

# Role of Fluorescent Pseudomonads Siderophore to Increase Bean Growth Factors

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## Abstract

Since the Iranian soils are almost limy, application of Fluorescent pseudomonas have a major potential to provide the microelements needed for crop plants due to their siderophore production ability. In this work three indigenous high siderophore producing strains, a reference strain 7NSK2 and its pyoverdine mutant, MPFM1, were employed. To explore their efficiency, siderophore production was investigated by CAS-agar method. Effect of bacteria inoculation on the shoot and root dry weight increase as well as chlorophyll content of the crop were assayed under greenhouse condition. Furthermore, synergistic effects of bacteria strains and iron chelates, Fe-EDDHA and Fe-EDTA, and zinc sulphate on promotion of common bean plants were estimated. Strain UTPF76 with 20.4 mm halo in CAS-agar medium had the highest siderophore production capability. The results of the greenhouse experiments revealed that three of strains, 7NSK2, UTPF5 and UTPF76 increased the plant growth factors significantly. Addition of iron chelates and zinc sulphate improved bacterial efficacy. Strains MPFM1 and UTPF61 not only did not have positive effect on the plant growth but also in some cases decreased the growth factors. In colonization survey UTPF76 and UTPF5 had the greatest colonization activity. In conclusion, our results showed that indigenous stains have a high potential in biotechnological applications and their industrial application is recommended.

**Keywords:** Common bean, Fluorescent pseudomonad's, Iron chelates, Siderophore

## 1. Introduction

Iron, is a critical micronutrients required for metabolism by many plant, due to its diverse role in chlorophyll biosynthesis, redox reactions and some physiological activities (Briat *et al.*, 1995). On the other hand iron in crops and vegetables plays a vital role in providing the required iron for human and animal. The availability of iron to plants in neutral and particularly in alkaline soils are severely limited (Banaei *et al.*, 2005). The amount of available iron at pH 7 is about  $10^{-17}$  mol/L that it is extremely lower than the amount required for soil microorganisms ( $10^{-6}$  mol/L)(7). Due to iron starvation, the quantity and quality of crops are dropping and this would lead to a severe damage to the respective economy. Currently, iron mineral salts are used as shoot spraying, and chelating-iron compounds like Fe-EDTA and Fe-EDDHA are applied as soil drenching (Alvarez-Fernandez *et al.*, 2004). Using these methods to compensating the lack of iron depends on an environmental and host plant condition and it should repeated annually. Furthermore, microbial siderophores are one of the major sources of iron in plants (Crowley, 2006). Siderophores are low-weight compounds

(approximately 600 to 1500 daltons) which produced under iron-limitation. They change iron to soluble and absorbable form for microorganisms and plants (Budzikiewicz, 1997). Application of high siderophore producer microorganisms in roots and fortifying them to soil has significantly amended the iron insufficiency in plants. Proving the effects of soil microflora in plant nutrition had been distinguished when the cultivated plants in sterile soil had a lower rate of chlorophyll and iron in comparison to the cultivated plants, potted in presence of indigenous microflora (Masalha *et al.*, 2000). Chen *et al.* (1998) demonstrated that adding composts to nutrition liquid, due to have varieties of microflora cause iron amelioration in different kinds of plants. Cereals are permitted to produce phytosiderophore which could chelate the iron directly or receive the iron from the microbial siderophores. The rate of phytosiderophore consumption is more in some plants like barely which are high phytosiderophore producers and the sign of iron shortage decreased in them totally (Marschner *et al.*, 1986). Kloepper *et al.* (1980) were the first to document the importance of Fluorescent pseudomonads siderophore for increasing plant growth. Fe-pyoverdine produced by Fluorescent pseudomonads cause significant growth, chlorophyll and iron increasement in comparison with Fe-EDTA in *Arabidopsis thaliana* (Vansuyt *et al.*, 2007). In a similar experiment, increase in Fe-pyoverdine stopped iron-chlorosis in peanut (Cornelis and Matthijs, 2006).

## 2. Material and Methods

### 2.1 Siderophore assay

In this test, siderophore production was evaluated qualitatively using Chrome Azurol (CAS) agar as modified by Alexander and Zuberer (1991).

For inoculating the plates, five microliter ( $\mu$ l) of bacterial suspension contained 24 hours bacterial culture was added to this medium. 7NSK<sub>2</sub> was used as positive control and MPFM1 (pyoverdine mutant) was used as negative control and the plates were incubated in 27 °C. Separated iron from CAS indicator due to siderophore production cause orange halo around bacterial colonies. The halo diameter was measured for 3 days.

### 2.2 Greenhouse experiment

Red bean seeds, NAZ cultivar, were disinfected with Sodium hypochloride solution (2.5%) for 3 minutes and then washed in distilled water 3 times. Seeds were treated with antagonistic strains following the method of Weller *et al.* (1983). For seed coating, a loop full of 48 hours culture of each bacterial isolate was taken in sterilized 100 ml erlenmeyer flasks containing liquid King's B Medium. Strains were grown at 27°C for 48 h on a rotary shaker. Cells were then collected by centrifugation at 10000 g for 10 min and thoroughly washed by physiological serum (NaCl, 0.85%). After that cells separated from this solution for the second time by centrifugation. The suspension ( $1 \times 10^9$ ) in carboxymethyl cellulose (CMC) was prepared by following formula:

$$D_1V_1 + D_2V_2 = D_f(V_1 + V_2)$$

Where  $D_1$ : primary suspension density,  $V_1$ : primary suspension volume,

$D_2$ : bacteria density in added suspension, (CMC doesn't contain bacteria so  $D_2 = 0$ ),  $V_2$ : added suspension volume,  $D_f$ : final suspension density.

Bean seeds were floating in bacterial suspension on a rotary shaker at 27°C for 30 minutes. CMC 0.1% was added as adhesive factor to non-bacterized control seeds. The inoculants coated seeds were placed in a cool and dry place under shade for drying.

### 2.3 Growth promotion by bacterial strains in presence of Iron and Zinc sources

Experimental design was a 4×4 factorial arranged in greenhouse condition. The factors were four bacterial applications (UTPF5, MPFM<sub>1</sub>, 7NSK<sub>2</sub> and untreated control) and four soil amendment (25 ml of 1 mM Fe-EDDHA and FeEDTA solution: 100 $\mu$ M ZnSO<sub>4</sub>: untreated control). 300 g of bean farm soil with the sign of iron deficiency, sterilized and it was added to 1/3 volume from perlite to pots. 3 bacterized bean seeds, NAZ cultivar, were planted in each pot. The pots were kept in greenhouse condition and were irrigated twice weekly to preserve moisture. Plants were grown for 35 days and then Plant growth factors were evaluated after harvesting by taking the total weight of plant. At this point, shoot and root weights were determined for plant growth promotion. The amount of chlorophyll, which is suitable indicator for iron absorption, was measured in 3 developed upper leaves by Chlorophyll meter SPAD-502 and the average of them was used in calculations.

### 2.4 Root colonization assay by strains

An experiment was conducted to determine the colonization pattern of the strains in rhizosphere. In brief, rhizosphere populations of the bacteria were isolated, by placing 1 gram of the roots with adhering soil in glass tubes containing 9 ml physiologic serum and they were shaken for 10 minutes. 100  $\mu$ l of serial dilutions of the

suspension were plated on to King's Medium B supplemented with 50 µg Kanamycin (All of the bacteria contained resistant gene against this antibiotic). All plates were incubated in a growth chamber (28°C) and After that bacterial colonies were counted after 48 hours and bacterial populations were expressed as colony-forming units (CFU)g<sup>-1</sup> of fresh root tissue.

### 3. Result and discussion

#### 3.1 Siderophore production in strains

Fe-CAS complex have blue color but sequestering of iron from this complex by bacterial siderophores turn its color to the orange that appears as halo surrounding bacterial colony. Each agent could break this connection by taking the iron cause orange color and the halo diameter indicated the amount of that agent. This medium has the same reaction against each material which has siderophore virtue. So halo diameter shows all of bacterial siderophores. Among the 3 strains tested for production of siderophore, UTPF76 recorded the highest siderophore activity (halo diameter= 20.4 mm) followed by 7NSK<sub>2</sub> (12.92), UTPF5 (9.26), UTPF61 (8.48) and MPFM<sub>1</sub> (6.28). MPFM<sub>1</sub>, which was pyoverdine mutant, produced siderophore in this method but it had the lower activity comparing to 7NSK<sub>2</sub> (Table 1). According to Buysens *et al* (1996) reports, this isolate produced at least two other siderophore, pyocheline and salicylic acid, in addition to pyoverdine. According to these results and also Sadaghiani *et al.* (2006), many Iranian isolates have high ability to produce siderophore in comparison with foreign isolates. This is the consequences of compatibility between Iranian isolates with poor and alkaline soils.

#### 3.2 Greenhouse experiments

Commercial chelators effects were compared with Fluorescent pseudomonads in this experiment. Zn element was selected due its role in Fluorescent pseudomonads stability and also in siderophore production incensement (Hofte *et al.*, 1993). The results indicated the strains had significant effect on growth factors (Fig 1&2). The greatest growth development was related to UTPF76 followed by UTPF5. The effect of these strains was more than commercial Fe chelators. UTPF61 and MPFM<sub>1</sub> not only didn't increase growth factors but also reduced them. Most of treatments and replications which contained these two strains showed some deformation symptoms that the reason of it couldn't be interpreted. Growth factors development in 7NSK<sub>2</sub> was significantly more than its siderophore mutant. This difference was noticeable for siderophore value (table 1). Application of Fe-chelators indicated significant difference in growth incensement. Fe-EDDHA chelator was more effective than Fe-EDTA chelator in increasing all growth factors. This chelator is the most effective and stable chelator in alkaline soils because the other Fe-EDTA in high lime soils are unstable (Lindsay, 1979). Fe-EDTA chelators are more effective than the other chelatores in acidic soils while soil lime and pH, that both of them can be seen in Iranian soils, increase, the use of these chelates is limited. Hence Zn-EDTA and Cu-EDTA chelators establish and Fe which is separated from chelator sediments as hydroxides (Lindsay, 1979). In this investigation, Fe-EDTA chelator didn't indicate significant effect on plant growth and chlorophyll content, too (Table 1&2). On the other hand, plant root development showed different response against chelate incensement and significant growth development was observed in most cases. Zn operates as auxin producer co-factor. Therefore zn addition to soil could enhance root development stimulation and plant growth (Galeston, 1994). Zn addition increases plant growth significantly while Zn addition to UTPF76 follows growth reduction. Zn is an essential co-factor for changing tryptophan amino acid to auxin (growth stimulator hormone). Galston (1994) reported extra auxin not only doesn't increase plant growth but also decrease it especially. Greenhouse experiments demonstrated that growth reduction in treatments was more obvious in roots. It is theorized that high auxin producers have a negative impression on plant growth. So it is better not to use Zn with these strains. Zn addition has a negative effect on strains activities and it could be seen among all of strains. But Zn increased root growth in untreated plants.

#### 3.3 Root colonization

Two necessary criteria should be met by successful growth stimulator bacteria. One of them is production of plant growth stimulator metabolites like siderophores, hormones and the other one is colonization of suitable places of root in appropriate time. In this experiment 2 native strains, UTPF76 and UTPF5 with 1×10<sup>9</sup> and 3×10<sup>9</sup> (CFU/g) respectively, had the most root colonization. UTPF61, 7NSK<sub>2</sub> and MPFM<sub>1</sub> with 8×10<sup>8</sup>, 9×10<sup>7</sup> and 6×10<sup>7</sup> (CFU/g) respectively followed them. It is understood from results that native strains have greater colonization; on the other hand, they could adapt themselves with Iranian soils rhizosphere condition. In this investigation, 7NSK<sub>2</sub> wild type, which has high ability to enhance growth factors (Hofte *et al.*, 1991; Hofte *et al.*, 1993), behave weaker than native strains. Surely this reduction is related to lack of ability of successful root colonization. At the end we can conclude that Iranian native Fluorescent pseudomonads have high ability to promote plant growth and utilization of them in stable agricultural is suggested as replacing chemical compound.

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Table 1. Siderophore production of different strains of fluorescent pseudomonad's in CAS Agar medium

Strains	Halo diameter in CAS Agar medium (mm)			
	First day	Second day	Third day	Means of three days
UTPF5	6.48 ± 0.62	8.18± 0.83	10.15± 1.2	8.27 ± 0.43
UTPF61	5.53 ± 0.75	8.23 ± 0.69	8.42± 0.64	7.48 ±0.91
UTPF76	14.48 ± 0.81	17.78 ± 0.92	20.4 ± 1.12	17.58 ±1.03
7NSK2	7.48± 0.35	10.98 ±1.05	12.92± 0.78	10.46 ± 0.52
MPFM1	5.40 ± 0.62	5.95 ± 0.84	6.28± 0.92	5.91± 0.84

Values in the column followed by ± standard error from mean

Table 2. Influence of integrated application of PGPR strains and ion sources on leaf chlorophyll content of common bean.

Strains	Leaf chlorophyll content			
	Fe-EDDHA	Fe-EDTA	Zinc sulphate	Control
UTPF5	43.66 a	39.96 abc	40.227 abc	42.66 a
UTPF61	29.23 de	41.52 ab	38.69 abc	37.34 abcd
UTPF76	42.16 ab	40.43 abc	37.5 abcd	39.33 abc
7NSK2	46.33 a	41.13 ab	41.73 ab	41.8 ab
MPFM1	37.4 abcd	37.46 abcd	33.5 bcde	33.3 bcde
Without bacteria	38.49 abc	31.66 cde	27.97 e	20.86 f

<sup>1</sup>Amendment contain 25 ml of 1mM Fe-EDDHA, 1mM Fe-EDTA and 100µM ZnSO<sub>4</sub> solutions per pot. <sup>2</sup>distilled water used as control. Values in the column followed by different letters indicate significant differences among treatments according to LSD tests (P < 0.05).

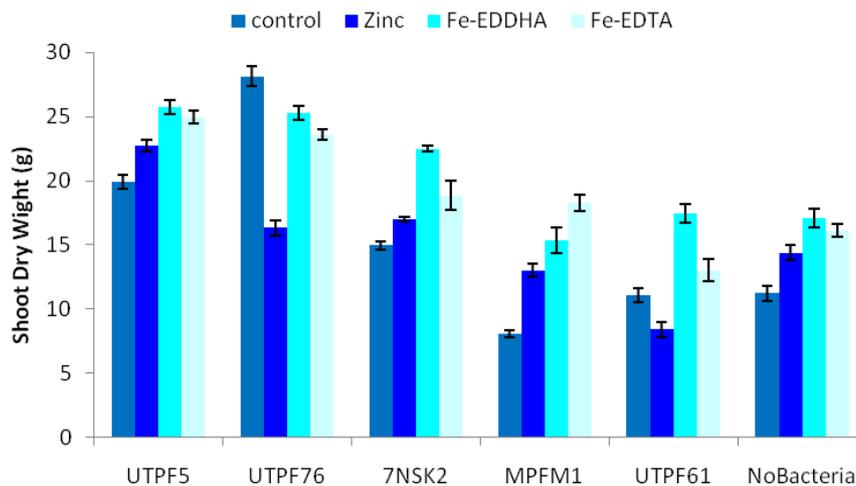


Figure 1. Effect of PGPRs strains with or without application of iron chelates ( 1mM Fe-EDDHA and FE-EDTA) and 100 µM zinc sulphate on dry Wight of common bean shoot. Error bars indicate standard error of the mean

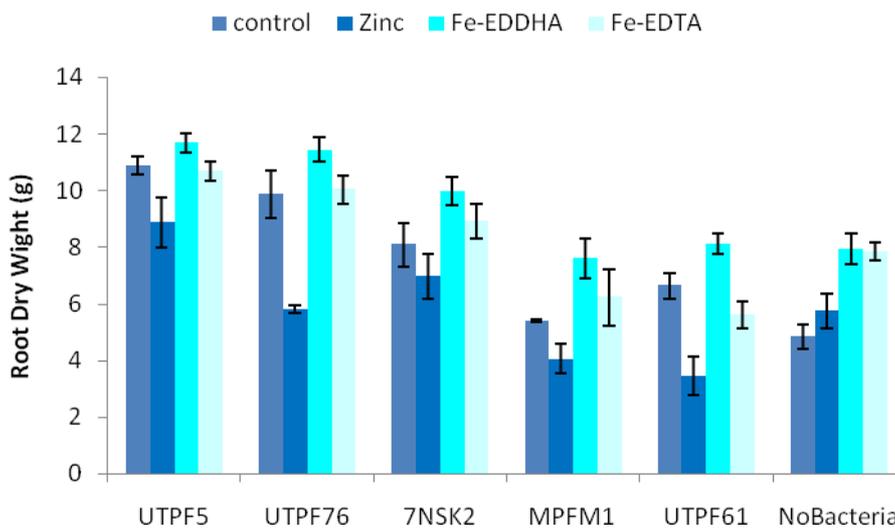


Figure 2. Effect of PGPRs strains with or without application of iron chelates and zinc sulphate on wet Wight of common bean roots. Error bars indicate standard error of the mean