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ORIGINAL RESEARCH ARTICLE

# Foliar Application of Iron Fortified Bacteriosiderophore Improves Growth and Grain Fe Concentration in Wheat and Soybean

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**Abstract** Iron (Fe) is one of the key micronutrients essential for plant growth, yield and quality. Wheat (*Triticum aestivum*) and soybean (*Glycine max*) are important food crops but have relatively low Fe content in grains/seeds. Foliar application of Fe-invigorated bacteriosiderophore might increase Fe content in grain as well as improve overall plant growth. From a preliminary experiment conducted on soybean using 20 bacterial strains, *Arthrobacter* sp. (low siderophore producing) and *Lysinibacillus fusiformis* (high siderophore producing) were selected based on amount of siderophore produced and response of plants. This result was validated on field grown soybean and wheat crops by applying bacteriosiderophore with or without Fe on foliage. Siderophore was applied at flowering stage in both crops and observations were recorded on the sixth day after foliar spray. Significantly higher shoot biomass, area of leaves or flag leaf and tissue Fe concentration was recorded by siderophore produced by *L. fusiformis* with Fe as compared to *Arthrobacter* sp. In comparison to control (water), application of Fe fortified bacterial siderophore resulted not only in increased grain yield by 45% and 28% in wheat and soybean, respectively but also enhanced Fe concentration in grains by 1.7-fold in soybean to 2.0-fold in wheat. Partitioning of Fe in grain was higher in wheat as compared to soybean after foliar spray. Thus, we reported for the first time that bacteriosiderophore with added Fe as foliar application could be an economical and targeted agronomic approach towards Fe fortification in crop plants.

**Keywords** Bacteriosiderophore · Chrome azurol sulphonate assay · Foliar fertilization · Iron fortification · Wheat and soybean

## Introduction

Iron (Fe) is an essential micronutrient to virtually all forms of life. Although Fe is the second most abundant metal element in earth's crust but its low solubility and high fixation in soil reduces its bioavailability to the plant roots [1]. Fe plays a major role in redox reactions in metabolic processes such as photosynthesis, respiration and nitrogen assimilation besides being involved in biosynthesis of chlorophyll and hormones [2]. Fe chlorosis is a widespread agricultural problem which reduces productivity as well as nutritional quality of crops. A continuous supply of Fe through roots and its remobilization from older to newly developing leaves is essential for optimum growth and yield. Availability of Fe in soil solution is largely pH dependent with higher concentration at lower pH but once

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inside the plant, Fe is immobile. The common strategies for curing Fe deficiency are the use of cultivars tolerant to Fe chlorosis or soil application of Fe fertilizer. However, farmers may not always have access to high yielding Fe deficiency tolerant cultivars or such cultivars may not be available for every crop. The rapid conversion of soil applied Fe into unavailable form as solid Fe(III) urges the need of an alternative method to treat Fe deficiency [3]. To overcome Fe deficiency in crops, foliar fertilisation is one of the most economic and targeted approach.

Various inorganic and organic Fe compounds have been used for foliar application but the results on Fe chlorotic plants were contradictory [4–7]. In comparison to inorganic salts, use of Fe chelates has been strongly recommended to treat Fe chlorosis [8]. A few studies have shown enhanced penetration and leaf re-greening with inorganic Fe salts over chelates [6, 9]. Fe(II) salts rapidly oxidise when exposed to air while Fe(III) salts forms a gelatinous hydrous polymers once the pH increases more than 2.0 [10]. So, inorganic Fe salts are not preferred for foliar application but chelated form of Fe when applied on foliage was easily translocated within the plants in comparison to other Fe-containing compounds [4]. Foliar application of Fe fertilizers such as ferrous sulphate ($FeSO_4$), $Fe^{III}$ diethylene triamine penta acetic acid (FeDTPA), $Fe^{III}$ ethylene diamine –*N*,*N*′-bis (2-hydroxyphenyl acetic acid) (FeEDDHA) and $Fe^{III}$ ethylene diamine tetra acetic acid (FeEDTA) to soybean significantly increased yield, though FeEDDHA was in effective in ameliorating Fe chlorosis [11, 12]. The varying results of foliar application of synthetic or inorganic Fe fertilizers might be due to the factors as mentioned above which affects the efficiency of foliar fertilisation. However, excessive use of synthetic chelates such as EDTA, EDDHA may raise the concern over environmental pollution as they are either non-degradable or poorly biodegradable [13]. Therefore, there is a need to identify and develop environment safe organic Fe chelators to prevent Fe chlorosis.

Under Fe deficient condition several soil microbes produce low molecular weight ($\sim$ 0.5 to 1.5 kDa) Fe-chelating compounds called 'bacteriosiderophore', which has high affinity for ferric ions [14]. Siderophore, in general, forms a highly spin and thermodynamically stable complex with Fe(III) and serve as a carrier for Fe transport into the plant cells [15]. Currently, 500 siderophore compounds have been isolated from different microorganism and based on the functional group, they are categorized as hydroxamates, catecholates and carboxylates [16]. Improvement in Fe uptake and plant growth was reported when seeds or root was inoculated with microbial inoculants producing siderophore [17]. Plants absorb Fe-siderophore complex by different mechanism such as direct uptake of Fe-siderophore complex, chelate and release Fe or by ligand exchange reaction [18]. Formation of Fe-siderophore complex is affected by the concentration of divalent or trivalent cations such as $Zn^{2+}$, $Cd^{2+}$, $Cu^{2+}$, $Ni^{2+}$, $Pb^{2+}$ - and $Al^{3+}$ in soil which competes with Fe for binding sites in siderophore there by reducing the chances of Fe binding. To overcome the limitations of soil application of siderophore, and because of aqueous soluble nature of bacterial siderophore, it becomes highly suitable for foliar application [19]. Earlier studies reported improved growth and yield due to foliar application of plant growth-promoting rhizobacteria (PGPR) in pea [20], tomato [21] and canola [22]. However, very little information is available on the effect of foliar applied siderophore extracted from bacterial culture in combination with Fe on growth and Fe content of crop plants. In the present study, the effect of foliar applied bacteriosiderophore, with or without addition of Fe, on various growth traits and Fe content in grains/seeds was investigated. For this purpose, experiments were conducted *first* to quantify the siderophore production by different bacterial strains and their evaluation on soybean, *second,* response of field grown soybean and wheat crops to foliar application of siderophore, produced by contrasting bacterial strains, fortified with or without Fe.

## Materials and Methods

### Experiment 1: Preliminary Experiment on Soybean

A preliminary experiment was conducted on soybean (*Glycine max,* var. DS-2614) in glasshouse. The temperature was maintained at an optimal 28 ± 2 °C/25 ± 2 °C Day/Night and relative humidity at 85% with natural light. Pots were filled with approximately 5 kg air-dried, well sieved soil collected from field at a depth of 0–30 cm. Recommended dose of fertilizers (NPK) was added and after germination one healthy plant per pot was maintained.

Twenty PGPR strains (Supplementary Table 1) procured from the Division of Microbiology, ICAR-IARI, New Delhi were streaked on Luria–Bertani (LB) agar plates and incubated at 37 °C for 24 h followed by subculturing in LB broth. For siderophore production, 1.0 mL culture of each bacterial strain was inoculated in 1.0 L of Fe deficient succinate medium (SM) [23] and incubated at 37 °C for 48 h with constant shaking. After sufficient growth, the bacterial culture was centrifuged at 800*g* for 15 min to pellet the cells. The supernatant containing siderophore was incubated with $FeCl_3$ (2.0 mM) for 90 min at room temperature [21]. Before foliar application, pH of spray solution was set to 6.0 using HCl or KOH with addition of Triton X-100 as surfactant. At flowering or R2 stage, soybean plants were sprayed with Fe-siderophore complex and distilled water as control. The above-ground

biomass was recorded on 6th day after foliar spray and the yield traits viz. number of pods, test weight (100-seed weight) and total seed yield per plant were recorded at maturity or R8 stage.

### Quantification of Siderophore Produced by Different Bacterial Strains

The amount of siderophore in culture solution was quantified by chrome azurol sulphonate (CAS) assay [24]. For this, 0.5 mL of supernatant was mixed with 0.5 mL of CAS solution and 10 µL of sulfosalicylic acid. After 20 min incubation in dark, absorbance was recorded at 630 nm. The change in colour of blue dye (CAS assay solution) to purple orange indicated the presence of siderophore. The blank was set using the SM medium and the reference solution contained SM medium, CAS dye and sulfosalicylic acid (Supplementary Fig. 1b). The quantity of siderophore released was determined using the following formula:

$$\text{Percent siderophore} = \frac{\text{Absorbance reference} - \text{Absorbance sample}}{\text{Absorbance reference}} \times 100$$

### Experiment 2: Field Experiment Using Siderophore Produced by Contrasting Bacterial Strains

Based on the quantity of siderophore production, two contrasting bacterial strains, *Lysinibacillus fusiformis* (high siderophore producing) and *Arthrobacter* sp. (low siderophore producing), were selected to validate the effect of foliar application of Fe fortified bacteriosiderophore on soybean and wheat (*Triticum aestivum* L., var. C 306) in field. Soybean was sown on 25th July 2016 while wheat was sown on 12th November 2016. For soybean, seeds were dibbled at a distance of 15 cm from plant-to-plant and 30 cm between the rows. Wheat was sown manually in six rows per plot at 22 cm spacing between rows. The net area per plot was 3.0 $m^2$ and 4.0 $m^2$ for soybean and wheat, respectively.

Foliar sprays were prepared by inoculating 5.0 mL culture of each strain in 5.0 L of Fe deficient SM medium and incubated at 37 °C with continuous shaking. After measuring the bacterial growth by absorbance at $OD_{600nm}$, culture solution was centrifuged at 800*g* for 15 min and siderophore content in the supernatant was checked by CAS assay as described above. The supernatant containing siderophore obtained from each bacterial strain was divided into two portions, one portion was incubated with $FeCl_3$ (2.0 mM) while the other portion was maintained without Fe. A total of six foliar treatments, namely (1) water as control (2) 2.0 mM $FeCl_3$ solution (3) *Arthrobacter* sp. supernatant without Fe (4) *Arthrobacter* sp. supernatant with 2.0 mM Fe (5) *L. fusiformis* supernatant without Fe and (6) *L. fusiformis* supernatant with 2.0 mM Fe, were included. For the ease of writing, the bacteriosiderophore fortified with Fe will be denoted as bacterial species +Fe. The foliar application was carried out at flowering stage in both crops. While spraying, the soil surface was covered with polythene to avoid dripping of excess solution from foliage into the soil and it was removed the next day. On the sixth day after spraying, plants were sampled for shoot dry weight, total leaf area, flag leaf area, and Fe content analysis in different organs. Another set of treated plants were harvested at physiological maturity, sun-dried, threshed and yield attributes were recorded. The Fe concentration in different tissue was estimated by wet digestion with di-acid mixture ($HNO_3$:-$HClO_4$) using atomic absorption spectrometer (ECIL, India). The partitioning of Fe in different plant tissue was calculated by multiplying the tissue Fe concentration with their dry weight and expressed as mg Fe per plant.

### Statistical Analysis

Field experiments were carried out in a randomized block design with five replications for each treatment. Data were subjected to one-way analysis of variance (ANOVA) and means were compared with critical difference (CD) at $P \leq 0.05$ level of significance. The data from experiment 1 was subjected to hierarchical cluster analysis based on Ward's method to group the bacterial strains. The statistical software R version 3.1.2 (R Foundation for Statistical Computing, Vienna) was used. Graphs were made using Graph Pad Prism version 6.00 (Graph Pad Software, La Jolla, CA).

## Results and Discussion

The bacterial strains varied significantly in terms of the quantity of siderophore production induced by Fe deficient condition in the media (Supplementary Fig. 1a). The effect of foliar applied siderophore fortified with Fe on growth, yield and Fe content depends upon the quantity of siderophore secreted by bacteria. The bacterial strains were grouped into three clusters based on the quantity of siderophore produced and the response of plant (Supplementary Fig. 2). Cluster 1 included twelve bacterial strains which produced low ($\leq$ 30%) siderophore whereas Cluster 2 and Cluster 3 contained four strains each. Cluster 3 included bacterial strains with highest ($>$ 80%) siderophore production capacity while Cluster 2 exhibited moderate siderophore producing bacterial strains. In all clusters,

siderophore quantity was positively related to the aboveground biomass, seed yield and 100-seed weight (Fig. 1a–c). In Cluster 3, bacteriosiderophore quantity was significantly correlated with biomass ($R^2 \geq 0.95$) and seed yield ($R^2 \geq 0.89$) while in Cluster 1, biomass and test weight were significantly correlated. A similar response was obtained for shoot dry weight, seed yield and 100-seed weight with different strains. Foliar application of *L. fusiformis* resulted in maximum shoot biomass and seed weight while strains producing low siderophore resulted in minimum biomass accumulation and seed yield. *L. fusiformis* belongs to the genus *Bacillus* and their occurrence is ubiquitous in soil [25]. It is reported that *Bacillus* sp. promotes plant growth in many ways like production of a diverse range of antibiotics [26], improvement of soil fertility and mobilization of nutrients [27] through increased nutrient solubility in soil. Our results are in agreement with findings of Vendan et al. [28] who reported that inoculants of *L. fusiformis* significantly promote growth of Ginseng plants. Likewise, Winkelmann [29] reported that *Arthrobacter* sp. belongs to low or no siderophore secreting bacterium group. Moreover, for their own siderophore requirement, *Arthrobacter* sp. depends on other siderophore producing bacterial strain [30]. Therefore, based on preliminary results contrasting bacterial strains, *Arthrobacter* sp. (low siderophore producing) and *L. fusiformis* (high siderophore producing) belonging to Cluster 1 and 3 respectively, were selected for further validation.

In the field, both soybean and wheat crops showed significant increase in leaf area, aboveground biomass, and yield due to foliar application of *L. fusiformis* +Fe but no effect was observed without Fe as compared to control. The effect of Fe alone and *Arthrobacter* sp. +Fe showed similar results for most of the traits in both crops. In soybean, the foliar application of *L. fusiformis* +Fe resulted in increased leaf area (22%), aboveground biomass (35%), pod number per plant (34%), total seed weight (45%) and test weight (21%) as compared to control (Supplementary Fig. 3a–d). However, application of Fe resulted in increased leaf area (5%), total biomass (20%), pod number per plant (17%), total seed weight (24%) and test weight (10%) as compared to control. Similar effect was exhibited by *Arthrobacter* sp. +Fe application on leaf area (8%), total biomass (19%), pod number per plant (24%), but the total seed weight (33%) and test weight (16%) were significantly higher as compared to control. In wheat, the flag leaf area, total chlorophyll concentration and total grain yield varied significantly (Supplementary Fig. 4a–d) due to foliar treatments. Foliar application of *L. fusiformis* +Fe resulted in increased flag leaf area (28%), total chlorophyll (27%), grain yield (28%) and test weight (9%) as compared to control. Similar to soybean, the foliar application of Fe and *Arthrobacter* sp. +Fe produced comparable results by increasing leaf area (18–20%), total chlorophyll (5–8%), grain yield (14–15%) and test weight (7%) as compared to control. Our results demonstrate that the foliar application of Fe alone or in combination with siderophore produced by *Arthrobacter* sp. had similar effect on plant growth and yield but the combination of Fe with siderophore produced by *L. fusiformis* significantly improved plant growth and yield.

Few reports are available on growth enhancement by foliar applied PGPR but the effect of foliar applied bacterial siderophore in combination with Fe is lacking. Previous studies showed that when *Bacillus* OSU142 strain applied as foliar spray in apricot during two successive years, increased yield by 30% and 90% respectively [31]. Further, foliar application of PGPR resulted in increased height and nitrogen status of canola plants [22] or improved photosynthesis and metabolite concentration in sesame by *Bacillus methyl otrophicus* KE2 [32]. However, our results on both wheat and soybean were in disagreement with earlier reports as the foliar application of siderophore without Fe showed no significant improvement in

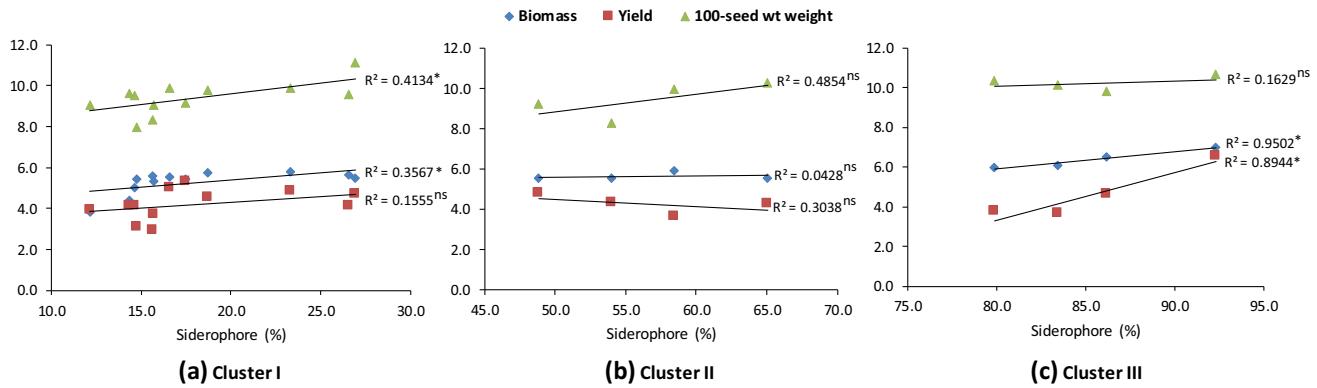


**Fig. 1** Relationships between percent siderophore production by bacterial strains versus total aboveground biomass, and 100-seed weight in different clusters. Each data point represents the effect of bacteriosiderophore produced by an individual bacterial strain. Data corresponds to mean ± SEm (n = 5). * and ns significant and non-significant ($P \geq 0.05$) respectively

growth and yield traits but when fortified with Fe, these traits were significantly improved. This might be due to difference in the methodology as we applied only siderophore present in the supernatant while in other studies, whole organism along with siderophore was applied which might induce several growth effects.

The concentration and partitioning of Fe in different organ of soybean and wheat were significantly influenced by foliar treatments (Table 1). In soybean crop at flowering stage, significant increase in leaf Fe concentration was observed by application of Fe (30%), *Arthrobacter* sp. +Fe (25%) and *L. fusiformis* +Fe (27%) as compared to control. However, the Fe concentration in stem was highest due to *L. fusiformis* +Fe (98%) followed by *Arthrobacter* sp. +Fe (45%) treatment. At maturity, *L. fusiformis* +Fe application resulted in doubling of Fe concentration in stem, while pod cover retained 40% and seed exhibited 70% increase as compared to control. Other treatments also resulted in increase in seed Fe concentration (30% by Fe and 40% by *Arthrobacter* sp. +Fe) in comparison to control. Although all foliar treatments improved seed Fe concentration in soybean but higher Fe retention in foliage at harvest was observed in Fe (20%) and *Arthrobacter* sp. +Fe (30%) treatments. In wheat crop, at anthesis stage, increase in leaf Fe concentration was more than doubled with foliar application of Fe, *Arthrobacter* sp. +Fe and *L. fusiformis* +Fe as compared to control (Table 1). The stem Fe concentration was also doubled with siderophore in combination with Fe as compared to control. At maturity stage, *L. fusiformis* +Fe treatment resulted in lowest Fe retention in leaf tissue as compared to other two Fe treatments while in stem and chaff, the Fe concentration was more than doubled. Moreover, the Fe concentration in wheat grain was obtained highest (102%) by *L. fusiformis* +Fe as compared to other treatments. Reduced leaf Fe concentration at harvest suggests efficient mobilization of Fe from leaf to developing seeds/grains as observed in both soybean and wheat, leading to higher Fe concentration which was noted in *L. fusiformis* +Fe treatment. Besides tissue Fe concentration, the total Fe content or accumulation in seed/grain in both crops was significantly increased by foliar treatments (Fig. 2). The Fe content obtained highest with *L. fusiformis* +Fe treatment in both soybean (2.5 folds) and wheat (2.6 fold) in comparison to control. In wheat, foliar application of Fe alone also resulted in more than two-fold increase in total Fe content in grain as compared to control.

Earlier reports suggest that foliar application of Fe fortified siderophore not only improved Fe absorption by foliage but also enhanced its mobilization towards grains. Our results are in agreement with Fernández et al. [8] who compared the Fe uptake as well as its mobilization into neighbouring leaf after application of Fe-siderophores, Fe–EDTA and $FeSO_4$ to the distal part of the leaf in *Vicia faba, Nicotiana tabacum* and *Citrus madurensis*. They observed maximum mobilization of Fe in neighbouring leaf in Fe siderophore complex while least translocation was obtained in $FeSO_4$. Similarly, Basiouny and Biggs [33] reported that Fe chelates are mobilised better than inorganic Fe-containing compounds into and throughout the plant system and the response of plants to organic Fe chelates might be due to improved antioxidant scavenging system [7]. In contrast to some commonly used synthetic chelates, the siderophores are not environmentally hazardous and may serve as an alternative means of supplying Fe to plants [34]. Mobilization of Fe from vegetative tissues into grains through phloem may be influenced by soil properties viz. nitrogen, moisture and pH or plant's physiological response such as transpiration rate and photosynthesis [35]. Therefore, application of Fe directly on the foliage plays an important role in grain Fe accumulation [36]. Foliar application of *Bacillus* sp. strain OSU 142 in apricot resulted in higher nutrient (N, P, K, Ca and Mg)

**Table 1** Influence of foliar application of bacteriosiderophore fortified with or without Fe on tissue Fe concentration in soybean and wheat during flowering and harvest stages

| Treatments | Soybean (mg Fe $g^{-1}$ DW) | | | | | | Wheat (mg Fe $g^{-1}$ DW) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Flowering | | Maturity | | | | Anthesis | | Maturity | | | |
| | Leaf | Stem | Leaf | Stem | Pod cover | Seed | Leaf | Stem | Leaf | Stem | Chaff | Grain |
| Control (water) | 0.925 | 0.205 | 0.813 | 0.140 | 0.240 | 0.09 | 0.443 | 0.127 | 0.373 | 0.120 | 0.160 | 0.137 |
| Fe | 1.198 | 0.219 | 0.976 | 0.252 | 0.266 | 0.12 | 0.900 | 0.260 | 0.696 | 0.260 | 0.230 | 0.218 |
| *Arthrobactor* sp. +Fe | 1.160 | 0.298 | 1.057 | 0.247 | 0.270 | 0.13 | 0.453 | 0.158 | 0.683 | 0.210 | 0.230 | 0.227 |
| *Arthrobactor* sp. | 0.908 | 0.220 | 0.841 | 0.183 | 0.260 | 0.11 | 0.921 | 0.357 | 0.330 | 0.170 | 0.220 | 0.171 |
| *L. fusiformis* +Fe | 1.173 | 0.405 | 0.823 | 0.283 | 0.337 | 0.15 | 0.430 | 0.178 | 0.450 | 0.270 | 0.350 | 0.277 |
| *L. fusiformis* | 0.933 | 0.203 | 0.830 | 0.273 | 0.310 | 0.12 | 0.913 | 0.187 | 0.307 | 0.330 | 0.270 | 0.222 |
| Significance | ** | ** | ** | ** | *NS* | ** | ** | ** | ** | ** | ** | ** |

*NS* non-significant

**Significant at $P = 1\%$

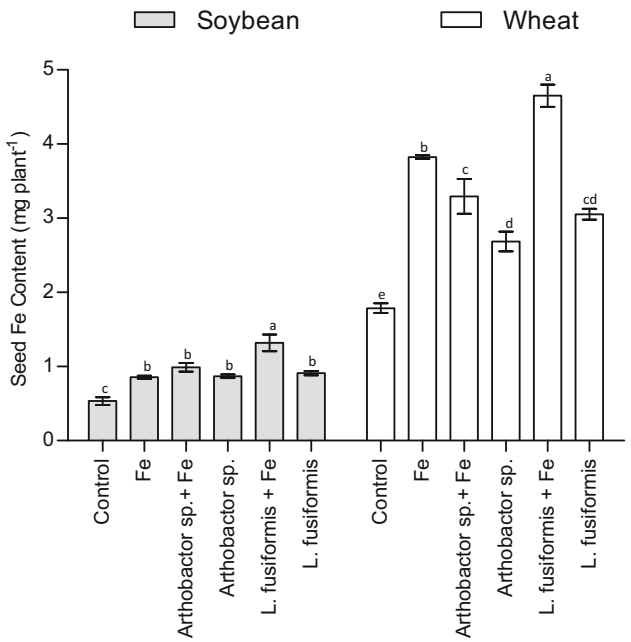


**Fig. 2** Influence of foliar application of bacteriosiderophore with and without Fe enrichment on Fe content in seed/grain per plant of soybean and wheat. Data corresponds to mean ± SEm (n = 5). Mean with the same letter are not significantly different at $P \leq 0.05$ according to least significant difference test

concentration in leaf tissue which might be due to it's a symbiotic N-fixing ability in the phyllosphere [37, 38]. However, in-depth physiological studies are needed to explore the translocation of Fe applied with bacterial siderophore, towards developing grain. Further, the siderophore produced by *L. fusiformis* fortified with Fe may be validated on other crops under field conditions which might provide an easy, economic and targeted strategy for biofortification.

## Conclusions

In this study contrasting bacterial strains, *L. fusiformis* (92.3%) and *Arthrobacter* sp. (12.1%) in terms of siderophore production were identified and validated on soybean and wheat crops. Results from this study strongly suggested foliar application of Fe with siderophore to enhance plant growth, yield and of Fe concentration of in grains. This is first report that the foliar application of Fe fortified bacteriosiderophore can represent a low cost, easy and sustainable method for enhancing Fe concentration in legume and cereal grains to help alleviate the Fe deficiency.

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**Author Contribution** Conceived of or designed study: RP, SS, AKS; Performed research: SS, AK, SC; Provided facility and research support: RP, PB, AKS; Analysed data: SS, RP, SC; Wrote the paper: SS, RP, PB, VP.

**Compliance with Ethical Standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Research Involving Human Participants and/or Animals** Not applicable.

**Informed Consent** Not applicable.

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