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BIOFORTIFICATION OF IRON IN CHICKPEA BY PLANT GROWTH PROMOTING RHIZOBACTERIA

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Abstract

Iron deficiency is a major nutritional disorder being responsible to affect millions of people around the globe. Its malnutrition may be reduced through biofortification: a process to produce micronutrient enriched staple food. Plant growth promoting rhizobacteria (PGPR) can fortify iron content within edible plant tissues by enhancing its availability through various mechanisms. In a pot study, five bacterial isolates (S_1, S_2, S_3, S_4 and S_5) were tested for improving plant growth and bioavailable iron (Fe) content in chickpea where Fe was applied in the form of iron sulphate solution. Results showed that inoculation with PGPR significantly enhanced the plant height, root length, root fresh and dry weights, shoot fresh and dry weights and Fe content compared to un-inoculated control plants. Application of $FeSO_4$ significantly improved the Fe content upto 100 and 173% in grain and shoot respectively, as compared to control. Application of PGPR along with iron showed 81 and 75% increase in grain and shoot iron contents, respectively, over control. These results suggested that PGPR can help plants to uptake extra Fe from soil, if soil is supplemented with additional Fe. These findings advocate that microbial assisted biofortification in grain can alleviate micronutrient deficiency in humans especially in resource limited countries.

Key words: Micronutrients, Siderophore, Bioavailable, Phytohormones.

Introduction

Micronutrient malnutrition is a serious problem to human health throughout the world, primarily in resource limited countries (Kennedy *et al*., 2003). According to estimates of World Health Organization (WHO), around two billion people are being suffered with vitamin A, iron and iodine deficiencies. Iron deficiency is a highly prevalent nutritional disorder afflicting 2.5 to 5 billion people around the globe (Yip, 2002) where poor households and pre-school children are severely affected due to high demand for iron (Benoist *et al*., 2008). Iron acts as co-factor for several enzymes performing basic functions in human body. Inadequate supply of iron contributes to disability, anemia and stunted mental growth (Sheftela *et al*., 2011). Its malnutrition may be reduced by enhancing the bio-available iron content through iron supplementation and food fortification (Rana *et al*., 2012). These attempts are usually expensive and difficult to sustain on daily basis particularly in undernourished countries (Best *et al*., 2011).Therefore, it seems most desirable that crop fortification with iron content would be cost effective approach to overcome the hidden hunger of iron.

Biofortification is a process to produce micronutrient enriched staple food through mineral fertilizer, conventional breeding and transgenic approaches but their success have not always been consistent (Murgia *et al*., 2012). Plant growth promoting rhizobacteria have been reported to fortify the iron contents in food crops besides improving the soil fertility and crop yield through siderophore production (Rana *et al*., 2012). Siderophores are low molecular weight organic compound having high affinity for iron. Typically, there are four major groups of siderophore namelycatecholates, hydroxamates, hydroxypyridonates, or aminocarboxylates (Neilands, 1977). Siderophores form very stable iron complexes and enhance the dissolution of iron bearing minerals by coordinating with iron atom at mineral surface. Plant growth promoting rhizobacteria synthesize siderophores, release those into surrounding environment, dissolute the iron by making iron chelate complex and translocate towards the plant by growing roots. These iron siderophores complexes are taken up by plant through transporter proteins that are located on plasma membrane of root (Boukhalfa & Crumbliss, 2002).Furthermore, these rhizobacteria enhance plant growth by conferring several beneficial effects such as increased nitrogen fixation, phosphorus solubilization, synthesis of phytohormones, production of organic acid and reduce susceptibility to disease(Ahemad & Kibret, 2014). Sharma *et al*. (2013) reported that iron content in rice grain was improved by the application of plant growth promoting bacteria (PGPR). Therefore, biofortification of plant through PGPR is considered as safe measure to improve iron content in different edible plant parts and to alleviate its malnutrition.

Chickpea has vital dietary importance in developing countries including Pakistan. Chickpea is ranked 3rd grain legume in world and first in South Asia for its production. It is a rabi pulse crop grown in rain fed areas (Shah *et al*., 2013). It is cultivated in 1.12 million hectares with 0.98 million tons production in Pakistan (Anon., 2014) Chickpea is cheapest and readily available source of fats (11.40%), protein (19.5%), ash (4.8%) and carbohydrates (57-60%) (Anon., 2014).So, it is imperative to biofortify the chickpea with iron through PGPR. To address this issue, present study was carried out to improve iron uptake and overall growth and yield of chickpea through rhizobacterial inoculation.

Materials and Methods

Five previously well characterized plant growth promoting bacterial strains (S_1, S_2, S_3, S_4 and S_5) having ability to produce siderophore were used in this study.

Inoculum preparation and seed treatment: The inocula of selected strains were prepared by growing them in glucose peptone broth medium. Flasks containing inoculums were incubated at 28± 1°C. Uniform cell density (10^7-10^8 CFU mL^{-1}) was maintained by measuring the optical density at 535 nm. Inoculum of each culture was injected into sterile peat (100 $mLkg^{-1}$) obtained from Chhanga Manga Forest Station and incubated for 24 hours at 28 ± 1°C.For seed inoculation, seeds were dressed with slurry that was prepared by mixing of clay, peat and 10% sugar solution. In case of un-inoculated control, seeds were coated with the same but autoclaved bacterial suspension.

Pot experiment: Pot trial was conducted to study the effect of five bacterial strains (S_1, S_2, S_3, S_4 and S_5) with and without iron on the growth and iron contents of chickpea. For this purpose, pots were filled with 10 kg soil having sandy clay loam texture, EC_e1.14dSm^{-1} and saturation percentage (32%). Five inoculated chickpea seeds were sown at 5cm depth in each pot. All the isolates were tested alone and in combination with the iron @ 28 ppm/pot (5.6 kg ha^{-1}) in the form of iron sulphate solution (soil application). A control treatment was also maintained where neither iron nor PGPR were applied. Another treatment was included where only iron was applied but un-inoculated. All thetreatments were arranged according to completely randomized design (CRD) with three replications. Recommended doses of NPK @ (20, 65 and 20 kg ha^{-1}) were applied in each pot as urea, diammonium phosphate and sulphate of potash, respectively. Pots were irrigated with water, meeting the irrigation quality criteria for crop. Five seeds were sown per pot, after germination, thinning was done to maintain the one plant per pot. Furthermore, plant protection measures were adopted against the attack of pests. Plants were harvested and the data related to yield contributing parameters were recorded at maturity. Root and shoot of plants were analyzed for Fe contents. Wolf (1982) method was followed for the digestion of plant samples. For this purpose, 0.1 g oven dried and ground plant samples were taken in a digestion tube and 2 mL of concentrated sulfuric acid was poured into it. The samples were left over night at room temperature. Then 1 mL 35% extra pure H_2O_2 was poured into the digestion tube along the sides and the tube was rotated. The tube was heated up to 350°C after mounting it in a digestion block for 20 minutes. Then the tube was removed, cooled and added with 1 mL of H_2O_2 and again heated for 20 minutes. Same procedure was continued until the material became colorless. Fifty mL volume of the colorless extract was made by using distilled water and stored for the determination of Fe contents. Samples from different plant parts (grain, shoot and roots) were analyzed for iron accumulation by atomic absorption spectrophotometer. Iron content in soil was determined by ammonium bicarbonate-DTPA (AB-DTPA) extractable method (Soltanpour *et al*., 1976).The data were analyzed by using statistical procedures (Steel *et al.,* 1997) and means were compared according to Duncan's Multiple Range Test (DMRT) (Duncan, 1955).

Results

Effect on plant growth and yield: Result regarding the plant growth and yield (Table1) revealed that selected bacterial isolates significantly improved the plant height compared to the un-inoculated control plant, whereas the performance of isolates was better in the presence of iron. Plant height was found to be enhanced with isolate $S_1$and S_4but maximum plant height was observed bycombined application of isolate S_4+Fe which was 97% more compared to untreated and uninoculated control plants. A significant increase in shoot fresh weight was also observed with PGPR inoculation compared to control and iron treated plants whereas maximum increase (94%) in fresh weight of shoots was recorded by the combined application of Fe and S_1. Similarly, all the PGPR isolates significantly improved shoot dry weight. The highest increase in shoot dry weight (78%) was observed with combined application of Fe and S_2over uninoculated control which was statistically different from other isolates. Results regarding root length of chickpea revealed that root length was significantly improved by all the isolates. The isolate S_2showed the highest improvement in root length (97%) when used in combination with iron and gave significant results compared to other isolates. Moreover, isolateS_2 significantly enhanced the root fresh weight but highest root dry weight was noted when isolate S_2 was used in combination with iron. Similarly, the highest root dry weight (84% more) was noted with combined inoculation of isolate $S_1$and iron. It was also observed that application of PGPR alone and in combination also improved the grain yield over control and Fe treated plants. Inoculation with S_1improved the grain yield upto 53% as compared to the treatment where only iron was applied. However, the maximum increase in grain yield (94%) was obtained by the combined application of $S_1$and Fe compared to control.

Effect on iron concentration in different plant parts: Data regarding the total iron concentration in grains (Table2) showed that PGPR inoculation significantly increased grain iron content compared to uninoculated iron treated plant. However, the maximum increase (81%) in Fe content of grains was observed with the combined application of Fe and isolate S_2 as compared to uninoculated iron treated plant which was statistically different from other isolates. Similarly, inoculation had positive effect on shoot Fe content. It was observed that shoot Fe content enhanced up to 75% by combined application of Fe and isolate S_2 over uninoculated iron treated plants. Iron content of root was increased up to 62% with isolate S_1as compared to uninoculated Fe treated plants whereas results were more pronounced (97%) when isolate $S_1$was used in combination with Fe compared to sole application of inoculation and Fe.

Table 1. Application of PGPR alone and in combination with Fe to improve growth and yield of chickpea.

Treatments	Plant height (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	Root length (cm)	Root fresh weight (g)	Root dry weight (g)	Grain yield (g/plant)
Neither iron nor inoculation	12.66f	4.79e	2.40e	13.33g	2.83g	1.03f	1.67g
Fe alone	16.66e	5.59d	2.83d	18.00f	3.23f	1.2ef	2.12f
S_1alone	21.00cd	7.34c	3.60c	22.00de	4.8cd	1.23e	2.50de
S_2 alone	19.66de	7.5c	3.66c	21.33e	4.8cd	1.26e	2.46de
S_3 alone	21.66b-d	7.63c	3.80c	21.66de	4.46e	1.3de	2.43e
S_4alone	21.66 b-d	7.8c	3.70c	21.33e	4.56de	1.33c-e	2.38e
S_5alone	21.00 cd	7.36c	3.73c	21.66de	4.63de	1.23e	2.57d
S_1+Fe	24.33 ab	9.33a	4.53b	22.66c-e	5.03bc	1.90a	3.24a
S_2+Fe	23.33 a-c	8.66ab	5.06a	26.333a	5.56a	1.60b	3.03b
S_3+Fe	23.00 a-c	8.86ab	4.63b	24.66b	5.13b	1.46b-d	2.86c
S_4+Fe	25.00a	8.76b	4.66b	23.00cd	5.1b	1.53b	2.91bc
S_5+Fe	22.00ad	8.83ab	4.70b	24.00bc	5.03bc	1.50bc	3.00bc

*Means sharing the same letter do not differ significantly at p≤0.05

Table 2. Application of PGPR alone and in combination with iron (Fe) on iron uptake indifferent plants parts of chickpea.

Treatments	Fe Concentration (mg 100 g^{-1})		
	Grains	Shoot	Root
Neither iron nor inoculation	1.20g	0.66g	0.14g
Fe alone	2.40f	1.80f	0.86f
S_1alone	3.26de	2.23e	1.40ce
S_2alone	3.30c-e	2.50cd	1.30e
S_3alone	3.36b-e	2.26e	1.33de
S_4alone	3.20e	2.36de	1.36ce
S_5 alone	3.40b-e	2.40b	1.30e
S_1+Fe	3.60bc	2.73b	1.70a
S_2+Fe	4.36a	3.16a	1.56ab
S_3+Fe	3.50b-e	2.80b	1.50bc
S_4+Fe	3.53b-d	2.70b	1.50bc
S_5+Fe	3.63b	2.63bc	1.46b-d

*Means sharing the same letter do not differ significantly at p≤0.05

Discussion

Iron deficiency is a major nutritional disorder throughout the world. Biofortification is a technique to produce the micronutrient-dense staple crops. Plant growth promoting rhizobacteria have potential to fortify the iron content within edible plant parts through various mechanisms. In the present study, five bacterial isolates were tested alone and in combination with iron to increase iron concentration in edible parts of chickpea. The results of our study revealed that inoculation with PGPR isolates improved the plant growth, yield and Fe uptake. However, combined application of Fe and PGPR showed more pronounced results. Growth promotion by PGPR may be attributed to increased nitrogen fixation, P-solubilization, production of phytohormones and synthesis of organic acids (Remans *et al*., 2008).It has been reported that PGPR inoculation enhanced the root and shoot growth due to nitrogen fixation, P-solubilization as well as their ability to produce certain compounds (Salantur *et al*., 2006). PGPR improved the growth of chickpea plant by enhancing P-solubilization and indole acetic acid production (Yadav *et al*., 2010). Moreover, siderophore production by rhizobacteria play important role in Fe nutrition of plant and thereby improve the plant growth. Husen (2003) found that siderophore produced by the bacterial isolates *Bacillus cereus* UW 85 and *Azotobactervinelandii* MAC 259 increased the plant growth. Inoculation of soybean and chickpea seed with siderophore producing fluorescent *Pseudomonas* improved the plant growth and yield (Kumar & Dube, 1992). Similarly, growth factors such as shoot and root dry weight were improved by siderophore producing strains in bean (Omidvari, 2010). Growth promotion and reduction in pathogenicity was observed due to siderophore production by *Bacillus megaterium* isolated from tea rhizosphere (Chakraborty *et al*., 2006). Gangwar & Kaur (2009) isolated the *E. coli* from rye grass and sugarcane which had ability to produce siderophore and ultimately improved the plant growth and yield. Moreover, it has been reported that soybean growth under non-sterilized soil condition improved due to siderophore production by PGPR (Cattelan *et al*., 1999).

In our study, results regarding Fe concentration in different plant parts (grain, shoot and root) showed that Fe contents were enhanced by PGPR application. It was

observed that combined application of Fe and PGPR inoculation gave best results compared to all other treatments. This enhancement in iron content might be due to siderophore production by PGPR which made this combination more competitive than control and Fe application alone. Many bacteria release ferric iron-specific ligands, known as siderophores, under conditions of Fe scarcity help in binding and transport of iron (Abd-Alla, 1998). Sharma *et al*. (2013) found that application of PGPR improved iron translocation from root to grain through siderophore production. Similarly, inoculation of wheat seed with three cyanobacterial strains (CW_1, CW_2 and CW_3) and one bacterial strain (PW_5) improved the protein content of grain and micronutrients (Fe, Zn and Cu) concentration (Rana *et al*., 2012). Furthermore, increase in iron content mightbe due to over expression of ferritin genes. Previous reports showed that increasing capacity for iron storage in plants by over expressing ferritin genes that induced iron uptake mechanism, resulted in enhanced iron content (Goto & Yoshihara, 2001; Curie & Briat, 2003).

Conclusion

Inoculation with PGPR along with Fe increased the iron uptake and overall growth and yield of chickpea. Therefore, it can be concluded that application of Fe with rhizobacteria could be the most promising and cost effective strategy for improving the iron contents of chickpea. However, more work is still needed to reveal the exact mechanism of biofortification of micronutrients by rhizobacteria.

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