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Optimization of cultural conditions for production of antifungal metabolites by Streptomyces gougeroti

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Abstract

Use of antagonist microorganisms against fungal plant pathogens is an attractive and ecologically alternative to the use of chemical pesticides. *Streptomyces* spp. are beneficial soil bacteria and potential candidates for biocontrol agents. Different *Streptomyces* species were isolated from different sources sites from soil in Damietta.

During primary and secondary screening, some actinomyctes isolates showed antifungal activity against tested phyto pathogenic fungi *(Penicillium italicum, Macrophomina phaseolina, Fusarium oxysporium, F. solani, Alternaria alternata, Sclerotina sclerotiorum* and *Rhizoctonia solani*). The results showed that the most active isolate against tested phyto pathogenic fungi *Macrophomina phaseolina* and *Alternaria alternata* was identified as *Streptomyces gougeroti*. *Streptomyces gougeroti* showed maximum antifungal activity against *Alternaria alternata* and *Macrophomina phaseolina* at 35°C ± 2, pH 6.5. Glycerol and beef extract were the best carbon and nitrogen sources used respectively.

Keywords: Streptomyces gougeroti, Macrophomina phaseolina, Alternaria alternata and Antifungal activities.

Introduction

Fungal plant diseases are a major concern to worldwide agricultural production. Plant diseases are a major problem facing plant cultivation and are responsible for the loss of 10 % of the total global crop production (Strange and Scott, 2005). Pathogenic fungi can generate thousands of microspores in lesions and lead to considerably increased propagation in similar hosts that could give way to split the diseases (Giraud et al. 2010). Fungi, one of the most aggressive plant pathogens, are conventionally destroyed with chemical fungicides. Their widespread use, which has tripled over the last 40 years, has accelerated environmental pollution and degradation. Moreover, chemical fungicides may be lethal to beneficial insects and microorganisms populating the soil and may enter the food chain (Budi et al. 2000). With all the problems associated with synthetic chemicals, many scientists are investigating biological pesticide solutions (Martinez 2012; Nega 2014). Biological pesticides include chemicals derived from microorganisms, plants and animal sources. Fungal infections are the main cause of postharvest rots of fresh fruit and vegetables during storage, transport and cause significant economic losses in the commercialization phase (Gatto et al. 2011).

Infections caused during postharvest conditions lowers the shelf life and adversely affect the market value of fruits (Tripathi et al. 2008). Moreover, mycotoxins the secondary metabolites produced by fungi have adverse effects on humans and animals (Zain 2011).

Biological control, the use microorganisms to control plant diseases, offers an alternative, environmentally friendly strategy for controlling phyto pathogens (Kishore et al. 2005; Prapagdee et al. 2008; Sharma et al. 2011). The potential use of microorganisms in the treatment of plant fungal diseases is based on the antagonistic nature of microbes towards the fungal pathogens. The results of experimental and field trials studies of microbial antagonistic against plant fungal pathogens are promising (Sharma et al. 2009; Williams 2009).

Various microbial antagonists have been investigated as potential antifungal biocontrol agents of plant diseases. Many species of actinomycetes, especially those belonging to the genus *Streptomyces* are well known as biocontrol agents that inhibit or lyse several soils borne and airborne plant pathogenic fungi (El-Tarabily 2006; Lee et al. 2008; Sousa et al. 2008).

Many strains of *Streptomyces* are known to suppress fungal growth in vitro (Crawford et al. 1993). *Streptomyces* species or their products have been used to suppress fungal plant diseases in vivo (You et al. 1996; Liu et al. 1995). The objectives of this study were to study the optimization for antifungal activities by *Streptomyces gougeroti* against *Alternaria alternata* and *Macrophomina phaseolina*.

Material and methods

Isolation of Streptomyces sp.

The dilution agar plating technique described by Johnson et al., (1959) was used for isolation of *Streptomyces* sp. Starch- nitrate medium was used for isolation and purification of *Streptomyces* sp. (Waksman, 1959). The plates were incubated at 28°C for seven days.

Growth of fungal strains

Fungal strains, *Alternaria alternata* was isolated and identified by Prof El-Fallal and *Macrophomina phaseolina*. These strains were grown in Potato- dextrose agar medium at 30 °C for five days. The discs of agar cultures (6 mm diameter) were used separately to inoculate 250 ml conical flasks containing potato- dextrose agar medium and poured in petri- dishes to examine the anti-fungal activities of tested *Streptomyces* strains.

Preparation of metabolites from the tested isolates

After the incubation period, cultures were harvested by centrifugation at 4,000 rpm for 10 minutes. Culture supernatants were immediately transferred and filtered through Millipore filter (0.45µm) to get cell free supernatant. The remaining supernatants were used for the determination of intracellular protein.

Determination of intracellular protein

Harvested pellets were washed three times with distilled water. Washed pellets were centrifuged at 4,000 rpm for 10 minutes, washed and dissolved in 20 mL of NaOH (1M), and boiled for 20 minutes. Boiled pellets were allowed to cool and then centrifuged at 4,000 rpm for 10 minutes. Dilution of the clarified solution was used to determine the intracellular protein concentration of the organisms using the Bio-Rad protein assay (Bradford 1976).

Environmental factors affecting production of antifungal metabolites by Streptomyces gougeroti

Effect of different media

The selected isolate was grown in different media including: starch nitrate broth, starch-ammonium sulphate broth, glycerol-yeast broth, glycerol-asparagine broth, starch-casein broth, Czapek-dox broth and CM-I broth. Flasks were incubated at 28°C ± 2 and at 150 rev min^{-1} for 7 days. Then the cultures were collected and centrifuged at 4000 rpm for 20 minutes and the supernatants were immediately transferred and filtered through Millipore filter (0.45µm) to get cell free supernatant. Antifungal activities were assayed, supernatant were subjected to agar diffusion assay against *Alternaria alternata* and *Macrophomina phaseolina* to select the most appropriate media for production the antimicrobial agent; (Rabah et al. 2007).

Effect of incubation periods

The selected isolate was grown in a liquid glycerol-yeast media (Waksman 1961) as a basal medium. Flasks were inoculated using spore suspensions and incubated at $28^{O}C \pm 2$ at 150 rev min^{-1}. After 3, 4, 5, 6, 7 and 8 days, three flasks were harvested for each interval. Antifungal activity was assayed as before.

Effect of different pH.

The selected isolate was grown in a liquid glycerol-yeast medium. The pH was adjusted at 5.0, 6.0, 7.0, 8.0 and 9.0. Flasks were inoculated with tested isolate and incubated at 28°C ± 2 for 5 and 4 days. Antifungal activity was assayed as before.

Effect of different temperatures

The tested isolate was grown in liquid glycerol-yeast medium at pH 6.5. Flasks were inoculating with spore suspensions and incubation was carried out at 25, 30, 35 and 40°C for 4 days . Antifungal activity was assayed as before.

5- Effect of different carbon sources

Starch, cellulose and colloidal chitin were added separately (1%) (w/v) to liquid glycerol-yeast media and autoclaved with the media. Glucose, maltose, fructose and sucrose (1%) (w/v) were added following sterilization by passing through Millipore filter (0.45 μm) to liquid media.
The pH of the media was adjusted to 6.5, then flasks were incubated at 35°C ± 2 for four days. Antifungal activity were assayed as before.

6-Effect of different nitrogen sources

A range of different nitrogen sources includes NH_4Cl, $(NH_4)_2SO_4$, $NaNO_3$ and asparagine were added in equimolecular nitrogen weights equivalent to the nitrogen content of 27 g L^{-1} peptone and yeast of glycerol-yeast media (Waksman 1961). Other nitrogen sources like peptone (14 % N), yeast (10 % N), casein (14.5 % N), tryptone (13.5 % N) and beef extract (12.5 % N) were added.

The pH of the media was adjusted to 6.5, then the flasks were inoculated and incubated at $35^{0}C$ at 150 rev min^{-1} for four days. Antifungal activity was assayed.

Results

We isolated some strains of actinomycetyes from soil, most of these isolates showed high antifungal activities against tested phyto pathogenic fungi. We select the tested isolate that showed high antifungal activity against *Macrophomina phaseolina* and *Alternaria alternata.* This isolate was identified in previous work according to Bergey's Manual of Systematic Bacteriology- volume five the actinobacteria (Good Fellow *et al*. 2012) as *Streptomyces gougeroti*. *Streptomyces* isolate was grown on glycerol-yeast agar medium at 35°C ± 2.
The most producing medium for bioactive metabolites was glycerol-yeast extract medium as it promoted the highest bioactive potential against *Alternaria alternata* (2.97 ± 0.02 cm) and *Macrophomina phaseolina* (2.9 ± 0.05 cm). Starch-ammonium sulphate, glycerol-asparagine, CM-I and Czapek-Dox media had no antifungal activity against the tested phytopathogenic fungi (Figure 1).

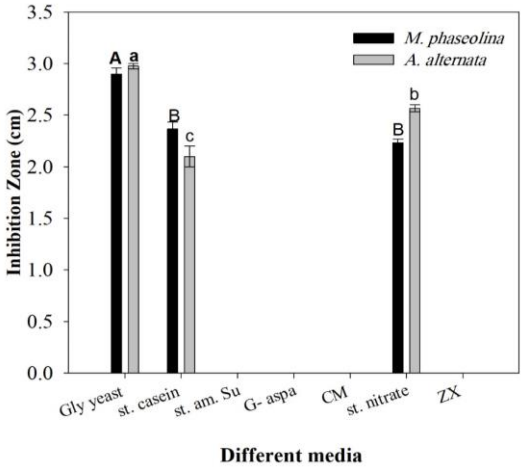

Fig. 1. Effect of different media on antifungal activity of *Streptomyces gougeroti* against *Macrophomina phaseolina* and *Alternaria alternata*

The time course of production of antifungal activity of *Streptomyces gougeroti* against *Macrophomina phaseolina* and *Alternaria alternata* started from the 3rd day then increased significantly to the maximum activity in 4th day (3.5 ±0.11 cm and 4.87 ± 0.24 cm) respectively. The intracellular protein content started from the 3rd day then increased significantly to the maximum growth in the 4th day (3.04 ± 0.08 mg/ml) (Figure 2 A & B).

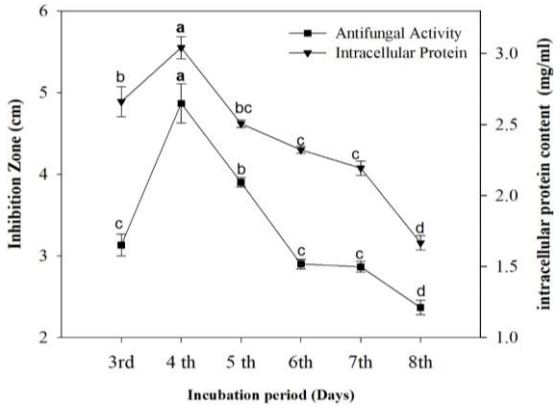

Figure 2.A. Effect of incubation period on antifungal activities and intracellular protein content of *Streptomyces gougeroti* against *A. alternata*

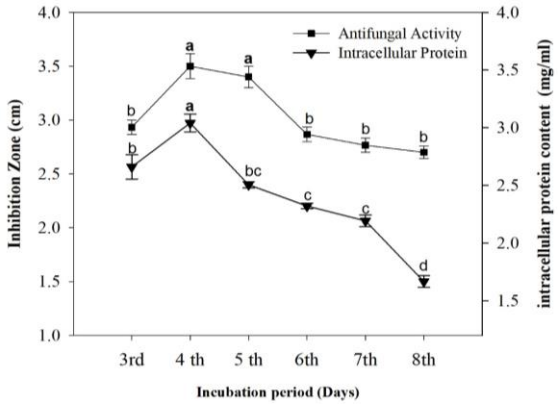

Figure 2.B. Effect of incubation period on antifungal activities and intracellular protein content of *Streptomyces gougeroti* against *M. phaseolina*

Antifungal activity of *Streptomyces gougeroti* against *Macrophomina phaseolina* and *Alternaria alternata* started from pH 6 then increased significantly to the maximum activity at pH 6.5 (3.67 ± 0.09 cm, 3.90 ± 0.06 cm). The intracellular protein content stared from the pH 5 then increased significantly to the maximum growth at pH 6.5 (3.20 ± 0.20 mg/ml) (Figure 3 A &B).

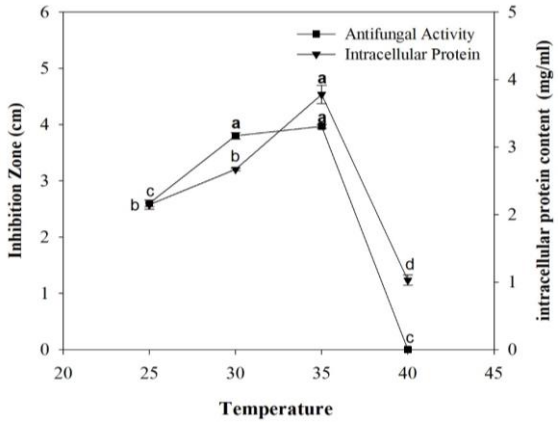

Figure 3.A. Effect of temperatures on antifungal activities and intracellular protein content of *Streptomyces gougeroti* against *A. alternata*

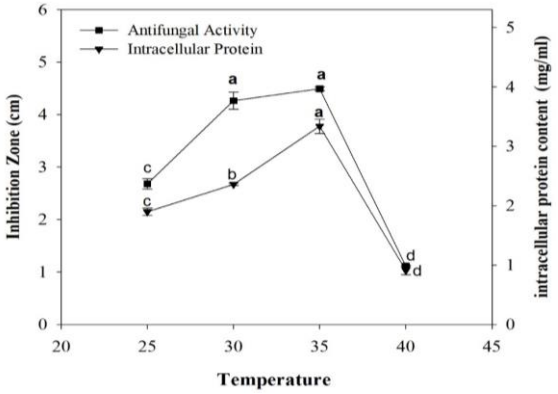

Figure 3.B. Effect of temperatures on antifungal activities and intracellular protein content of *Streptomyces gougeroti* against *M. phaseolina*

The antifungal activity against *Macrophomina phaseolina* and *Alternaria alternata* increased from 25°C to 35°C with optimum temperature at 35 °C (4.00 ± 0.03 cm and 3.96 ± 0.03 cm) respectively, then sharply decreased by increasing temperature at 40°C. The intracellular protein started at 25°C then increased significantly to the maximum temperature at 35 °C (3.80 ± 0.14 mg/ml) (Figure 4 A & B).

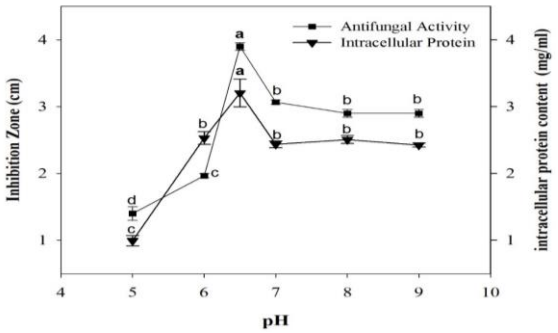

Fig. 4.A. Effect of pH on antifungal activities and intracellular protein content of *Streptomyces gougeroti* against *A. alternata*

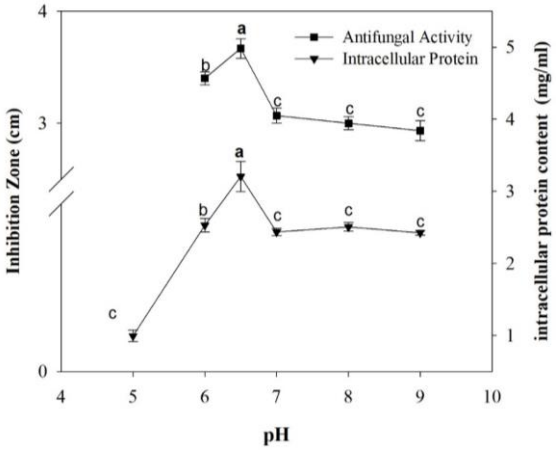

Figure 4.B. Effect of pH on antifungal activities and intracellular protein content of *Streptomyces gougeroti* against *M. phaseolina*

The highest antifungal activity of *Streptomyces gougeroti* against *Macrophomina phaseolina* and *Alternaria alternata* was observed in presence of glycerol (3.93 ± 0.07 cm, 4.00 ± 0.06 cm). Colloidal chitin and cellulose had the minimum antifungal activity. The maximum intracellular protein content in presence of glycerol was 4.15 ± 0.05 mg/ml. (Figure 5 A & B).

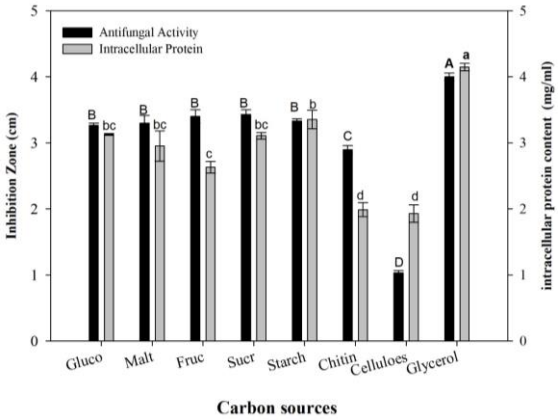

fig 5. A. Effect of different carbon sources on antifungal activities and intracellular protein content of *Streptomyces gougeroti* against *A. alternata*

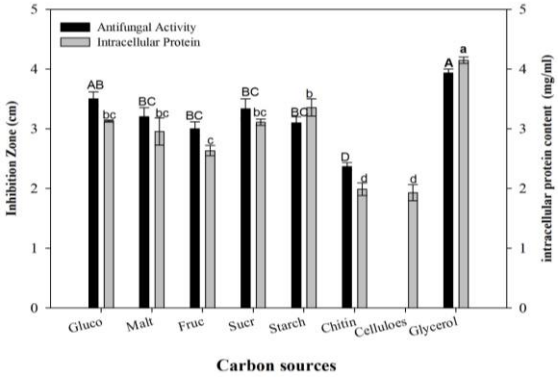

Fig 5. B. Effect of different carbon sources on antifungal activities and intracellular protein content of *Streptomyces gougeroti* against *M. phaseolina*

The highest antifungal activity against *Macrophomina phaseolina* and *Alternaria alternata* were recorded when beef extract was used as the nitrogen sources (4.16 ± 0.09 cm and 4.56 ± 0.03 cm respectively).

The maximum intracellular protein content of *Streptomyces gougeroti* was occured in presence of beef extract (3.46 ± 0.03 mg/ml). Casein could not stimulate the growth of *Streptomyces gougeroti* (Figure 6 A & B).

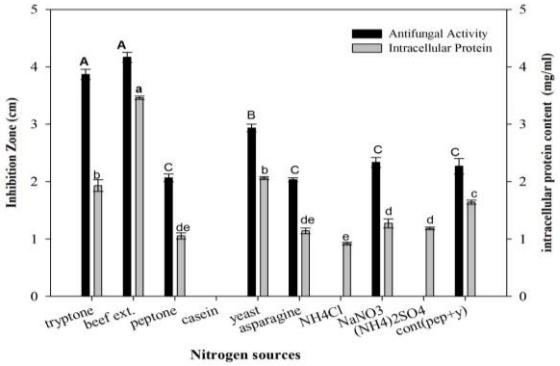

Fig 6.A. Effect of different nitrogen sources on antifungal activities and intracellular protein content of *Streptomyces gougeroti* against *A. alternata*

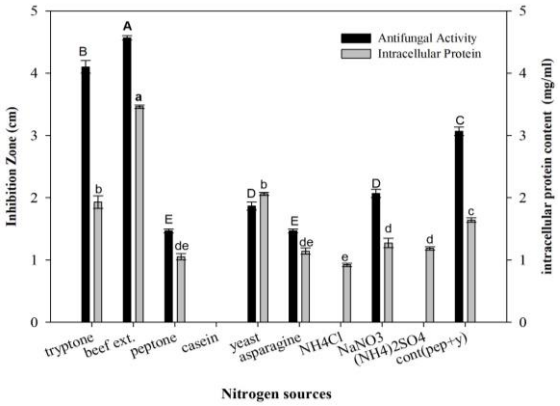

Fig 6.B. Effect of different nitrogen sources on antifungal activities and intracellular protein content of *Streptomyces gougeroti* against *M. phaseolina*

Discussion

Biological screening of *Streptomyces* sp. is carried out throughout the world for the determination of their antifungal activity (Bressan and Figueiredo 2003; Cao et al. 2005; Bonaldi et al. 2014 and Zhang et al. 2020). This study was performed to isolate and screen *Streptomyces* isolates from soil possessing antifungal activities and optimize their antifungal activities.

Starch nitrate agar medium used for isolation of *Streptomyces* sp. from soil samples (El-Naggar 2015; Atta et al. 2009; El-Naggar et al. 2009; Korayem et al. 2015). Also, Starch-casein agar medium was used to isolate actinomycetes (El Karkouri et al. 2019; Hasani et al. 2014). Some *Streptomyces* isolates could grow on glycerol yeast-extract agar media (Bawazir et al. 2018; Duddu and Guntuku 2016).

Production of antifungal activity from *Streptomyces gougeroti* against *Alternaria alternata* and *Macrophomina phaseolina* increased reaching maximum values after four

days, after that, the production of antifungal activity was decreased. This result is similar to that of Abdelwahed et al., (2012) and Singh et al. (2017) who reported that antimicrobial activity of *Streptomyces* sp. against *Macrophomina phaseolina, Fusarium oxysporum, Colletotrichum truncatum* and *Rhizoctonia solani* was after four days. In this study, *Streptomyces gougeroti* showed maximum antifungal activity at 35 °C. The present result was agreed with James et al. (1991); Sajid et al. (2011) and Bundale et al. (2015) who reported that *Streptomyces thermoviolaceus*, *Streptomyces malachitofuscus* and *Streptomyces coeruleorubidus* showed maximum antifungal activity at 35 °C. The highest yield of antifungal activity of *Streptomyces gougeroti* was when pH of the medium was from 6.5 to 7.0. This result is in accordance with Khair (2011) and Sajid et al. (2011) for *Streptomyces malachitofuscus* against *Candida albicans* and *Mucor miehei*.

The best carbon source for production of antifungal activity from *Streptomyces gougeroti* against *Alternaria alternata* and *Macrophomina phaseolina* was glycerol. This result is in agreement with that obtained by Grahovac et al. (2014) and Hasani et al. (2014). Glucose is certainly one of the best sources of carbon in the medium for antifungal production, this result is similar to Tarhan et al. (2011) and Khair (2011). On the other hand, maltose and sucrose showed the highest antifungal activity from *Streptomyces albidoflavus* against *Rhizoctonia solani* Islam et al. (2009) and Laidi et al. (2008). The optimal production of antifungal activity from *Streptomyces gougeroti* against *Alternaria alternata* and *Macrophomina phaseolina* were obtained when beef extract used as nitrogen source as recorded by Oskay (2011). Organic sources of nitrogen as peptone, yeast extract, and soybean meal gave better antifungal activity than other inorganic sources (Mitrović et al. 2017). In contrast to these results, maximum antimicrobial activity was obtained in culture supplemented with asparagine and potassium nitrate as nitrogen sources according to Thakur et al. (2009).

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الملخص العربي

عنوان البحث : الظروف المثلى لاستربتوميسس جوجيرتى كعوامل تحكم بيولوجية محتملة ضد مسببات الأمراض الفطرية للنباتات

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تعتبر الأكتينوميسيتات من المصادر الهامة لانتاج مركبات ايض حيوية عديدة و بخاصة من جنس استربتوميسس، هذه الدراسة تهدف لعزل بعض انواع الأكتينوميسيتات من التربة، و تصفية الكائنات المعزولة التى لها نشاط بيولوجى ضد بعض الأنواع من الفطريات الممرضة للنبات، أوضحت النتائج أن استربتوميسس جوجيرتى أقوى الكائنات المنتجة للمواد الضد فطرية، و كانت الظروف المثلى للانتاج كالتالى، أفضل وسط غذائى هو glycerol-yeast**، فترة التحضين ٤ ايام، رقم الأس الهيدروجينى ٦,٥، درجة الحرارة المثلى ٣٥°م وثبت أن الجليسرول و مستخلص اللحم أفضل مصدر كربونى و نيتروجينى على التوالى.**