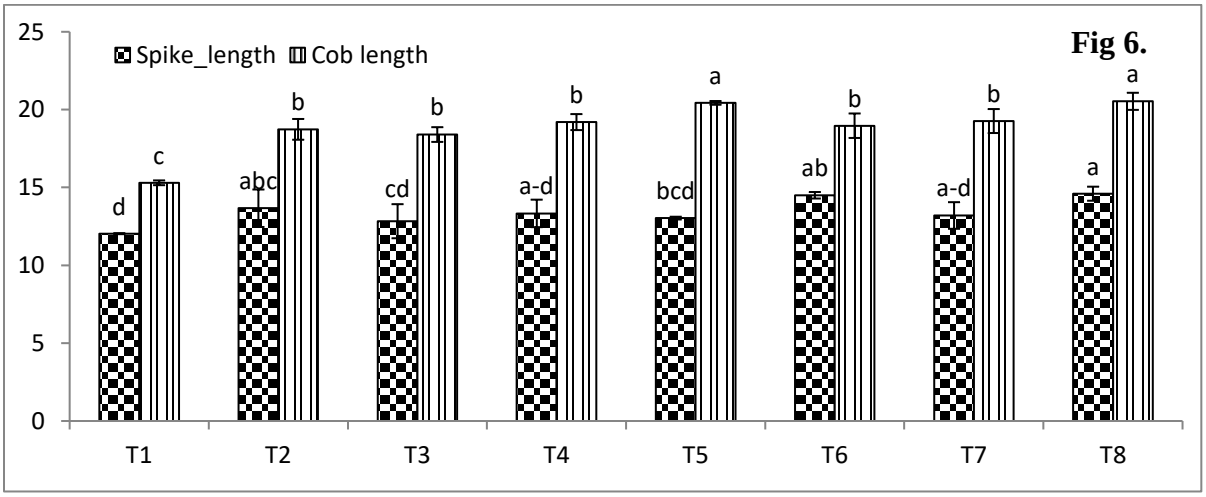


Figure 6. Effect of fluorescent producing Pseudomonas bacteria on spike and cob length of wheat and maize at p<0.05 level of significance.

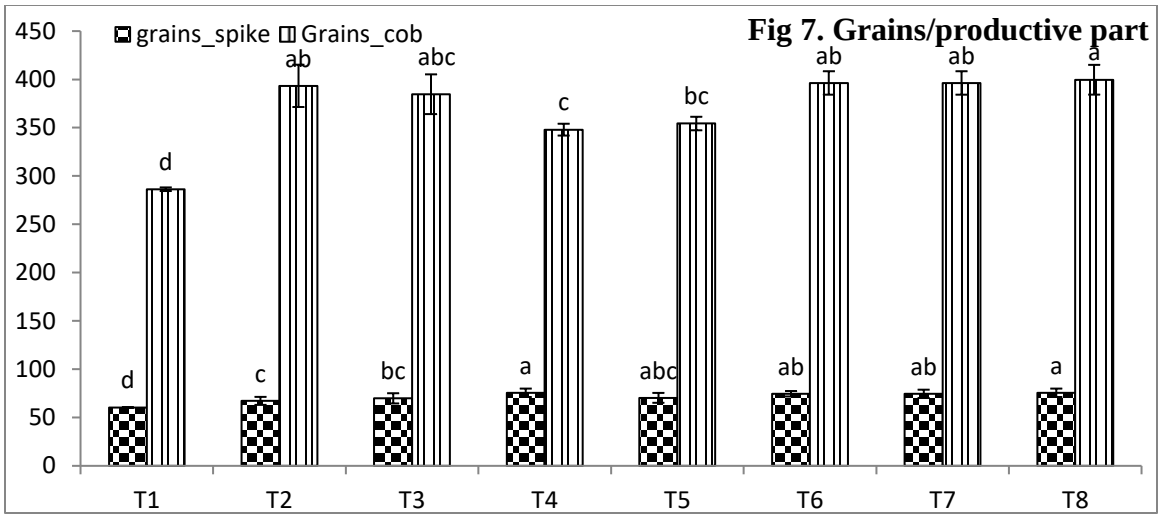


Figure 7. Effect of fluorescent producing Pseudomonas bacteria on grains per spike of wheat and cobs of maize at $p<0.05$ level of significance.

Conclusions

The findings of this study perceived that biofortification of cereals through seed inoculation with siderophore (SP) producing microbes having fluorescent producing character can increase the solubilization of insoluble (Fe) and bring about improvement in growth and development of the cereals in alkaline calcareous soil.

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