

Iron acquisition in maize (*Zea mays* L.) using *Pseudomonas* siderophore

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Received: 2 December 2016 / Accepted: 11 February 2017 / Published online: 31 May 2017
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Abstract Siderophore facilitates iron availability in soil, but its assistance in iron transportation to different plant parts is not reported till date. Therefore, it is worthwhile to study the effect of siderophore produced by *Pseudomonas* for iron acquisition in the presence and absence of iron. To study these effects, two siderophore-producing *Pseudomonas aeruginosa* strains RSP5 and RSP8 were selected. RSP5 and RSP8 produced the highest and lowest amounts of siderophore, respectively. Iron (Fe) concentration of stem, leaf, seed, and shoot length, root length, cob length, and number of grains parameters were analysed. It was observed that the plants treated with RSP5 were sturdier and taller than RSP5 + Fe > RSP8 > RSP8 + Fe > Fe > Control plants. Iron content of RSP8 vs. RSP8 + Fe, RSP8 + Fe vs. Control, and RSP8 + Fe vs. RSP5 + Fe was significantly different ($P < 0.01$). Analysis of variance (ANOVA) proves that RSP5 was able to transport higher amount of iron to maize plant than other treatments. Increase in shoot length, root length, cob length, grain number and iron content of stem, and leaf and seed of maize plant inoculated with RSP5 suggests that the strain can be used as an inoculant for increasing iron transportation in maize plant. (Indian Patent Filed: 40163/DEL/2016).

Keywords *Pseudomonas* · Plant growth promoters · Siderophore · Iron fortification

Introduction

Seventeen essential plant food nutrients or minerals are required for optimal growth and development of crops. All are equally important to the plant, though required in different amounts. This difference has led to the grouping of these essential minerals into three categories; primary (macro) nutrients (nitrogen, phosphorus, potassium, carbon, hydrogen, and oxygen), secondary nutrients (calcium, magnesium, and sulphur) and micronutrients (boron, chlorine, copper, iron, manganese, molybdenum, and zinc). Inadequate balance of these minerals with other nutrients may lead to growth suppression or even complete inhibition (Mengel et al. 2001). Micronutrients are involved in the key physiological processes of photosynthesis and respiration (Marschner 1995; Mengel et al. 2001) and their deficiency can impede these vital physiological processes thus limiting yield gain.

Iron (Fe) is an essential element for all living microorganisms and human beings, and for plant growth (Kobayashi and Nishizawa 2015), as it plays a major role in oxygen transport, oxidative metabolism, cellular proliferation, oxygen metabolism, electron transfer, DNA, and RNA synthesis, and acts as a catalyst in enzymatic processes (Aguado-Santacruz et al. 2012). Although iron is present in abundance, it is unavailable to plants due to its prevalence as insoluble iron oxyhydroxide polymers under aerobic conditions. Bacteria present in soil tend to produce siderophores under iron limiting conditions to enhance the bioavailability of Fe in the environment. The existence of siderophore-producing microorganisms in all the

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rhizosphere has led to the hypothesis that plants would become Fe deficient in the absence of siderophore as siderophore chelate the insoluble iron (Fe^{3+}) in different pH condition and make it available for plants (Kloepper et al. 1980; Crowley et al. 1991).

Iron in human and animal occurs in two forms (dietary iron): heme and non-heme. The primary sources of heme iron are hemoglobin and myoglobin from consumption of meat, poultry, and fish, whereas non-heme iron is obtained from cereals, pulses, legumes, fruits, and vegetables (Abbaspoura et al. 2015). In agriculture, Maize occupies a prominent position and each part of the maize plant is put to one or the other use and nothing goes as waste. Maize as a crop has multiple uses but is chiefly grown for human and Livestock consumption. In most of the developing countries, maize is consumed directly as food but is iron deficient. Therefore, the purpose of the study was to utilize the siderophore-producing ability of *Pseudomonas* strains to transport iron to the seeds of maize plant from soil. The research could be beneficial to enhance iron content in the plant/seed to enhance the dietary heme in food and feed components.

Material and method

Isolation of siderophore-producing *Pseudomonas*

Presumptive *Pseudomonas* were selected from lab culture isolated from Pine and Oak forest and Agricultural land of Uttarakhand, India (Sah and Singh 2016).

Quantification of siderophore production with Fe

Quantification of siderophore was done for 14 isolates on sodium succinate medium (SSM). SSM supplemented with 20 μM Fe was inoculated with overnight grown culture of *Pseudomonas* and incubated for 24 h at 28 °C in incubator shaker. Sample was withdrawn after 24 h from SSM and centrifuged at 8000 rpm for 8 min. Absorbance of supernatant was measured at 400 nm (Meyer and Abdallah 1978; Sah and Singh 2016)

Siderophore conc. = $\text{OD} \times 1500$
 $\times 1000/16,500$ (mg/L) or ($\mu\text{g/ml}$).

Plant growth experiment

On the basis of siderophore production, RSP5 and RSP8 were selected to observe the iron transport in maize plant. Seeds of maize (*Zea mays* L.) were obtained from G. B. Pant University of Agriculture and Technology, Pantnagar, India. These seeds were surface sterilized in 0.1%

HgCl_2 , followed by thorough washing with autoclaved distilled water. Bacterization of seed (seed load; 10^5 CFU per seed) was performed using carboxy methyl cellulose (CMC) as matrix (Sharma and Johri 2003). This was followed by sowing seeds in pots (500 g soil volume/pot). Standard Hoagland's solution was used to irrigate the pots once a week; autoclaved double distilled water was used during rest of growth period. Standard Hoagland's solution supplemented with 20 μM Fe was used to irrigate the pots for observation to stop siderophore production. The observations for root/shoot length, number of leaves, length of corn, number of grains per corn, number of rows per corn, and iron content in leaf, stem, and seed, and were taken after 70 days of sowing. Different combinations were designed in triplicates as: (a) soil with Bacteria; (b) soil with Fe (20 μM); (c) Soil with Bacteria and Fe (20 μM); and (d) Control: Without bacteria and Fe (20 μM). The results were analysed biometrically using SPSS Statistics for Windows, Version 20.0. (Armonk, NY: IBM Corp.).

Atomic absorption spectroscopy (AAS) of the plant parts (stem, leaf, and seed)

Stem, leaf, and seeds were collected separately for all the sample plants and labeled. Equal weight of each sample was weighed and burnt to ash in hot oven. Aqua regia was prepared taking HCl and HNO_3 in the ratio of 3:1.15 ml of aqua regia; 10 ml water and sample were mixed in separate beakers followed by boiling on hot plate. The mixture was boiled till the volume reduced to 8 ml followed by filtration. The filtrate was then properly diluted and iron content was measured by AAS (Analytical Iena vario-6 cold Vapour-HG-AAS) at NPL, Delhi. One-way repeated-measures analysis of variance (ANOVA) and post hoc analysis (Holm-Sidak method) were performed for further exploration of data through pairwise multiple comparisons through professional statistical software, i.e., Sigma Plot (Systat Software, San Jose, California, USA) and IBM SPSS Statistics for Windows, Version 20.0. (Armonk, NY: IBM Corp.).

Result and discussion

Isolation of siderophore-producing *Pseudomonas*

On the basis of molecular studies, the *Pseudomonas* strains isolated from Pine and Oak forest and Agricultural land of Uttarakhand, India were identified as a *P. aeruginosa*. The GenBank/NCBI accession number of the strain *P. aeruginosa* strains was as RSP5–KR051490 and RSP8–KR051488 (Sah et. al 2017).

Quantification of siderophore-producing *Pseudomonas* with and without Fe

Siderophore production for 14 isolates having a zone size more than 2 cm was quantified with and without adding Fe (20 μM) to SSM. Supernatant of SSM medium after 24 h of inoculation with *Pseudomonas* was scanned spectrophotometrically and clear peak was observed in the range of 367–400 nm confirming production of siderophore (Meyer and Abdallah 1978). SSM medium without addition of iron was accompanied by siderophore

production in the range of 75.00–210 $\mu\text{g mL}^{-1}$, while after supplementing SSM medium with iron, siderophore production was repressed with a range between 10.00 and 160 $\mu\text{g mL}^{-1}$. The highest siderophore producer was identified as *P. aeruginosa*, RSP5 (134, 210 $\mu\text{g mL}^{-1}$ with and without iron, respectively) and the lowest siderophore producer was identified as *P. aeruginosa* RSP8 (10, 75 $\mu\text{g mL}^{-1}$ with and without iron, respectively). Colquhoun and Sorum (2001) confirm that *P. fluorescens* growing in iron-limited conditions (less than 160 g L^{-1}) should induce siderophore production. It was observed that

Table 1 Effect of different bacterial treatments on the plant growth characteristics

S. no.	Bacterial treatments	Shoot length (cm) mean \pm SE	Root length (cm) mean \pm SE	Cob length (cm) mean \pm SE	Number of grains mean \pm SE
1	RSP5	156 \pm 1.52	21.66 \pm 0.88	21.66 \pm 0.881	395.66 \pm 44.29
2	RSP5 + Fe	149.66 \pm 2.33	8.66 \pm 1.33	12 \pm 1.52	156 \pm 12
3	RSP8	123.33 \pm 3.33	12.33 \pm 4.09	18 \pm 1.15	344 \pm 22.27
4	RSP8 + Fe	113.66 \pm 0.88	8.33 \pm 1.66	8.66 \pm 0.66	173.66 \pm 3.17
5	Fe	106.33 \pm 7.57	9.33 \pm 0.66	8.33 \pm 1.2	173.66 \pm 3.17
6	Control	103.33 \pm 4.80	7 \pm 0.57	10 \pm 0.57	120.66 \pm 11.02

The table represents the results of the plant growth experiment in the presence and absence of 20 μM iron. The results were based on the shoot length, root length, cob length, and number of grains per cob. The results are a mean of triplicates

Fig. 1 Effect of different bacterial treatments on cob length, number of rows/cob, and number of grains/row

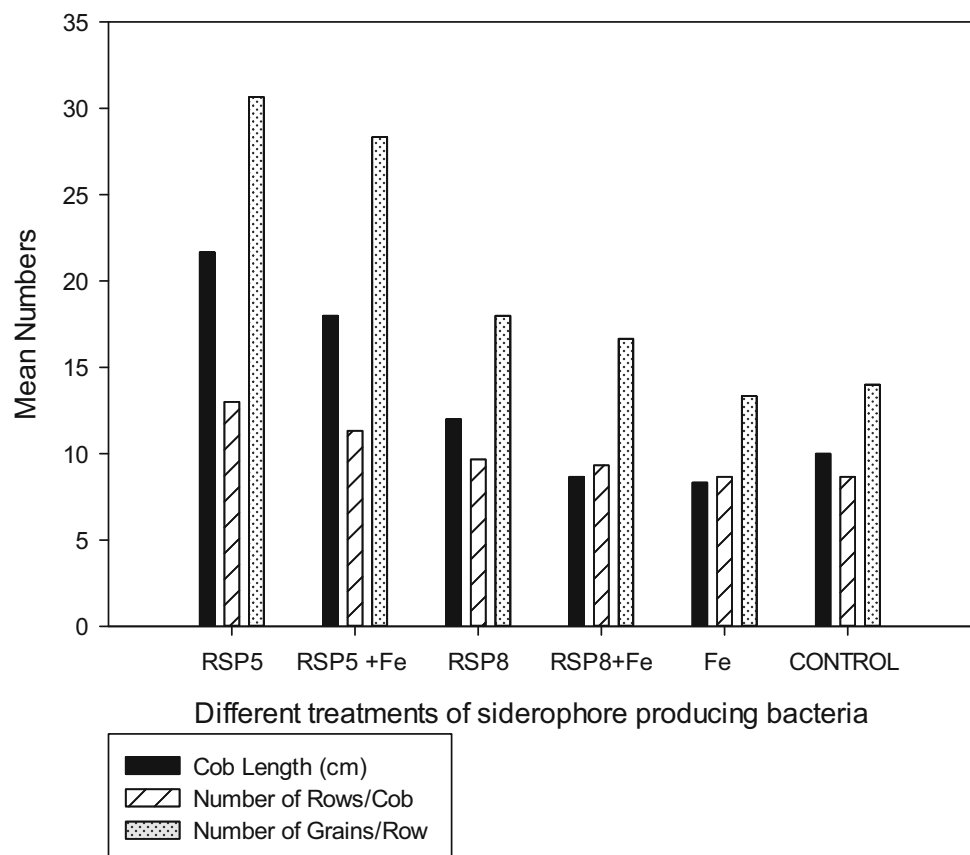
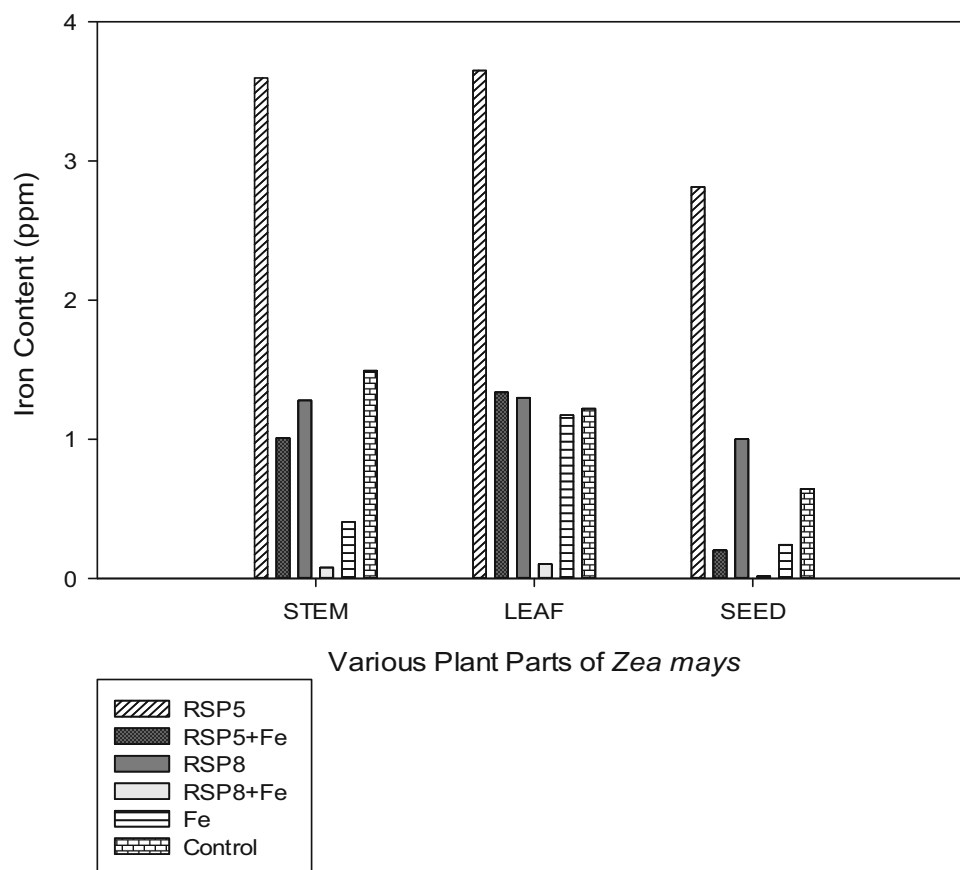


Fig. 2 Iron transported to stem, leaf, and seed with the help of siderophore using different treatments



siderophore production and iron concentration are inversely proportional. Threshold level of iron which repressed siderophore production was found to be 20 μM . It was reported earlier also that Siderophore production impedes at a concentration $\geq 20 \mu\text{M}$ (Nair et al. 2007; Bholay et al. 2012).

Plant growth experiment

Biometrical analysis

Maize seeds were sown and different treatments were given, according to the experiment design. After 70 days, the shoot length, root length, cob length, and number of seeds of maize plants were analysed (Table 1). It was observed that the plants treated with RSP5 were sturdier and taller than the other combinations (RSP5 + Fe > RSP8 > RSP8 + Fe > Fe > Control plants). The shoot length (SL) and root length (RL) of control plant were observed to be 103 and 7 cm, respectively. An increase of 51 and 44% was observed in the shoot length of maize plants inoculated with RSP5 and RSP5 + Fe, respectively. The shoot enhancement was recorded to be 19.35 and 9.99% by RSP8 and RSP8 + Fe respectively. A negligible increase of 3% was observed in

shoot length of plants uninoculated by any bacteria. A comparable difference of 21% was observed in shoot length of plants inoculated with RSP5 and RSP8. The SL and RL of plants inoculated with RSP5 > RSP8 as RSP5 produce more siderophore than RSP8. Siderophore produced by RSP5 and RSP8 was lowered when 20 μM Fe was added, and hence the SL and RL of RSP5 + Fe < RSP5 and RSP8 + Fe < RSP8 treated plants.

Some species of *Pseudomonas* are considered as the plant growth promoting rhizobacteria (PGPR). *Pseudomonas* bacteria improve plant growth and yield through direct and indirect mechanisms (Crowley et al. 1991). *Pseudomonas* produce siderophore under Fe deficiency conditions which act like chelates and transport Fe into plant roots and help in growth of plants (Sharma and Johri 2003).

Corn data analysis

Figure 1 and Table 1 represent the variation in Cob length and grains per cob from soil inoculated with RSP5, RSP8, RSP5 + Fe and RSP8 + Fe. The cob length (CL): 21 cm, rows per cob (RC): 18 and grains per cob (GC): 395 were highest when plants were treated with RSP5, while the CL and GC of Control plants were 10 cm and 120. The CL and

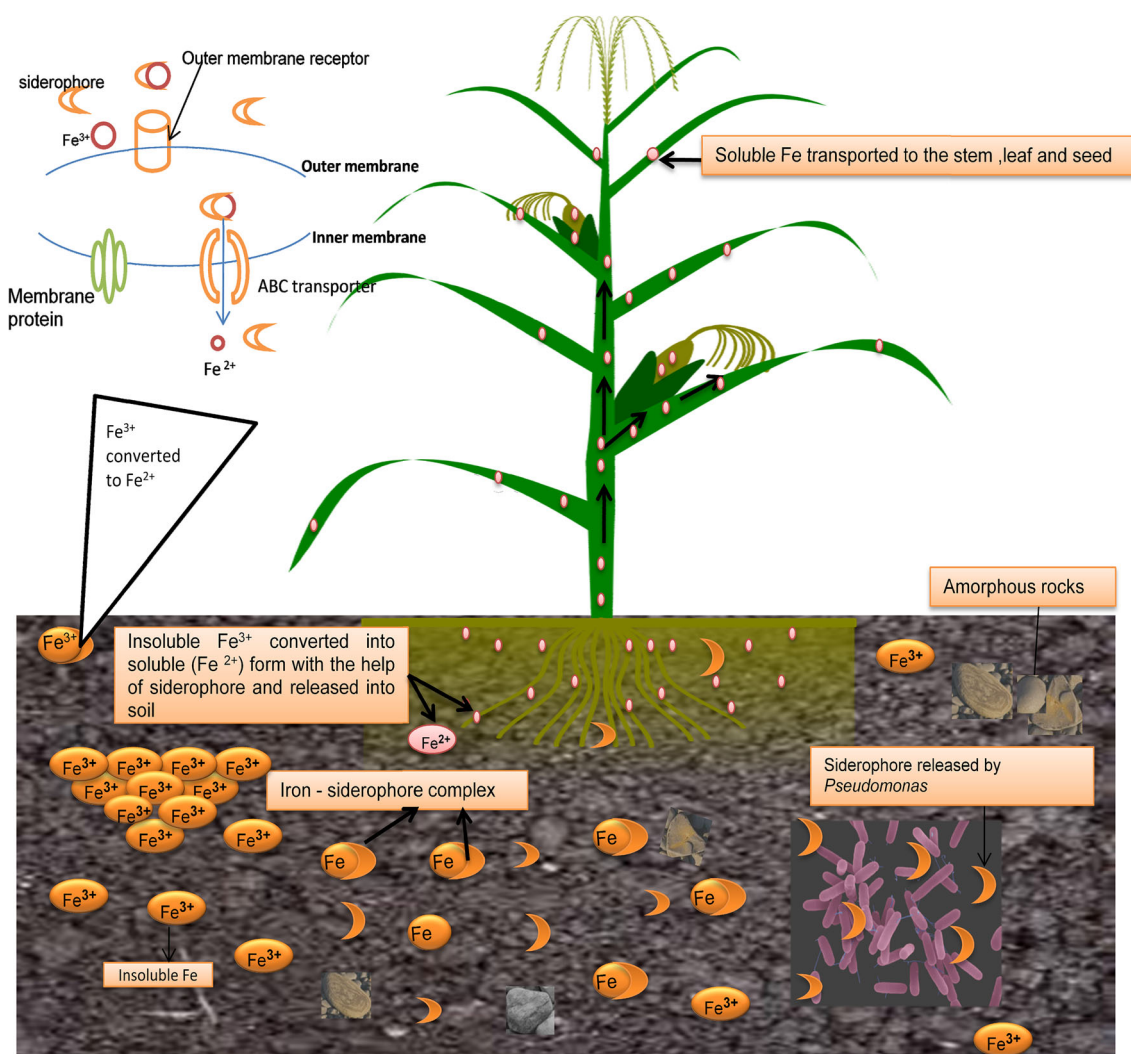


Fig. 3 Schematic representation of iron transportation to stem, leaf, and seed with the help of siderophore

GC of RSP5 + Fe treated plants was 12 cm and 156, respectively; the values were less than RSP5 but more than Control. The CL and GC of plants inoculated with RSP8 (18 cm and 344 respectively) was more than the control plant but less than the plants treated with RSP5. The CL and GC of the various treatments was observed to be highest in RSP5 followed by RSP8 > RSP5 + Fe > RSP8 + Fe ≥ Fe > Control. The reason for the above results may be higher siderophore production by RSP5 than RSP8. The absence of RSP5 and RSP8 in Fe treated and Control plants leads to least CL and GC as no siderophore is produced in either of the treatments.

Iron transportation with the help of siderophore

The AAS results revealed highest level of iron in stem, leaf, and seed (SLS) (3.59, 3.65, and 2.81 ppm, respectively) of plants treated with RSP5. The iron transportation

in plants treated with Fe and RSP8 + Fe was almost equal. Iron content in SLS of plants uninoculated with bacteria and supplied with Fe (20 μM) was recorded to be 0.40, 0.75, and 0.24 ppm, respectively, and for the plants treated with RSP8 + Fe (1.17, 0.09, and 0.14 ppm, respectively). The iron content of SLS increased by 301.6, 328, and 487.5%, respectively, when treated with RSP5 and 71.87, 63.56, and 92.89% when plants treated with RSP8 (Fig. 2). The graph clearly depicts the relation between siderophore and iron in seed.

Iron transportation in plant through leaf to seeds represented in Fig. 3 and was better understood when analysed statistically.

One-way repeated-measures analysis of variance (ANOVA) exhibited highly significant differences in iron content among various treatments of stems, leaves, and seeds of maize plant ($df = 5$; $SS = 19.216$; $MS = 3.843$; $F = 55.475$; $P < 0.001$). A post hoc analysis (Holm–Sidak

Table 2 Post hoc analysis for comparing the iron content in stem, leaf, and seed amongst the various treatments

S. no.	All pairwise multiple comparison procedures (Holm–Sidak method): overall significance level = 0.05 comparisons for factor	Diff. of means	<i>t</i>	<i>P</i>	<i>P</i> < 0.050
1	RSP5 vs. RSP8 + Fe	3.288	15.300	<0.001***	Yes
2	RSP5 vs. Fe	2.746	12.779	<0.001***	Yes
3	RSP5 vs. RSP5 + Fe	2.504	11.651	<0.001***	Yes
4	RSP5 vs. Control	2.234	10.396	<0.001***	Yes
5	RSP5 vs. RSP8	2.161	10.054	<0.001***	Yes
6	RSP8 vs. RSP8 + Fe	1.127	5.246	0.004**	Yes
7	Control vs. RSP8 + Fe	1.054	4.904	0.006**	Yes
8	RSP5 + Fe vs. RSP8 + Fe	0.784	3.648	0.035*	Yes
9	RSP8 vs. Fe	0.586	2.725	0.140	No
10	Fe vs. RSP8 + Fe	0.542	2.521	0.169	No
11	Control vs. Fe	0.512	2.383	0.178	No
12	RSP8 vs. RSP5 + Fe	0.343	1.597	0.456	No
13	Control vs. RSP5 + Fe	0.270	1.255	0.557	No
14	RSP5 + Fe vs. Fe	0.242	1.127	0.490	No
15	RSP8 vs. Control	0.0735	0.342	0.740	No

The results are a mean of triplicates

* Significant

** Highly significant

*** Extremely significant

Method) was performed for further exploration of data through pairwise multiple comparison (Table 2) which showed a significant effect of various treatments on the transportation of iron on the plant through leaf to seed. Analysis of variance proves that RSP5 was able to transport higher amount of iron to maize plant than other treatments (RSP8, Fe, RSP5 + Fe, RSP8 + Fe, Control). A highly significant difference $P < 0.001$ was observed when iron content of stem, leaf, and seed of plants supplied with RSP5 was compared to Fe, RSP5 + Fe, RSP8, RSP8 + Fe, and Control with a mean difference of 2.746, 2.504, 2.161, 3.288, and 2.234, respectively (Table 2). The statistical difference observed in iron content of plants treated with RSP5 and RSP8 represents the difference in iron transported with both the treatments. Significant difference ($P < 0.01$) was observed when iron content of RSP8 vs. RSP8 + Fe, RSP8 + Fe vs. Control, and RSP8 + Fe vs. RSP5 + Fe were compared. Highly significant difference in iron level, $P < 0.001$, and a difference of mean 2.504 in RSP5 vs. RSP5 + Fe suggest that there is a comparable difference in iron content of both the treatments and excess of iron hindered production of siderophore as well as transport. A highly significant difference was observed when soil supplemented with RSP5 and Control was compared. The iron content in RSP8 and Control was not significantly different ($P > 0.05$) with a minor difference of mean (0.740), while iron content of RSP5 vs. RSP8 + Fe have a high difference of mean

(3.288). The iron content in treatments Control vs. Fe, RSP5 + Fe, RSP8 and Fe vs. RSP5 + Fe, and RSP8 + Fe was not significantly different suggesting that the difference in their iron content was not comparable. The statistical analysis results also prove that the iron content in stem, leaf, and seeds of maize plant grown in uninoculated iron sufficient and iron-deficient soil were not significantly different ($P > 0.05$).

RSP5 produces higher amount of siderophore than RSP8; therefore, the iron transported with the help of siderophore is more in seeds coated with RSP5 than RSP8. Siderophore production reduced after inoculating the soil with Fe and it was interesting to note that iron transportation was also reduced when inoculated with RSP5 and RSP8 along with iron supplementation. The absence of *Pseudomonas* strains (RSP5 and RSP8) in Control plant resulted in low iron transportation and plant growth.

Soil microbes play an important role in favoring plant iron (Fe) uptake under Fe-limiting conditions (Jin et al. 2010); similarly, siderophore released by RSP5 was capable of solubilising the insoluble iron present in soil making it available to plant and transporting it to the plant (stem, leaf, and seed). Masalha et al. (2000) suggested that the microbial community present in rhizosphere plays an important role in Fe acquisition, affecting the composition of siderophore secreting microbes in the rhizosphere (Jin et al. 2010; Camejo et al. 2013). Under Fe-deficient

conditions, it has been reported that the pyoverdines (siderophores secreted by *Pseudomonas*) show a high affinity for Fe(III) (Meyer and Abdallah 1978; Boukhalfa and Crumbliss 2002), and thus, when the Fe(III) content in soil was increased more than 20 μM , the siderophore production by (*P. aeruginosa*) RSP5 and RSP8 was negligible affecting the iron transportation to the maize plant (stem, leaf, and seed). Bacterial siderophore is not absorbed by the plant, but Fe released by siderophore through a reduction-based mechanism (Cesco et al. 2002; Hordt et al. 2000; Romheld and Marschner 1986) is absorbed by the plant.

Conclusion

Siderophore production by different *Pseudomonas* species has widely been studied and is found to be dependent on iron content present in soil. Our experimental findings reveal that *Pseudomonas* isolates RSP5 and RSP8 yield different amount of siderophore in iron deficient ($\leq 20 \mu\text{M}$) and iron sufficient ($\geq 20 \mu\text{M}$) soil. RSP5 produces higher amount of siderophore than RSP8 in iron sufficient as well as iron-deficient soil. The plant growth experiment suggests high amount of siderophore produced by RSP5 solubilised more iron from soil and transported iron more efficiently to various plant parts of Maize (dietary food) than RSP8. The increased iron content in stem, leaf, and seed of Maize will be beneficial for supplementation of dietary iron (non-heme) in human and animal food and feed.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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