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Fungal Siderophores Production in Vitro as Affected by Some Abiotic Factors

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ABSTRACT

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Siderophores production *in vitro* was declined with increasing iron concentration in culture media and varied according to fungal strains and irradiation. Irradiated *Aspergillus niger* strain was superior over irradiated *Penicillium oxalicum* strain in siderophores production. The highest value of siderophores produced by *A. niger*, and *P. oxalicum*, respectively was achieved at pH 5.5 and pH 4.5 value. This holds true with both fungal strains either irradiated or not. Despite of fungal strains, the overall means of culture media reflected the superiority of Sabouraud agar (SA) culture medium on producing siderophores. The maximum siderophores production (73.31) was achieved at 25°C for both tested fungi. Irradiated or non-irradiated strains of *A. niger*, and *P. oxalicum* resulted in the highest siderophores with sucrose as a carbon source and sodium nitrate as nitrogen source. Generally, the two irradiated strains surpass the non-irradiated ones and no significant difference between *A. niger* and *P. oxalicum* strains either irradiated or not.

Introduction

In addition to its role in increasing quality and quantity yield of agricultural products, micronutrients have a significant effect on human and animal health that feed of plant materials. Deficiency of micronutrients such as iron (Fe), zinc (Zn) and manganese (Mn), has a global expansion (Mousavi *et al.*, 2007; Mousavi, 2011). Iron is the fourth frequent element in earth's crust often appearing as goethite, hematite and ferrihydrite. Because of the very low solubility of these minerals, ionic concentrations of Fe³⁺ and under toxic conditions also the one of Fe²⁺ are very low.

Oxic systems, with a pH in the neutral region often show concentrations of dissolved iron of about 10⁻¹⁰M to 10⁻⁹M (Poole and McKay, 2003; Kraemer, 2004). However, low availability of soil-Fe to plant roots is generally restricted in alkali soils because of their high pH and high bicarbonate concentrations that lower the solubility of Fe and reduce its uptake by plant roots especially for Strategy I plant species dependent on inducible ferric reductases for cellular Fe transport (Lucena *et al.*, 2007; Jeong and Connolly, 2009).

In most calcareous soils with high pH solubility of micronutrient is low and cause decline uptake of these elements and finally requirement of plants to these elements is increasing (Mousavi *et al.*, 2011; Alloway, 2008). Iron chlorosis in plants is an old worldwide problem occurring in areas of calcareous and/or alkaline soils (Mengel *et al.*, 2001; Tagliavini and Rombolà, 2001; Pestana *et al.*, 2003).

Under iron-limited conditions, most prokaryotic cells, certain fungi and plants elaborate multiple low-molecular-weight (often < 1000 Da), high-affinity chelating agents (although some siderophores have lower affinities), which solubilizes ferric iron in the environment and transport it into the cell (Crosa and Walsh, 2002; Ong and Neilands, 1979; Pérez-Miranda *et al.*, 2007). These compounds are generically known as siderophores and they are typically found in iron-deficient cultures (Dimkpa *et al.*, 2009). Fungi exploit the surrounding soil environment for nutrients primarily by hyphal extension. In this way, fungi secrete siderophores into the soil to chelate or bind tightly iron that is subsequently brought back into the cell by specific uptake mechanisms. This iron will be used for diverse processes that are essential to the organism's survival (Winkelmann, 2007).

Therefore, this work aimed to follow up the optimization of fungal siderophores production conditions and studying the impact of siderophores producer on iron availability (*In vitro*).

Materials and Methods

Experimental Organisms

Aspergillus niger (10618 AUMC) and *Penicillium oxalicum* (10619 AUMC) previously isolated from Mashtool and

Inshas Al-raml, sharkia, Egypt and identified at Assiut University Mycological Center (AUMC), Egypt were found to be best isolates in between 16 species tested for siderophores production.

Irradiation process

The source of irradiation was cobalt-60 gamma cell 220 located at Cyclotron, Nuclear Research Center, Atomic Energy Authority. The dose rate was 1.09 kGy at the time of experiment. Agar slants of the tested strains were subjected to dose levels 0.5 to 10.0 kGy.

Media: the following media were used:

Cas blue solution (CAS): (Schwyn and Neilands (Shin *et al.*, 2001)) using 60.5 mg Chrome azurol S (CAS) dissolved in 50 ml water distilled, deionized, and mixed with 10 ml iron (III) solution (1 mmol/l FeCl₃ .6H₂O, 10 mmol/l HCl). Under stirring, this solution was slowly added to 72.9 mg HDTMA dissolved in 40 ml water. The resultant dark blue mixture was autoclaved at 121°C for 15 min.

Czapek-Dox's agar medium (g/l): (Czapek 1902-1903 and Dox 191)) (Thom and Raper, 1945) Sucrose, 30.0; NaNO₃, 2.0; KH₂PO₄, 1.0; MgSO₄.7H₂O, 0.5; KCl, 0.5; FeSO₄.5H₂O, 0.001; CaCl₂, 0.001; Bacto-agar, 15.0 and rose bengal (Merck) as a bacteriostatic agent (0.065).

Potato Dextrose Agar (PDA) (g/l): (Leslie and Summerell, 2006) Potato slices, 250; Glucose, 20; Agar, 20.

Czapek's Yeast Extract Agar (CYA) (g/l): (Onion's *et al.*, 1981) Sucrose, 30; NaNO₃, 2.0; K₂HPO₄, 1.0; MgSO₄.H₂O, 0.5; KCl, 0.5; FeSO₄.7H₂O, 0.01; agar, 15; Yeast extract, 5.

Lignocellulose Agar (LCA) (g/l): (Miura and Kudo, 1970) Glucose, 1.0; KH₂PO₄, 1.0; MgSO₄.7H₂O, 0.2; KCl, 0.2; NaNO₃, 2.0; Yeast Extract, 0.2; agar, 13.0.

Sabouraud agar (SA) (g/l): (Sabouraud, 1896) Peptone, 10; glucose, 40; agar, 15.

Malt extract agar (MEA) (g/l): (Raper and Thom, 1949) Malt extract, 15; peptone, 1.0; glucose, 20.0; gar, 20.0.

Hagem's nutrient solution (g/l): (Szaniszlo *et al.* 1981) KH₂PO₄, 0.5; MgSO₄.7H₂O, 0.5; NH₄Cl, 0.5; glucose, 5.0; Malt extract, 5.0; FeCl₃ % 1 solution 10 drop, H₂O (distilled) 1000.0 ml from which FeCl₃ was omitted (= low iron medium) . Its phosphate content was increased to 2g K₂HPO₄ and 1.0g KH₂PO₄ L⁻¹.

Low Iron Media for siderophores production (g/l): (Abd-allah and Mahmoud, 2001): Glucose, 10; NaHPO₄, 7; KH₂PO₄, 3; NH₄Cl, 1; NaCl, 0.5; MgSO₄.7H₂O, 0.25; CaCl₂.2H₂O, 0.015; ZnCl₂, 0.015; Thiamine, 0.005 at pH 7. Iron was added as 0 or 10 µmol/l FeCl₃.

Determination of Siderophores Production in Broth Media

All glassware used was cleaned in 20% HCL to remove iron and rinsed in deionised water

Fungal growth: for siderophores production the tested isolates were grown on the following low iron medium. Sterilized Erlenmeyer flasks 100 ml capacity containing 50 ml medium supplemented with or without iron were inoculated with the tested microbes and incubated in orbital shaker incubator at 1.8 Hz and 28°C for 6 days. Mycelia were harvested by centrifugation at 67 Hz for 10 min and dried at 70°C (Abd-allah and Mahmoud, 2001).

Siderophores detection: A 1.0 ml aliquot of supernatant of fungal liquid cultures was mixed with 1.0 ml CAS assay solution. A reference was prepared with exactly the same medium used for growing the fungi, but uninoculated. The sample (s) and reference (r) absorbance at 630 nm were measured after 1 h of incubation at room temperature. The percentage of iron-binding compounds of the siderophores type was calculated by subtracting the sample absorbance values from the reference. Siderophores units are defined as [(Ar - As)/Ar]. 100 = % siderophores units. Percentages of siderophores units less than 10 were considered as negative and in this case no change in the blue color of CAS solution was observed.

Abiotic Factors Studied

Culture Medium of Inoculum Preparation

Siderophores production was examined by two irradiated and non irradiated fungal strains (*A. niger* and *P. oxalicum*) grown on PDA, CYA, LCA, SA and MEA Disc of 0.5 cm of fungal growth was transferred to CAS blue agar media which prepared according to Schwyn and Neilands (1987) and incubated at 28°C for 6 days and diameter of clear zone were measured.

Effect of pH: One disc of agar mycelium (0.5 cm diameter) obtained from stock plates was transferred in to 50 ml of Hagem's nutrient solution. The initial pH of Hagem's nutrient solution was adjusted to 2.5, 3.5, 4.5, 5.5, 6.5 or 7.5 using 1.0 N HCL or 1.0N KOH before sterilization (Federspiel *et al.*, 1991) and incubated in orbital shaker at 1.8 Hz and 28°C. Culture were harvested after 6 days and centrifuged at 67 Hz for 10 min (Abd-allah and Mahmoud, 2001). Siderophores detection in liquid media was measured at 630 nm as mentioned above.

Effect of Temperature: The effects of temperature on siderophores production were studied by growing fungal cultures at 15, 20, 25, 30, 35, 40 and 45°C in liquid low iron media prepared according to (Abd-allah and Mahmoud, 2001).

Effect of iron concentration: To study the effect of iron concentration on siderophores production by the fungal isolates, Malt extract (ME) medium without (control) and with iron (2 and 4 mmol l⁻¹) were prepared. One disc of agar mycelium (0.5 cm diameter) obtained from stock plates was transferred in to 50 ml of 2% liquid malt extract (Machuca and Milagres, 2003). Flasks incubated and siderophores detection in liquid media was measured at 630 nm as mentioned above.

Effect of carbon source: The following carbon sources were separately added into the base medium (30 g l⁻¹) of CY instead of sucrose to evaluate the effect of carbon source on siderophores production: dextrose, fructose, lactose, galactose or maltose (Zhao *et al.*, 2010).

Effect of nitrogen source: The following nitrogen sources were separately added into the nitrogen free base medium (equimolecular weights) to evaluate the effect of nitrogen source on siderophores production: peptone, sodium nitrate or ammonium sulfate (Zhao, *et al.*, 2010).

Results and Discussion

Effect of Culture Medium: It is obvious that siderophores doesn't significantly vary with different culture media when non-irradiated *A. niger* was considered (Fig. 1). To some extent, high siderophores production was induced by using CYA culture media. The irradiated *A. niger* (fig. 1: ii) showed lower values of siderophores

than those of non-irradiated one (fig. 1: i). In this regard, the highest value (3.85) of siderophores was detected with LCA culture media. Either irradiated (fig. 1: iv) or non-irradiated (fig. 1:iii) *P. oxalicum* resulted in lower siderophores values than those recorded with *A. niger*. Likewise *A. niger*, the non-irradiated *P. oxalicum* was superior over irradiated one in siderophores production.

Despite of fungal strains, the overall means of culture media reflected the superiority of SA culture medium on producing siderophores.

Effect of initial pH: Generally the highest value of siderophore was achieved at pH 5.5 and pH 4.5 value for *A.niger* and *P. oxalicum*. This holds true with fungal strains either irradiated or not. Siderophores produced by non-irradiated *A. niger* or *P. oxalicum* was gradually increased with increasing pH up to 5.5 and 4.5, respectively (Fig. 2). Irradiated *A. niger* showed siderophores values nearly closed to each other as affected by changes in pH values but resulted in higher siderophores value at the lowest pH value 2.5 (87.24) than those of non-irradiated strain (51.1). Reversible trend was noticed with *P. oxalicum* where non-irradiated strain induced siderophores values higher than those produced by irradiated strain specially at pH 4.5.

Various organisms evolved active strategies to overcome this lack of iron. These strategies usually contain active exudates which can increase the total soluble iron concentration by several magnitudes (Kraemer, 2004). Typical of these exudates are protons, increasing the solubility by decreasing the pH, reductants, increasing the iron solubility by reduction of insoluble Fe (III) to more soluble Fe (II) and the exudation of chelating agents such as

organic acids and siderophores which form soluble iron complexes. In this regard, experiments by (Srivastava, *et al.*, 2013) were conducted *in vitro* on three strains *Trichoderma* MPPLUNS1, *Trichoderma* MPPLUNS2 and *Trichoderma* MPPLUNS3, *Trichoderma* MPPLUNS1 was proved efficient strain, which exhibited higher reaction rate during C.A.S. (Chrome Azurol S) Blue Agar assay for qualitative assessment of siderophores production. Best siderophores production has been shown by *Trichoderma* MPPLUNS1 at 1mM concentration of iron and 4.5 pH.

Effect of Temperature: Gradual increase in incubation temperature resulted in fluctuated response of fungal strains to siderophores production (Fig.3). The overall means of siderophores reflected no significant difference between irradiated and non-irradiated *A. niger* strains. Non-irradiated *P. oxalicum* surpass the irradiated strain and both of them were superior over *A. niger* strains in siderophores production. As indicted by overall means of incubation temperature, the maximum siderophores production (80.06) was achieved at 25 °C but lowered at 15 °C and 45 °C when grown in chemically defined low iron medium. Bendale *et al.* (2010) also check the influence of temperature on siderophores production by growing *Streptomyces fulvissimus* at various temperatures. At 28 °C, maximum siderophores production was observed i.e. 93.47 % siderophores units which was little bit lowered at 37 °C but no siderophores production was induced at 20 °C and at 44 °C when grown in chemically defined low iron medium.

Effect of iron concentration: Among environmental factors, iron concentration is the most important that mainly regulates the biosynthesis and secretion of siderophores (De Villegas *et al.*, 2002). Since iron

requirements differ for various organisms (Stintzi and Meyer, 1994), it is necessary to investigate the iron requirement of the test strains. Iron concentration in growth medium seems to be effective on siderophores production by irradiated or non irradiated tested fungi (Fig. 4). The influence of exaneously added Fe (III) in standard medium (low iron medium) on siderophores production was observed. In the absence of exogenous iron / less than 2 mmol l⁻¹ irradiated and non irradiated *A. niger* produce more Siderophores than irradiated and non irradiated *P. oxalicum*. While, presence of 4 mmol l⁻¹ in growth medium significantly decrease siderophores production by the tested strains especially in case of non irradiated *A. niger* isolates. The best percentage of siderophores produced by the two different fungal strains was detected with presence of 2 mmol l⁻¹ iron in growth medium. Siderophores production was decreased proportionally with the increase in iron concentration. Iron more than 2 mmol l⁻¹ enhanced the growth of fungus strains but responsible for repression of siderophores production. Increased iron concentration favours growth but represses siderophores production (Meyer, 2000). Similar results have been reported by Bendale *et al.* (2010) for siderophores production by *Streptomyces fulvissimus* ATCC 27431. In deferrated succinate medium (dSSM), siderophore level reached 216.23µg/ml for PRS after 42 h, followed by that in *P. chlororaphis* ATCC 9446 (189.91 µg/ml) and fluorescent *Pseudomonas* spp. GRP3A (66.74 µg/ml). Compared to standard succinate medium (SSM), siderophore levels were lower in standard citrate medium SCM. This was also true for dSCM where the levels were smaller compared to dSSM (Sharma and Johri, 2003). In order to assess the influence of iron concentration on siderophores release, Sharma and Johri (2003) demonstrated that *Pseudomonas* GRP3A strain was used as a

model system. They found that gradual increase in iron concentration (0.6, 1.2, 2.4 and 6.0 μM), resulted in decrease of siderophores production. Also, siderophore production was maximum in standard succinate medium SSM after 72 h (51.03 $\mu\text{g/ml}$) and lowest in SSM supplemented with 6 μM Fe (11.98 $\mu\text{g/ml}$).

In deferrated SSM (dSSM), siderophores production increased in comparison to SSM. However, with gradual increase in iron supplementation (0.6–2.4 μM Fe^{3+}), The siderophores production declined in a manner similar to SSM. Lowest siderophore level was recorded at 6.0 μM Fe^{3+} (19.59 $\mu\text{g/ml}$) and maximum in normal dSSM (74.81 $\mu\text{g/ml}$). Generally, the above results are consisted with our data. In *vitro* condition, results obtained by Srivastava *et al.* (2013) the radial growth of *Trichoderma* MPPLUNS 1 was observed in mm/day. Same methodology was followed in respect of iron requirement of micro-organisms. In iron deficient conditions, *Trichoderma* MPPLUNS 1 has demonstrated higher reaction rate at 1mM iron concentration which was lowest among all provided iron doses. This was also proved in context of *Pseudomonas*, *Nurospora crassa*, *Fusarium dimerum* and *Mucor* sp, siderophores production as reported by (Dave and Dube, 2000).

Effect of different carbon source: Data presented in (Fig. 5) notified that carbon source play an important role that affects siderophores production by fungal strains either exposed to radiation or not. The effect of irradiation of *A. niger* on siderophores production was fluctuated according to carbon sources. For example, irradiated *A. niger* resulted in higher siderophores production than the non-irradiated one when

sucrose was applied while reversible was detected with dextrose source. Use of lactose, and maltose didn't reflects significant difference in siderophore production between irradiated and non-irradiated *A. niger* strains. Irradiated *P. oxalicum* showed higher response to carbon sources than the non-irradiated strain in growth medium. Like that of *A. niger*, *P. oxalicum* resulted in the highest siderophores with sucrose source.

Different sources of carbon and fungal strains at several incubation times were examined earlier (Sharma and Johri, 2003). They found that deferration of growth media resulted in increased siderophore production and lower growth, irrespective of carbon source.

Effect of different nitrogen source: The overall means of different nitrogen sources in the growth medium were nearly closed to each other. Considering the irradiation effect, the overall means indicated the superiority of irradiated strains over the non-irradiated one. In the same time, there was no significant difference in siderophores production between *A. niger* and *P. oxalicum* strains. Nitrogen is one of the major components of important cellular elements including proteins, nucleic acid and cell wall. Amino acids are particularly good sources of nitrogen, generally inducing an increased growth rate. Glutamic acid as the sole source of carbon and nitrogen has been reported to improve the production of siderophores (Casida, 1992). Bultreys and Gheysen (2000) have reported high siderophores production in strains of *P. syringae* with all the 20 amino acids when used as the sole source of both carbon and nitrogen.

Fig.1 Siderophores on CAS medium by 0.5 cm discs of *A. niger* and *P. oxalicum* on the tested media.

i: *A. niger*, ii: *A. niger*(irr), iii: *P. oxalicum*, iv: *P. oxalicum*(irr). a,b,c,d: disc of four strains growing on five different media as following:- a: on LCA medium, b: on PDA medium, c: on CYA medium, d: on SA medium, e: on ME medium

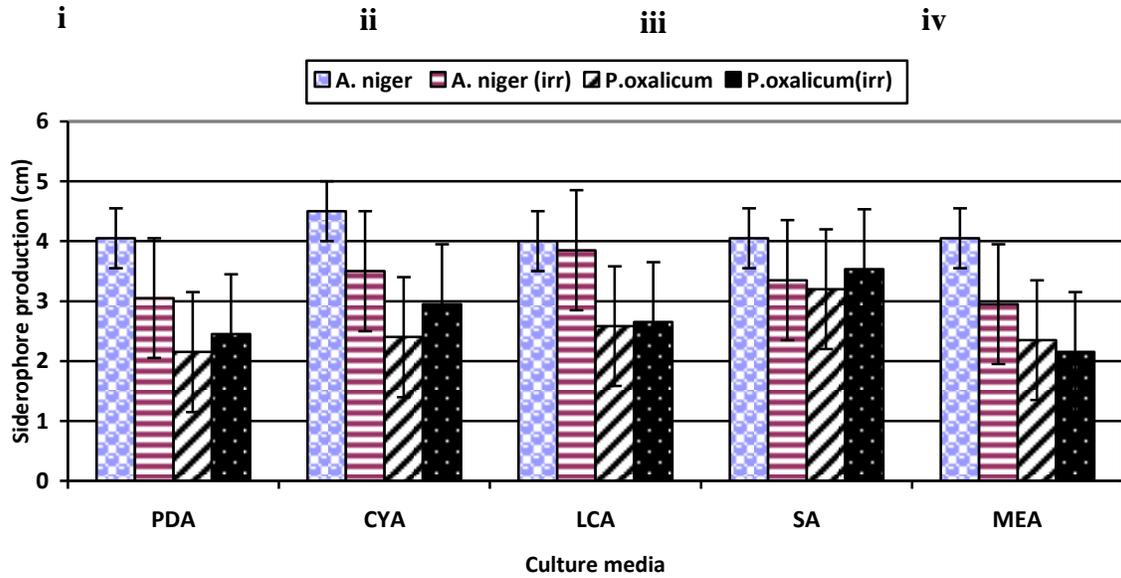
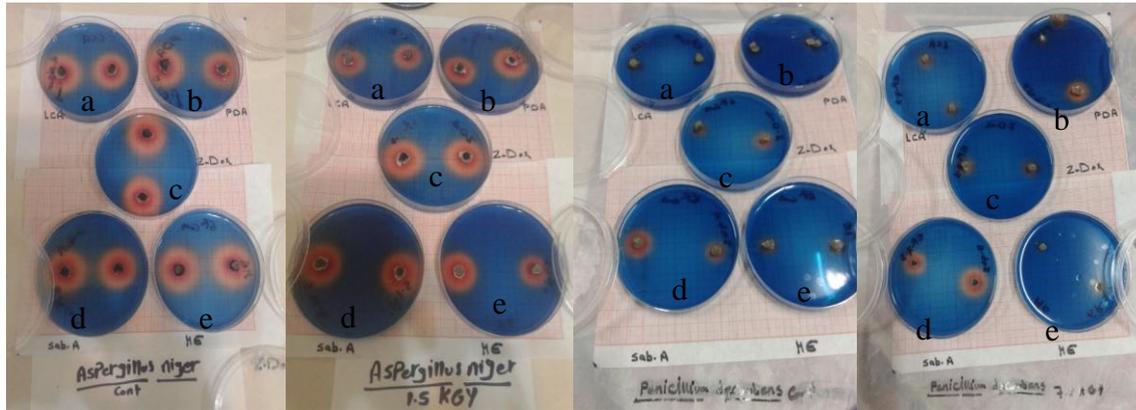


Figure 1. Effect of culture media on siderophore produced by irradiated and non-irradiated fungi

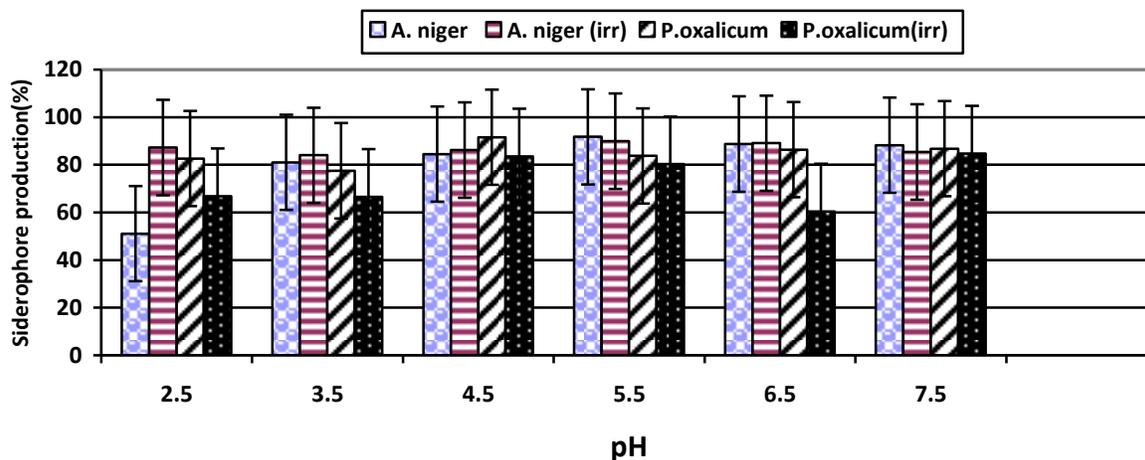


Figure 2. Effect of pH on siderophore produced by irradiated and non-irradiated fungi

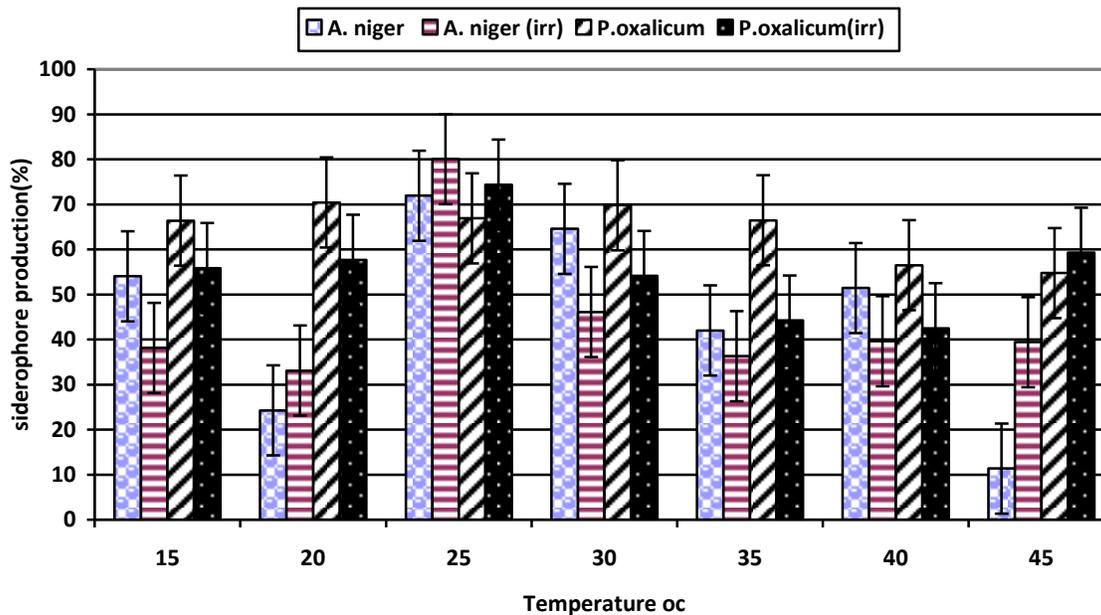


Figure 3. Effect of temperature on siderophore produced by irradiated and non-irradiated fungi

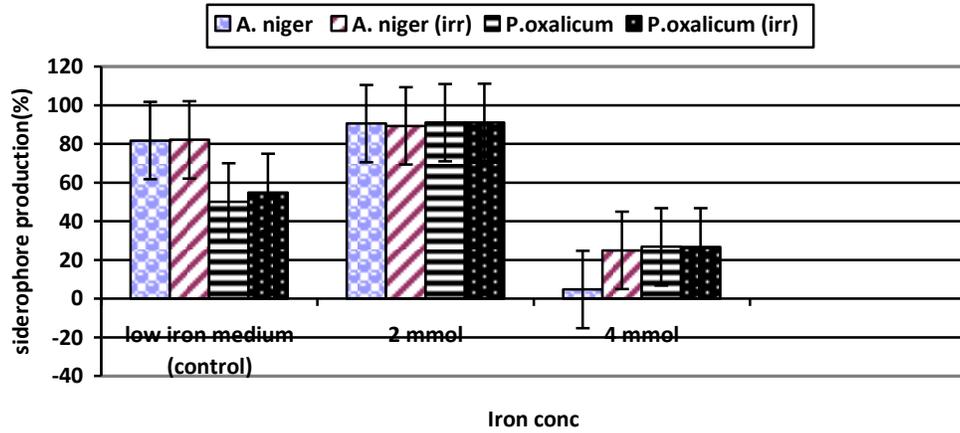


Figure 4. Effect of iron concentration on siderophore produced by irradiated and non-

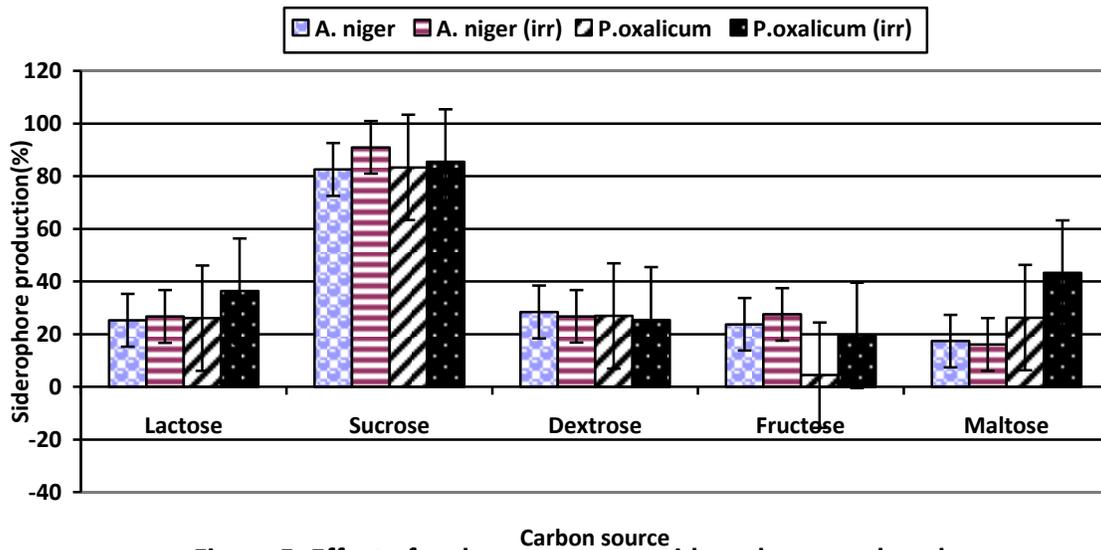


Figure 5. Effect of carbon sources on siderophore produced by irradiated and non-irradiated fungi

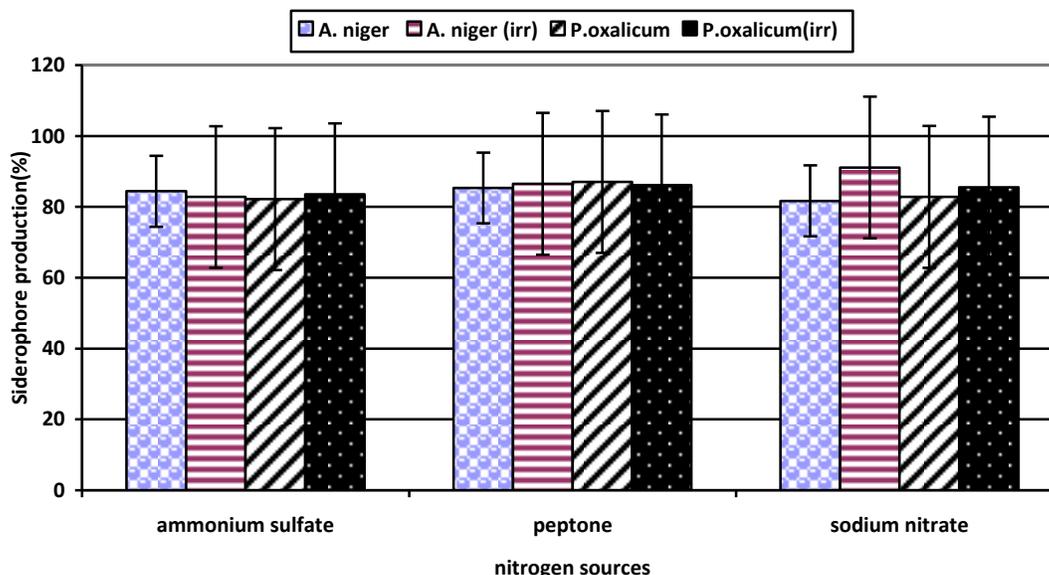


Figure 6. Effect of nitrogen sources on siderophore produced by irradiated and non-irradiated fungi

Solid and liquid culture media containing asparagine are reported to be highly effective for the induced production of siderophores by strains of *P. syringae* (Bultreys and Gheysen, 2000). This amino acid is usually combined with sucrose in the medium known as sucrose-asparagine (SA) for the production of siderophores in *Pseudomonas* (Laine *et al.*, 1996).

In conclusion, siderophores production was significantly affected by iron concentration in medium. Sources of carbon and nitrogen in culture media have a remarkable role in increasing siderophores production. In the same time, the best values of siderophores productions were achieved at pH 5.5, 4.5 and 25°C temperature. Both fungal strains have a meaningful potential in producing siderophores. These fungi were affected, to some extent, by irradiation process.

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