JCHR (2018) 8(3), 199-207

Journal of Chemical Health Risks

www.jchr.org

ORIGINAL ARTICLE

# Identification of Bacterial Isolates of *Pseudomonas fluorescens siderophore* from Rhizosphere of Corn Fields and Evaluation of Iron Absorption by Corn

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*(Received: 14 June 2018 Accepted: 26 August 2018)*

**KEYWORDS**

Iron;

Specific primer;

Single cross 704;

PFG5 isolate

**ABSTRACT:** Iron is one of the most important elements for plant growth and metabolism. Plant growth promoting bacteria such as *Pseudomonas fluorescens* isolates play an important role in the absorption of iron by plants under iron deficiency by production of microbial siderophore. In this study, 25 bacterial isolates were isolated from corn plants rhizosphere in Khorasan Razavi Province, Iran in 2017. Based on physiological-biochemical and molecular diagnostic methods, by using 16SPSEfluF and 16SPSER specific primers, bacterial isolates were identified as *P. fluorescens*. Among these isolates, the highest amount of siderophore production was related to the isolate PFD1 and the lowest to the isolate PFD3. Moreover, in the study of iron absorption by corn (Single Cress 704), the highest iron absorption was obtained from PFG5 treatment (283 ± 2/3 ppm) and the lowest from control treatment (7/1 ± 6/107 ppm). Isolate PFG5 showed the highest yield increase on fresh and dry weight of root and aerial parts among treatments. PFG5 isolates could be introduced as an effective isolate in iron deficiency conditions.

## INTRODUCTION

Iron is an essential element for growing plants which affects the physiological functions of the plant. This element has a key role in oxygen transfer, oxidation metabolism, oxygen metabolism, electron transfer, RNA and DNA synthesis [1, 2]. Iron (trivalent iron form) is one of the most abundant elements in the earth's crust, but due to its low solubility in aerobic conditions, its absorption is very low by plants [3].

Under iron deficiency, PGPRs, especially *Pseudomonas* spp., can produce low molecular weight substances called siderophore. Siderophores are low-molecular-weight compounds released by bacteria under the condition of iron deficiency. These compounds combine ferric ion (Fe3 +) with high specific activity and transfer into bacterial cells [4]. Several studies have been carried out on the absorption of microbial siderophores, such as fricorum a, agrobactin, podobactin and ferrioxamine B, by plants [5-8]. Iron absorption through ferredoxin-B microbial siderophore was reported by corn and cotton plants [9].

RSP5 and RSP8 strains of *P. aeruginosa* produced the highest and lowest amounts of siderophore, respectively. In this study, the number of seeds, stem length, root and corn, and iron content in stems, leaves and seeds in RSP5 strain treatment was more than other treatments. Corn seed treatment with various strains of *Pomunos* strain in iron deficiency conditions significantly increased seed germination and plant growth [10]. The siderophore produced by strain *Chryseobacterium* spp. separated

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from the rhizosphere of rice, iron was required to supply tomato in iron deficiency conditions [11].

Considering the importance of corn plants in supplying human and animal feed and also supplying some non-essential iron to the body, we aimed to isolate, biochemical and molecular identification of *P. fluorescens* from corn rhizosphere in Khorasan Razavi Province, northeastern Iran as well as evaluating the amount of production Seidophore was studied by different isolates of this species and its effect on corn growth.

## MATERIALS AND METHODS

### *Isolation and identification of bacterial isolates of P. fluorescens species*

In order to isolate bacterial isolates, sampling was performed in summer of 2015 from the rhizosphere of maize fields of Mashhad, Chenaran, Torbat Heydarieh, Gonabad, and Dargaz. Soil samples were transferred to the lab in plastic bags. After the dilution series was prepared from soil samples in sterilized distilled water, soil extracts were cultivated in Petri dishes containing nutrient agar medium (NA). Petri pancakes were kept in an incubator at 28 °C for 48 h. After the passage and create a single colony, biochemical tests – physiology, warm, oxidase, catalase, fluorescent pigment production on KB, Lavon, arginine dehydrogenase, urease and bacterial motility were performed (Table 1).

DNA extraction of bacterial isolates was done [12]. The solutions used were Tris 0.1 M, a NaOH2/0 normal Leaking buffer, and 1% SDS. For the denatured of phenol, chloroform, and isovalyl alcohol, a volume of 1:24:25 was used. After extraction of DNA, concentration of suspension was measured by nanotrop (Thermo 2000) and 50 µl of each sample with 40 ng / µl concentration in separate microtubes was prepared for use in the diagnostic PCR test. In order to identify the bacterial isolates of the *P. fluorescens* species, the 16SPSEfluF (5'-CGTCAACACATGGTGGAAC-3') and 16SPSER (5'-CGGAGGCATTGTCGAAGGAA-3') primers were designed based on the 16rDNA gene [13]. The materials and amount used for the PCR reaction included: 2.5 µl of the reaction buffer (10X PCR buffer), 1.5 µl MgCl2 (2.5 mM), 0.5 µl dNTPs (10 mM), 25/0 1 µl of Taq DNA polymerase (10 µl), 1 µl of initiator (10 µM), 1 µl of back primer (10 µM), 3 µl of DNA and 15.25 µl of sterilized water. Propagation in thermocycler with thermal program initial denatura 5 min priming at 94 °C, 30 cycles with 1 min secondary denaturant at 94 °C, 1 min connection at 55 ° C, 2 min synthesis at 72°C and final synthesis 7 min at 72°C.

**Table 1.** Characteristics of *P. fluorescens* bacteria isolates collected from the Khorasan Razavi Province, northeastern Iran

| Number | Straincode | Region of sampling | Number | Straincode | Region of sampling |
|---|---|---|---|---|---|
| **1** | PFD1 | Dragas | **14** | PFH4 | Torbat Heydrich |
| **2** | PFD2 | Dragas | **15** | PFG1 | Gonabad |
| **3** | PFD3 | Dragas | **16** | PFG2 | Gonabad |
| **4** | PFD4 | Dragas | **17** | PFG3 | Gonabad |
| **5** | PFD5 | Dragas | **18** | PFG4 | Gonabad |
| **6** | PFD6 | Dragas | **19** | PFG5 | Gonabad |
| **7** | PFM1 | Mashhad | **20** | PFC1 | Chenarran |
| **8** | PFM2 | Mashhad | **21** | PFC2 | Chenarran |
| **9** | PFM3 | Mashhad | **22** | PFC3 | Chenarran |
| **10** | PFM4 | Mashhad | **23** | PFC4 | Chenarran |
| **11** | PFH1 | Torbat Heydrich | **24** | PFC5 | Chenarran |
| **12** | PFH2 | Torbat Heydrich | **25** | PFC6 | Chenarran |
| **13** | PFH3 | Torbat Heydrich | | | |

***Determination of siderophore production by bacterial isolates P. fluorescens***

Chromosazole-sulfonate (CAS) agar test was used to evaluate the quality of siderophore production by bacterial isolates. The medium contained chromium azersulfonate, iron (III), hexadecyltrimethylammonium bromide, and nutrient agar medium NB. After autoclaving the culture medium, 20 µl of $10^8$ CFU / ml suspensions of each isolate was placed in a spot in the middle of the petri dish (0/45=600 OD) and incubated at 28 °C for 4 days. The incubator was kept. The color change of the medium from dark blue to orange (yellow) indicates the production of siderophore by bacterial strains [14]. Sucrose medium was also used to compare the production of siderophore by bacterial species. A 24-h culture of bacterial isolates was made with $10^8$ CFU/ml suspension and 20 µl of added 50 ml falcon containing succinate was added. The Falcons were kept on an incubator for 48 h. After 48 h of incubation, the absorption coefficient of each culture sample was determined at a wavelength of 400 nm [15]. This experiment was performed for each isolate in three replications and the results were calculated using SAS 9.2 software using Duncan's mean comparison test.

***The effect of siderophore on iron absorption of maize cultivar Single Cros 704 in greenhouse conditions***

Corn Seed Cultivar Single Cros 704 from the Agricultural Jihad Center of Razavi Khorasan Province was prepared. Sucrose medium was used to prepare a solution containing siderophore. Three bacterial isolates with the highest amount of siderophore production were selected in the catshot test and one isolate with the least amount of siderophore production. At first, 48 h of bacterial isolates were cultured; standard $10^8$ CFU / ml population was prepared by spectrophotometer. Then, the 20 µl suspension of each isolate was added to the sucrose medium and stored for 26 h on a shaker-incubator for 72 h. In order to remove bacterial cells inside a solution containing bacterial siderophore, bacterial suspension was centrifuged for 15 min at 10000 rpm, and 22.0 µm filters were used to remove bacterial cells completely [16]. Method was used to prepare the mixture of siderophore and iron III by mixing a solution containing siderophore and FeCl3. The treatments in this experiment consisted of four iron II complexes prepared from bacterial isolates PFG5, PFG1, PFD1 and PFD3 with maximum and minimum amount of siderophore, FeCl3 and Fe-EDTA solution. Moreover, a non-iron-cooled Hoagland diet was used as control and strain of *P. fluorescens* (prepared from the National Center for Genetic and Biological Diseases of Iran) as a positive control. The nutrient solution was adjusted using calcium carbonate at 0.1 gr per L [17]. This experiment was conducted in a completely randomized design with eight treatments in three replications. The results were evaluated after 45 d of vegetative period and emergence of deficiency symptoms. Moreover, using the atomic absorption device (Shimadzu AA-670), iron concentration in the samples was calculated after digestion and extraction of the air organs. The fresh and dry weight of the air and root organs was measured. Results were calculated using SAS 9.2 software and Duncan's mean comparison test.

## RESULTS AND DISCUSSION

***Identification based on biochemical characteristics - physiology and molecular characteristics of P. fluorescens isolates***

Study of biochemical-physiological characteristics of 54 bacterial isolates showed that gram-negative and aerobic isolates are compulsory. All isolates were able to produce fluorescent dye on KB medium. Positive catalase, negative oxidase, positive urease, and positive and mobile arginine dehydrogenase were the isolates. Sixteen SPSE fluF and 16SPSER were used to identify accurate and safe isolates of *P. fluorescens*. Of the 54 bacterial isolates, 25 isolates were able to produce 850 paired pieces of the PCR reaction (Figure 1).

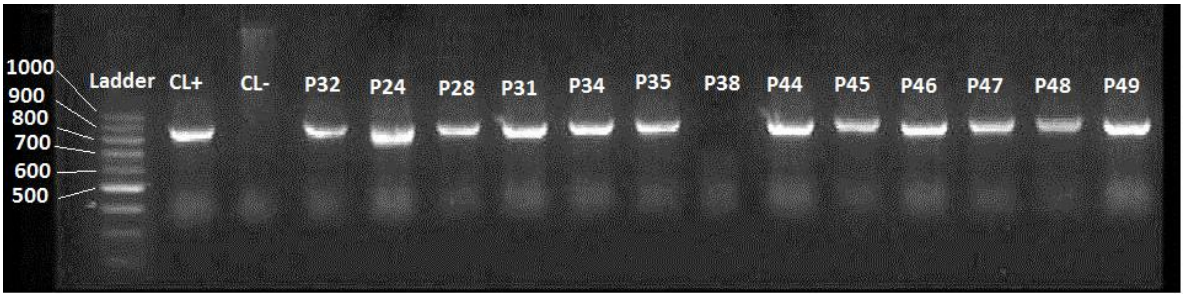


**Figure 1**. PCR product electrophoresis pattern of *P. fluorescens* isolated from corn fields of Mashhad, Chenaran, Dragas, TorbatHeydarieh, and Gonabad

Ladder: Marker, $CL^+$ :P. fluorescens, $CL^-$ :P. putida, P32 : FD2, P24 :PFM1, P28 :PFD5, P31: PFG3, P34, PFG1, P35 :PFM4, P38 :PFM12, P44 :PFG4, P45 :PFG5, P46 : PFM2, P47 :PFC1, P48 :PFC2, P49 :PFC4.

The use of specific primers for the rapid and accurate detection of useful and pathogenic bacteria is used as one of the necessary methods for the identification and management of bacteria [18]. Due to the heterogeneity of the *P. fluorescens* species, this species is divided into several biovares and biotypes based on the physiological and biochemical characteristics [19]. On the other hand, these differences between beavers and the similarity to similar species such as *P. putida* cause an error in the detection of this species. Therefore, the 16SPSEfluF and 16SPSER proprietary initiators were used in this study. This initiator is able to identify all *P. fluorescens* biotypes and species.

***Determination of siderophore production by bacterial isolates P. fluorescens***

The 25 bacterial isolates examined by chromosome orange coloring were able to produce siderophore in a test of chromosogrelsulfonate (CAS) agar. The average diameter of the orange halo between the strains was between 24 ± 1.8 and 37 ± 1.9 mm. Two isolates of PFD6 (37 ± 1.9 mm) and PFG4 (35.6 ± 1.7 mm) had the highest diameter of the halo. Moreover, two isolates of PFD3 (24 ± 1.8 mm) and PFH1 (25 mm) formed the smallest diameter of the halo. In the quantitative test of siderophore content, the absorbance intensity (OD) of bacterial isolates at 400 nm wavelengths was between 0.11 ± 0.122 and 0.306±0.007. The highest amount was related to the isolate PFD1 and the lowest was related to the isolate PFD3 (Figure 2). The results were not consistent with Chromosazole sulfonate (CAS) agar test (qualitative) and quantitative test for siderophore production among isolates. This complete mismatch could be due to the different diameters of the chromosomes Azurul-Sulfonate (CAS) agar in Petri's panes or the speed of moving bacterial isolates on the medium.

The results of a study of different fungal and bacterial isolates indicated that isolated isolates were more siderophore in chromosomal azole sulfonate (CAS) media [20]. The production of bacterial siderophore was investigated in isolates of *Pseudomonas* spp., including two isolates of GRP3A and PRS9, and isolate *P. chlororaphis* ATCC 9446 in two standard sustenance (SSM) and citrate (SCM) environments. The succinate environment is more suitable for the production of siderophore. The authors also examined the absorption rate of bacteria in the CAS medium at various wavelengths. Peak absorption of each of these isolates was 400 nm in length. Moreover, the highest amount of siderophore obtained from this fluorescent pseudomonas was at the end of the growth logarithmic phase. The production of hydroxylate group systrophores was reported in *P. fluorescens* 15.5 µg/ml [21]. The amount of siderophore production of strains *P. aeruginosa*, *P. fluorescens* and *P. putida* in casumino acid culture media at a wavelength of 400 nm was reported to be 1.5, 0.5 and 0.2 respectively [22]. The amount of siderophore production in different isolates of *P. fluorescens* in this study was between 0.11 ± 0.028 and 0.306 ± 0.007.

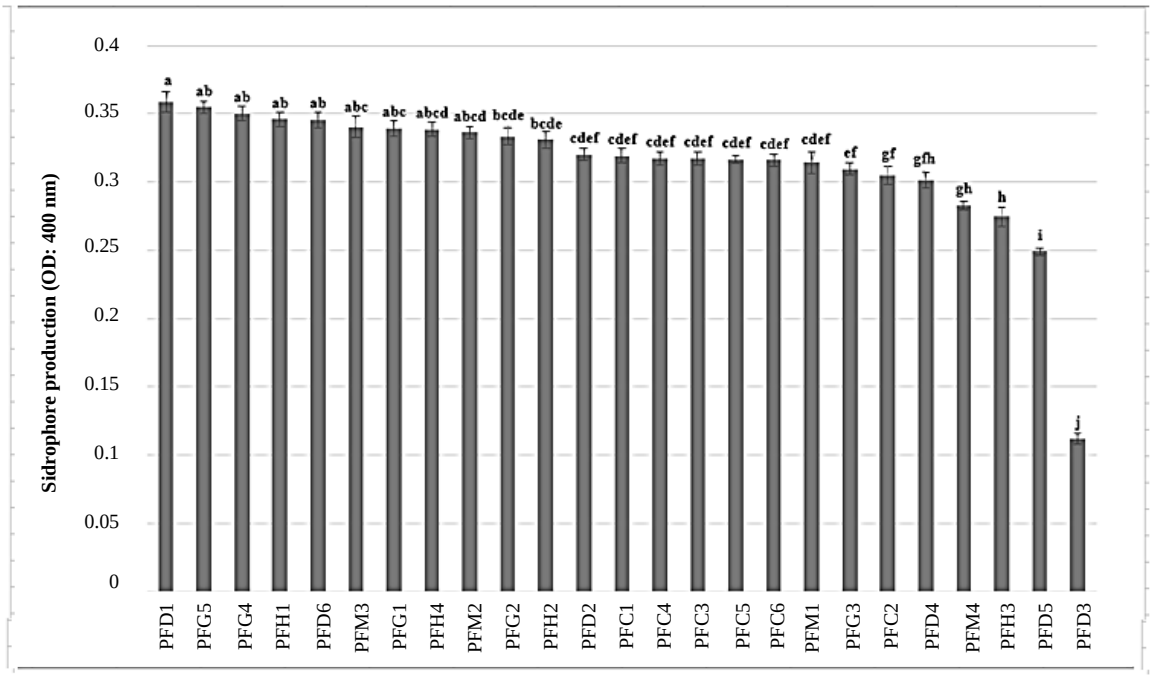


**Figure 2.** A quantitative study on the producing siderophore by P. fluorescens isolates using spectrophotometer at 400 nm wavelength

***The effect of different chalk Iron-siderophore on the rate of iron absorption by corn***

The highest iron absorption in PFG5 treatment (283 ± 2/3 ppm) and the lowest amount of iron absorption in the control treatment (107.6 ± 1.7 gpm) were observed in different treatments (Figure 3). Pewaredine iron chelate-siidophore chelate showed higher iron absorption efficiency in Arabidopsis than EDTA iron chelate [23]. In this study, iron absorption in maize plants under the treatment of PFG5 isolates was more than EDTA iron chelate and FeCl3 chelate. Carroll et al. reported five microbial hydropharmate hydroxamate, ferricom, coprogen, ferrocyanemic and tetracyclic iron oxide [24].

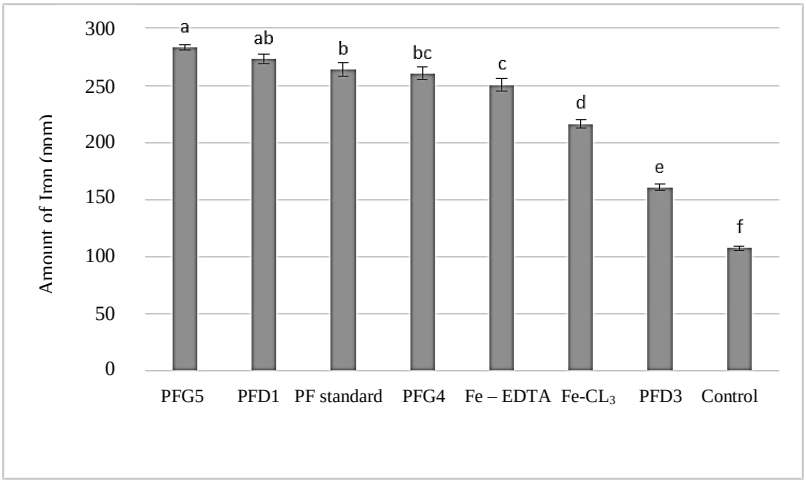


**Figure 3.** Iron absorption rate of corn plant under the influence of different treatments of iron-siderophore chelate.

According to the results of the comparison of mean fresh root weight in different treatments, PFG5 treatment with the highest amount of fresh weight was 24.93 ± 1.9 grams. The control group (14.53 ± 0.89 gr) had the lowest root fresh weight among the treatments (Figure 4).

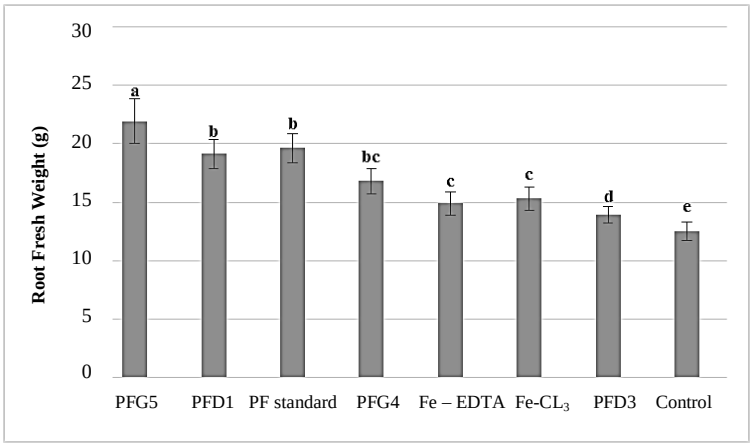


**Figure 4.** Mean comparison of root fresh weight under different treatments of bacterial isolates, Fe-EDTA and Fe-$Cl_3$

The mean of root dry weight in the treatments, PFG5 treatment, and control treatment with 3.93 ± 0.6 gr and 2.9 ± 0.2 gr, respectively, were the lowest and the highest (Figure 5).

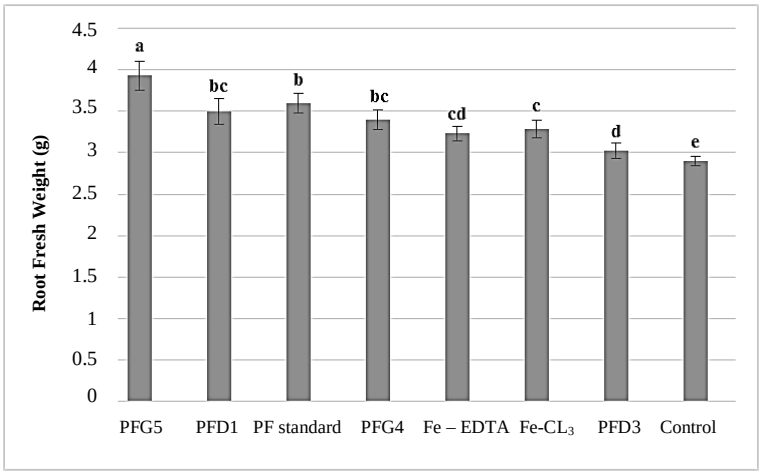


**Figure 5.** Mean comparison of root dry weight under different treatments of bacterial isolates, Fe-EDTA and Fe-$Cl_3$.

Potato seedlings with strains producing *P. siderophore* were studied. Flourescense (WCS374) and *P. putida* (WCS358) have contributed to the development of the rooted system in comparison with the mutations in the production of siderophore. The highest root dry weight was related to P16 isolate *P. flourescense*. In this study, the fresh and dry weight of root was higher in Seed or f-Bacterium treatments than other treatments. The results of comparison of mean fresh weight of aerial parts showed that PFG5 and PFD1 treatment had the highest amount of fresh weight in the treatments with 32.33 ± 1.8 and 31.76 ± 0.7 mg, respectively gave. Moreover, control treatment with the mean value of 18.88 ± 0.77 showed the lowest amount in the treatments (Figure 6).

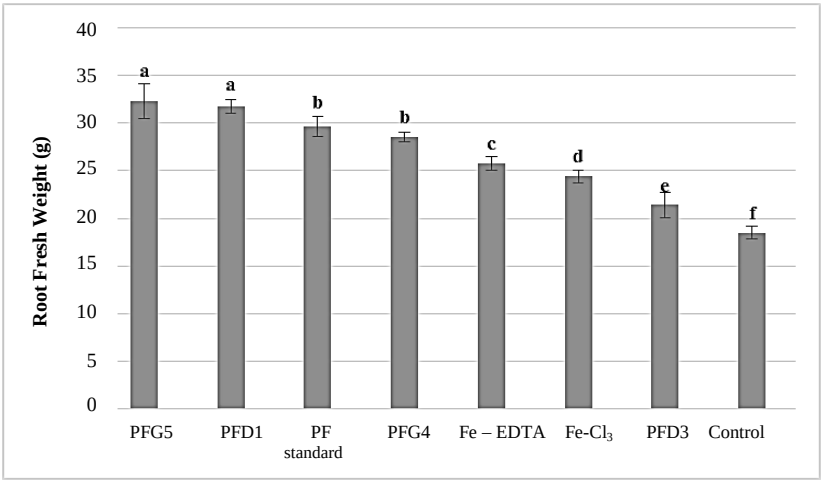


**Figure 6.** Mean comparison of aerial parts fresh weight under different treatments of bacterial isolates, Fe-EDTA and Fe-$Cl_3$.

PFG5 treatment with 1.5 ± 0.15 gr compared to other treatments had the highest dry weight of the aerial parts. The control treatment with the mean of 2.86 ± 0.88 grams had the lowest dry weight among the treatments (Figure 7). In another study, tomato seeded treatments with C138 siderophore strain Chryseobacterium C138, C138 siderophore with bacteria caused a significant increase in stem diameter, dry weight and height of tomato aerial parts compared to the total Hoagland nutrition solution [25]. Also, the highest dry weight of aerial parts was related to PF standard isolate treatment of *P. flourescense* [16].

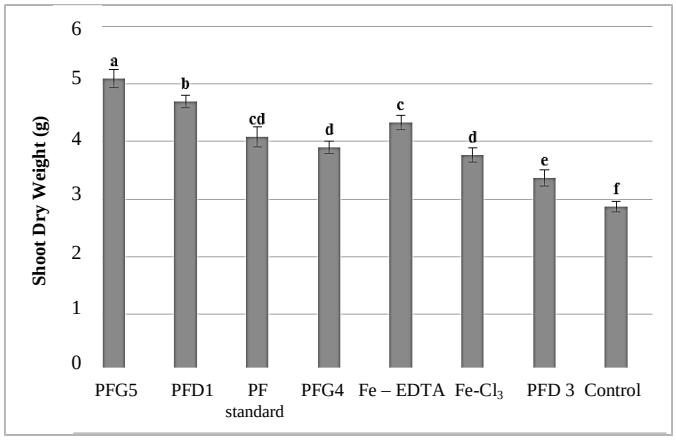


**Figure 7**. Mean comparison of aerial parts dry weight under different treatments of bacterial isolates, Fe-EDTA and Fe-$Cl_3$.

So Increasing fresh and dry weight of aerial parts and roots in bacterial isolates of *P. fluorescens*, in addition to the production of siderophore, can be due to increased production and secretion of various hormones [24].

## CONCLUSIONS

Regarding the heterogeneity of *P. fluorescens*, it is necessary to use molecular methods to detect different biotypes of this species. Moreover, the higher absorption of corn iron in microbial seed treatment shows the high efficiency of different microbial susceptibility in interaction with plants. The use of plant growth promoting bacteria is an appropriate option for regeneration of agricultural land and increasing yield of products, especially in iron-deficient areas.

## CONFLICT OF INTRESTS

Non-declared.

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