J. Plant Production, Mansoura Univ., Vol. 9 (9): 717 - 721, 2018

Siderophore Production by Rhizosphere Inhabiting Bacteria and Fungi

Osman, Y. [1]; A. M. Mowafy[1]; A. S. Gebreil[1]*and S. M. Hamed[1]
Botany Department, Faculty of Science, Mansoura University, Mansoura 35516, Egypt
***Email: biobotany2552007@gmail.com**

ABSTRACT

The production of siderophores is greater in rhizosphere inhabiting fungi and bacteria. The present work was designed to isolate the rhizosphere inhabiting bacteria and fungi from the rhizosphere of *Phaseolus Vulgaris, Pisum sativum, Vicia faba* and *Alfa alfa*. The obtained isolates has been screened for siderophores production using Chrome Azurol Sulfonate assay (CAS). The highest siderophore producer bacterium and fungus has been molecularly identified as *Bacillus* MG214652 and *Aspergillus niger* MH844535 respectively. They were able to produce 70.4% and 87% units of siderophore respectively. *Bacillus* MG214652 siderophores was characterized as catecholate type with maximum absorbance at 495 nm. *Aspergillus niger* MH844535 siderophore was characterized as hydroxymate type with maximum absorbance at 450 nm.

Keywords:*Aspergillus niger; Bacillus amyloliquefaciens*; catecolate siderophores; hydroxymate siderophores.

INTRODUCTION

Rhizosphere is the part of the soil surrounding the plant roots and this region was characterized by root exudations in intention to stimulate beneficial microbes growth that would be highly important for plant physiology in addition to their ability to inhibit pathogen growth and infection to plant (Leong, 1986; Höfte *et al.*, 1994). One of the important elements is iron, which is nearly essential for all forms of life (Clark, 2004), because iron have a diverse role in the redox reactions, the biosynthesis of chlorophyll, the detoxification of oxygen radicals and many other physiological activities(Alexander and Zuberer, 1991; Khamna *et al.*, 2009). Under aerobic conditions, Ferrous ions forms very insoluble mineral precipitates, which severely restrict the bioavailability of iron (Neilands, 1995).

Siderophores are iron scavenger-chelating agents, secreted by many organisms under iron stress conditions for iron chelation (Ali and Vidhale, 2013), it has low molecular weight "600-1500 Daltons" (Clark, 2004) with extraordinarily high complex formation constants for ferric iron (Matzanke, 1994). The iron-siderophore complex is recognized by a corresponding outer membrane receptor protein, which successively transports the complex into the cell (Lankford and Byers, 1973).

Based on the oxygen ligands for Fe^{+3} co-ordination, siderophores are classified in to catecholate, hydroxymate and carboxylate (Alexander and Zuberer, 1991; Khamna *et al.*, 2009). Catecholate type siderophore is mostly produced by the bacteria (Dave *et al.*, 2006), Each catecholate group chelate iron and form a hexadentate octahedral complex. On the other hand, most of the Fungal siderophores are belonging to hydroxymate type siderophore (Khamna *et al.*, 2009). Each hydroxamate group forms a bidentate ligand with iron (Winkelmann, 2007).

In this study, the ability of some legume rhizosphere-inhabiting bacteria and fungi to produce siderophores was explored. The siderophore producers were identified and the siderophores type were qualified and quantified.

MATERIALS AND METHODS

- **Samples collection and isolation of rhizosphere inhabited Bacteria and fungi**

The isolates for this study has been obtained from the rhizosphere region of Phaseolus vulgaris, Pisum sativum, Vicia faba and Alfa alfa. Plants and their associated root material were placed individually in a plastic bags and brought to laboratory. The soil dilution plate method was used as isolation technique (Kumar et al., 2015). Both solid Luria-Bertani medium (LB) and solid Potato dextrose medium (PDA) were used for bacteria and fungi isolation.

- **Screening for siderophoresproduction**

Because siderophores produced only under iron deficient conditions (Sharma and Johri, 2003), All glassware was rinsed with 6 N HCl for 24 h then washed with de-ionized water to get rid of iron contamination (Hu and Xu, 2011). The Chrome Azurol Sulfonate assay (CAS) was used for screening for the bacterial siderophores production (Schwyn and Neilands, 1987), while the modified method Chrome Azurol Sulfonate half plate was used for screening for the fungal siderophores production (Milagres *et al.*, 1999). In both methods, the CAS agar medium containing the blue dye complex of CAS, Fe^{+3} and HDTMA, the colour change from blue into orange or dark purplish-red color indicates the Fe-binding compound production. In this study, CAS Indicator Solution and the basal agar medium was prepared as (Nailwal *et al.*, 2014). During the preparation of CAS dye PIPES buffer was replaced with (0.1 M) HEPES buffer as a modification described in 2006 (Mirabello, 2006).

- **Siderophores production estimation**

The bacterial isolates were grown for 3 days on Iron-restricted minimal salt medium (MM9) at 37ºC (Murugappan *et al.*, 2011), while the isolated fungi were grown for 14 days on PDA medium at 28º C (Srivastava *et al.*, 2013). The culture supernatant of both types of microbiota was further subjected to Chrome Azurol Sulfonate liquid assay, for quantitative assessment of siderophore production. After CAS solution preparation, an equal volume was added to the culture supernatant, the positive result is the change of the blue color to orange or dark purplish-red and spectrum was observed at 630 nm. The results were expressed as a Siderophores unit which was calculated with following formula:

$$\%\text{Siderophore unit} = [(Ar - As)] / Ar \times 100$$

Where Ar is the reference absorbance and As is sample absorbance, both of them at 630 nm(Payne, 1994).

- **Bacterial molecular identification**

Based on the siderophore unit result, the most siderophores producer bacterium (Ph3) was molecularly

identified by 16S rRNA analysis. Firstly, the bacterial genomic DNA was isolated according to the manufacturer's instructions using the PrepMan™ Ultra Sample Preparation kit (PN 4322547). The isolated DNA was then used as a template for the PCR containing the universal bacterial primers the forward primer 5'-AGTTTGATCATGGTCAG-3' and reverse primer 5' GGTTACCTTGTTACGACT 3' (Tork *et al.*, 2010). The thermal cycler was programmed as follow: 95 °C for 10 min, 95 °C for 30 sec, 60 °C for 30 sec, 72 °C for 45 sec and 72 °C for 10 min (30 cycles). The PCR product was purified by Montage PCR Filter Unit (Millipore PN UFC7 PCR50) and confirmed to present in sample by running a 2% agarose gel. The sequencing reaction was performed in the 9700 thermal cycler at a total volume of 20 µL (7 µL of the purified PCR product and 13 µL of the Sequencing Module) by adjusting the thermal cycling conditions to 96 °C for 10 sec, 50 °C for 5 sec and 60 °C for 4 sec (25 cycles). Then the excess dye terminators and primers were removed from the cycle sequencing reaction using DyeEx™ 2.0 Spin Kit (Qiagen PN 63204). The generated sequence were analyzed by Finch TV (version 1.4.0) software and the phylogenetic tree was generated via MEGA software version 6software using the closest published type strains sequences. Then the sequence of the isolatewere submitted to the GenBank on NCBI.

- **Fungi molecular identification**

All the isolated fungi ASG1, ASG2, ASG3, ASG4 and ASG5 were molecularly identified by 18S rRNA analysis. Firstly, the fungal DNA was extracted by employing the Fast DNA® Spin Kit according to the supplier's instructions. The ITS1 region from the DNA samples were used as templates for PCR containing the following primers 18 FITS1 (5'-CTTGGTC ATTTAGAGGAAGTAA-3') and the reverse primer 18RITS4 (5'- TCCTCCGCTTATTGATATGC-3'). The PCR reactions was performed using a thermo-cycler at the following temperature programs: 94 °C for 5 min, 94 °C for 40 s, 55 °C for 45 s, 72 °C for 1.5 min, 72 °C for 7 min (35 cycles). The sizes of the PCR products was determined by electrophoresis on 1.5 % agarose gel. Then, the desired products were excised and purified by the Qiagen II Agarose Gel Extraction Kit according to the supplier's instructions. The sequencing reactions were performed using a thermo-cycler (Master cycler, Eppendorf) at a total volume of 10 µl by using the following temperature program: 96 °C for 1 min, 96 °C for 30 s, 60 °C for 10 s, 60 °C for 4 min, 72 °C for 5 min (25 cycles). The purified sequencing reaction products were dried under vacuum and then analyzed using Applied Biosystems (ABI PRISM Big Dye Terminator v1.1). The obtained sequences were annotated using the Sequencher™ 4.8 Software. DNA similarity search was performed using the BlastN program and the databases of European Molecular Biology Laboratory (EMBL) and GenBank from the National Center for Biotechnology Information website (NCBI). The generated sequence were analyzed by Finch TV (version 1.4.0) software and the phylogenetic tree was generated via MEGA software version 6software using the closest published type strains sequences. Then the sequence of the isolate were submitted to the GenBank on NCBI.

- **Detection of siderophores chemical nature**

The most siderophores producer isolates bacterium and fungus were tested for their siderophores function group. Catecolate and hydroxymate type siderophores were differentiated by $FeCl_3$test (Neilands, 1981). In this test, the formation of wine colored ferric catecolate with λ_{max} at 495nm indicates the presence of catecolate siderophore. While the formation of orange colored ferric hydroxamate, showing λ_{max} ranging from 420 - 450nm indicates hydroxymate siderophore (Neilands, 1981).Carboxylate type siderophore was detected by the spectrophotometric test (Shenker *et al.*, 1992). In this test spectra from 190-280 was monitored for the siderophores-Cu complex (Shenker *et al.*, 1992).

RESULTS AND DISCUSSION

In the present research from the rhizosphere region of *Pisum sativum*, *Phaseolus vulgari*, *Vicia faba* and *Alfa alfa*, a totally 25 morphological different bacterial colonies and 5 fungi mycelium were recovered, purified and preserved for further experiments. According to CAS assay, 11 bacteria strains and 3 fungi developed an orange or dark purplish-red color. Figure 1-I shows the change in the blue colour in response to iron chelation by bacteria and figure 1-II shows that for the fungal isolates.

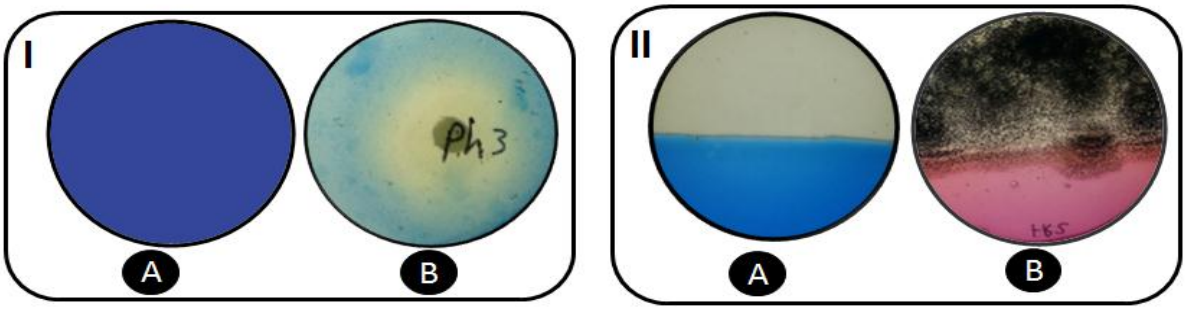

Figure 1. The production of siderophores by bacterial and fungal isolates, I) CAS assay for the bacterial isolate. A; blue CAS before inoculation. B, the orange halo around the well containing bacterial siderophore. II) Half plate CAS assay for the fungi isolate. A; blue CAS before inoculation. B; shows the growth of the fungal isolate and the change of the blue colour to the purplish-red colour.

Several rhizosphere bacteria and fungi synthesized and secreted siderophores in response to iron depletion condition (Crowley, 2006; Khamna *et al.*, 2009). It has been found that, siderophores production is greater in bacteria and fungi isolated from soil near plant roots than in heavy metal soil (Hussein and Joo, 2014). Thus, because the siderophores of the rhizosphere microorganism influence the ability of plants to acquire iron from soil, and inhibiting the pathogen from root colonization (Rajkumar *et al.*, 2010). Table one shows the siderophores unit (%) produced by the obtained rhizosphere bacteria and fungi. The bacterial isolate ph3 that has been obtained from *Phaseolus vulgaris* rhizosphere and the fungal isolate ASG4 that has been isolated from Vicia faba rhizosphere produced 70.4 and 87 respectively.

Table 1. Siderophore unit for the positive CAS assay isolates.

	Plant	Isolate code	Siderophores unite %
Bacteria	*Vicia faba*	V1	19
		V2	55
		V3	62.5
		V8	18.1
	Phaseolus vulgaris	Ph3	70.4
		Ph5	55
	Pisum sativum	P4	51.1
		P5	38.6
	Alfa alfa	A1	48.8
		A2	65.9
		A3	63.6
Fungi	*Vicia faba*	ASG4	87
	Pisum sativum	ASG1	35
	Alfa alfa	ASG3	79.1

The molecular identification of the bacterial isolate Ph3 showed that the strain is a member of the genus Bacillus, and the tree topology (Fig. 2) showed high level of sequence identity (99 %) to *Bacillus amyloliquefaciens*, it has been given the name *Bacillus MAP3 MG214652*. *Bacillus amyloliquefaciens* is recognized as a rhizosphere colonizing bacteria that is used as a plant growth promoting bacteria (Chen *et al.*, 2007) as well as a bio-control against phyopathogens (Arguelles-Arias *et al.*, 2009).

The selected fungi ASG4 was molecularly identified to be a member of genus Aspergillus as the tree topology (Fig. 3) showed high level of sequence identity to members of the Genus *Aspergillus niger* which is accounted as one of the rhizosphere fungus and crop growth promotion (Yadav *et al.*, 2011). Its accession number on the data base is MH844535.

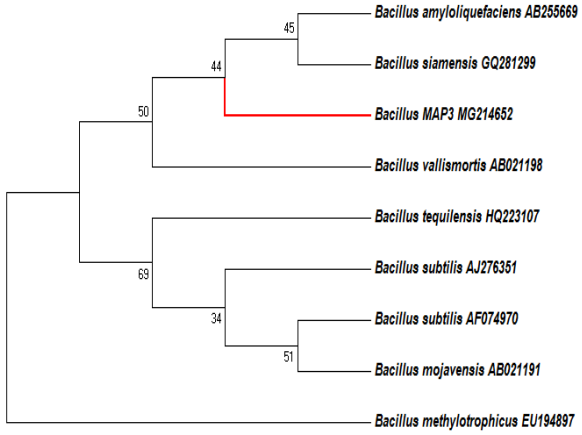

Figure 2. Phylogenetic analysis of the 16S rRNA gene sequences of *Bacillus MAP3 MG214652*, and the related published sequences.

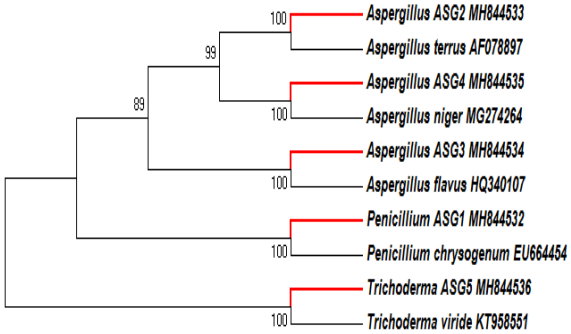

Figure 3. Phylogenetic analysis of the 18S rRNA gene sequences of the isolated fungi (ASG1, ASG2, ASG3, ASG4, ASG5) and the related published sequences.

In the $FeCl_3$ test, the spectral analyses of *Bacillus MAP3* ferrated siderophores, show a maximum absorbance at 495,which confirm the catecolate nature of the siderophores (Fig. 4-I), this result was consonance with a previous study (Clark *et al.*, 2014), that indicated the predominate of catecholate type siderophores in the Bacillus genus, specifically Bacillibactin catecolate type siderophores. However, in the study of (Murugappan *et al.*, 2011) they reported that its impossible to characterize the siderophores based on the bacteria species, because siderophores of varying nature have been produced by the same genus. In the same test for the siderophore of *Aspergillus niger MH603592*, its show maximum absorbance at 450 which is confirmed the hydroxymate nature of siderophores (Fig. 4-II) and is in accordance with the report of (Patel *et al.*, 2017), Moreover, (Neilands, 1981) have observed that the iron hydroxamate complex is more stable and therefore more significant in the rhizosphere. While no evidence for carboxylate in the spectrophotometric test for the both isolates.

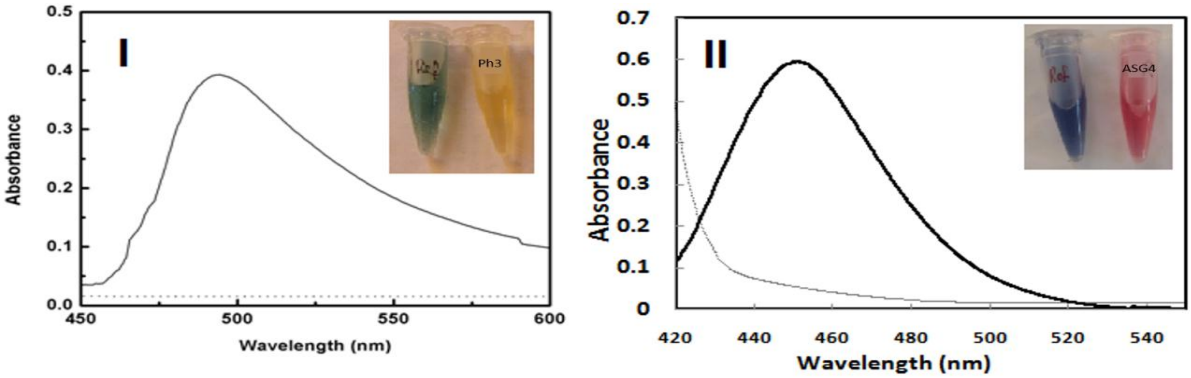

Figure 4. Types of siderophores produced by the obtained isolate. I) *Bacillus MAP3* MG214652 ferreted siderophore spectroscopic analysis that shows maximum absorbance at 495 nm. The Iron-catecolate type siderophore complex (bold), and FeCl3 alone (light). II) *Aspergillus niger MH844535* ferreted siderophore spectroscopic analysis that shows the maximum absorbance at 450 nm. iron-hydroxymate type siderophore complex (bold), and $FeCl_3$ alone (light).

REFERENCES

Alexander, D. and D. Zuberer (1991). Use of chrome azurol S reagents to evaluate siderophore production by rhizosphere bacteria. Biology and Fertility of soils 12(1): 39-45.

Ali, S. S. and N. Vidhale (2013). Bacterial siderophore and their application: a review. Int J Curr Microbiol Appl Sci 2(12): 303-312.

Arguelles-Arias, A., M. Ongena, B. Halimi, Y. Lara, A. Brans, B. Joris and P. Fickers (2009). Bacillus amyloliquefaciens GA1 as a source of potent antibiotics and other secondary metabolites for biocontrol of plant pathogens. Microbial cell factories 8(1): 63.

Chen, X. H., A. Koumoutsi, R. Scholz, A. Eisenreich, K. Schneider, I. Heinemeyer, B. Morgenstern, B. Voss, W. R. Hess and O. Reva (2007). Comparative analysis of the complete genome sequence of the plant growth–promoting bacterium Bacillus amyloliquefaciens FZB42. Nature biotechnology 25(9): 1007.

Clark, B. L. (2004). Characterization of a Catechol-Type Siderophore and the Detection of a Possible Outer Membrane Receptor Protein from Rhizobium leguminosarum strain IARI 312.

Clark, D., C. Youngblood, M. Taplin, E. Brown, B. Williams, C. Phillips and B. Garner (2014). Impact of iron availability on Bacillus amyloliquefaciens growth. Advances in Microbiology 4(13): 962.

Crowley, D. E. (2006). Microbial siderophores in the plant rhizosphere. Iron nutrition in plants and rhizospheric microorganisms. Springer, Netherlands: 169-198.

Dave, B., K. Anshuman and P. Hajela (2006). Siderophores of halophilic archaea and their chemical characterization.

Höfte, M., Q. Dong, S. Kourambas, V. Krishnapillai, D. Sherratt and M. Mergeay (1994). The sss gene product, which affects pyoverdin production in Pseudomonas aeruginosa 7NSK2, is a site-specific recombinease. Molecular microbiology 14(5): 1011-1020.

Hussein, K. A. and J. H. Joo (2014). Potential of siderophore production by bacteria isolated from heavy metal: polluted and rhizosphere soils. Current microbiology 68(6): 717-723.

Khamna, S., A. Yokota and S. Lumyong (2009). Actinomycetes isolated from medicinal plant rhizosphere soils: diversity and screening of antifungal compounds, indole-3-acetic acid and siderophore production. World Journal of Microbiology and Biotechnology 25(4): 649.

Kumar, P., G. Hemanth, P. Niharika and S. Kolli (2015). Isolation and identification of soil mycoflora in agricultural fields at Tekkali Mandal in Srikakulam District. International Journal of Advances in Pharmacy, Biology and Chemistry 4(2): 484-490.

Lankford, C. E. and B. R. Byers (1973). Bacterial assimilation of iron. CRC Critical Reviews in Microbiology 2(3): 273-331.

Leong, J. (1986). Siderophores: their biochemistry and possible role in the biocontrol of plant pathogens. Annual Review of Phytopathology 24(1): 187-209.

Matzanke, B. F. (1994). Iron storage in fungi. Metal ions in fungi 11: 179-214.

Milagres, A. M., A. Machuca and D. Napoleao (1999). Detection of siderophore production from several fungi and bacteria by a modification of chrome azurol S (CAS) agar plate assay. Journal of Microbiological Methods 37(1): 1-6.

Mirabello, S. A. (2006). Influence of siderophore producing bacteria and organic ligands on phase distribution of cadmium and its uptake by Brassica napus in the presence of goethite, Cornell University.

Murugappan, R., A. Aravinth and M. Karthikeyan (2011). Chemical and structural characterization of hydroxamate siderophore produced by marine Vibrio harveyi. Journal of industrial microbiology & biotechnology 38(2): 265-273.

Nailwal, S., M. S. Anwar, K. K. Budhani, A. Verma and T. K. Nailwal (2014). Burkholderia sp. from rhizosphere of Rhododendron arboretum: isolation, identification and plant growth promotory (PGP) activities. J Appl Nat Sci 6: 473-479.

Neilands, J. (1995). Siderophores: structure and function of microbial iron transport compounds. Journal of Biological Chemistry 270(45): 26723-26726.

Neilands, J. B. (1981). Microbial iron compounds. Annual review of biochemistry 50(1): 715-731.

Patel, D., S. Patel, P. Thakar and M. Saraf (2017). Siderophore Producing Aspergillus spp as Bioinoculant for Enhanced Growth of Mung Bean. International Journal of Advanced Agricultural Science and Technology 6(1): pp. 111-120.

Payne, S. M. (1994). [25] Detection, isolation, and characterization of siderophores. Methods in enzymology 235: 329-344.

Rajkumar, M., N. Ae, M. N. V. Prasad and H. Freitas (2010). Potential of siderophore-producing bacteria for improving heavy metal phytoextraction. Trends in biotechnology 28(3): 142-149.

Shenker, M., I. Oliver, M. Helmann, Y. Hadar and Y. Chen (1992). Utilization by tomatoes of iron mediated by a siderophore produced by Rhizopus arrhizus. Journal of plant Nutrition 15(10): 2173-2182.

Srivastava, M., R. Tiwari and N. Sharma (2013). Effect of different cultural variables on siderophores produced by Trichoderma spp. Int J Adv Res 1: 1-6.

Tork, S., M. Aly and L. Nawar (2010). Biochemical and molecular characterization of a new local keratinase producing Pseudomonas sp., MS21. Asian J Biotechnol 2(1): 1-13.

Winkelmann, G. (2007). Ecology of siderophores with special reference to the fungi. Biometals 20(3-4): 379.

Yadav, J., J. P. Verma and K. N. Tiwari (2011). Plant growth promoting activities of fungi and their effect on chickpea plant growth. Asian Journal of Biological Sciences 4(3): 291-299.

احتماليه انتاج السيديروفور بواسطه بكتيريا و فطريات تستوطن منطقه الريزوسفير

يحي عثمان اللازق ،عمرو محمد موافي ، أحمد شوقي جبريل و سمر محمد حامد

قسم النبات – كليه العلوم - جامعة المنصوره

منطقة الريزوسفير هي جزء من جذور النبات و التربه المحيطه و تتميز بوفرة من الإفرازات الجذريه و التي تحفز نمو مجموعه متنوعه من الكائنات الميكروبية، و هذه الكائنات بدورها تلعب دورا مهما في فسيولوجيا النبات حيث تساعده علي مقاومه الأمراض الميكروبية و إمداده بالعناصر و المعادن المهمه، احدى هذه العناصر هو الحديد. و هو عنصر مهم لأي خليه حيه لأنه يدخل في تفاعلات الأكسده و الإختزال،و في عمليتي التنفس و البناء الضوئي، ايضاً يعمل كعامل مساعد لبعض الإنزيمات. بالرغم من أن الحديد هو عنصر شائع في القشره الأرضيه لكن في الظروف الهوائيه يكون موجود بصوره معادن غير قابله للذوبان لذلك لا تستطيع النباتات و لا الكائنات الميكروبيه استهلاكها بخاصيه الإنتشار كما هي الحاله في باقي العناصر. لذلك تلجأ هذه الكائنات الي انتاج السيديروفور و هي مركبات عضويه يتم افرازها خارج الخليه الحيه حيث تقوم بإذابه الحديد من المعادن و تقدمه للخليه الحيه بصوره تمكنها من استهلاكه. في هذه الدراسة قمنا بعزل بكتيريا و فطريات من منطقه الريزوسفير لنبات الفاصوليا و البرسيم و الفول و البازلاء، ثم اختبرنا قدرة هذه الكائنات علي انتاج السيديروفور ثم بعد ذلك الكائنات التي أثبت انتاجها للسيديروفورقمنا بتعيين نسبه انتاجها و اخترنا البكتيريا و الفطر الأعلي انتاجيه لتعريفهما جينيا، بعد مقارنه نتيجه السكستين إس و ايتين إس مع قاعدة بيانات إن سي بي أيي تبين أن البكتيريا تنتمي لجنس الباسيلس و الفطر لجنس الأسبرجلس ثم قمنا بدراسه نوع السيديروفور لهذان الكائنان لنجد أن البكتيريا تنتج سيديروفور مجموعته الوظيفيه هي الكاتيكوليت و السيديروفور المنتج بواسطه الفطر مجموعته الوظيفيه هيدروكسيميت.