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Isolation, Evaluation and Molecular Identification of *Streptomyces* Isolates with Antimicrobial Activities

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ABSTRACT

Out of sixteen *Streptomyces* sp. isolates were isolated from root rhizosphere soil of tomato plants. Four isolates coded; C1, 8SW, 30SW and 11SA were selected as the highest antibiotic productivity. The highest records of the antibiotic productivity were 21 and 25 mm as inhibition zones obtained from the original strains 8SW and 11SA after 10 days using *E. coli* DH-5α as a tester. The highest records of the antibiotic productivity were 12 mm and 11 mm when used *Aspergillus niger* and *Saccharomyces cerevisiae* as testers. Three of the four tested *Streptomyces* isolates i.e. C1, 8SW and 11SA exhibited highly inhibited (31 mm) of the two fungal plant pathogens. Using RAPD and SRAP marker the clustering of tested isolates were different from each other, RAPD marker could be grouped isolates (C1 and 8SW), (30SW and 11SA) to two clusters. However, SRAP grouped isolates (C1, 8SW and 30SW) in one cluster, however isolate 11SA clustered separately. Based on DNA sequences using 16S rDNA the data showed that isolates C1 and 8SW belonged to *Streptomyces albidoflavus* and isolates 30SW and 11SA were belonged to *Streptomyces bobbili.* For more accuracy the isolate C1 aligned with all *Streptomyces* in gene bank and confirmed in belonged to *S. albidoflavus.*
Keywords: Antimicrobial, DNA sequences, RAPD, SRAP, *Streptomyces*, 16S rDNA.

INTRODUCTION

Streptomyces sp. a saprophytic gram-positive bacterium as a kind of Actinomycetes, it used as bio-control tools (Raaijmakers *et al.,* 2002; Qiu *et al.,* 2009; Zarandi *et al.,* 2009). It has antagonistic activity against a large number of plant pathogenic microbes (Oliveira *et al.,*2012). Also, it can reduce the extent of infection caused by pathogens (Neeno-Eckwall *et al.,* 2001).One of the isolated antifungal products was characterized as a cyclic depsipeptide, valinomycin by spectroscopic assay. The species of *Streptomyces* are the origin of thousands of bioactive materials and screening programs have shown that secondary metabolites can be extracted (Kom-Wendisch and Kutzner, 1992; Santos *et al.,* 2012). *Streptomyces* species can often be classified from the other threadlike actinomycetes on the colonies morphological characteristics (Korn-Wendisch and Kutzner, 1992), especially by mycelium aerial spore and color obtained by soluble pigment.

The species of *Streptomyces* were identified according to their cultural features and spore chain morphology and surface of spore. *Streptomyces* spores were investigated by scanning and transmission electron microscopic system (Skujin, *et al.,* 2002). The successful use of scanning electron microscope in studies of actinomycetes was made by (Williams and Davies, 1967). The characters chosen and used in that work Shirling and Gottlieb,(1966) are as follows: spores morphology, sporophores morphology, soluble pigment production, reverse side color of the vegetative mycelium, melanin reaction, aerial mycelium color, and nine different sugars utilization. These characteristics were assessed as reliable and constant, based on an analysis of a previous international cooperative study Kuster, (1961).

PCR has rapidity, sensitivity and specificity for fingerprinting and proved extremely useful in detecting the variability in many organisms, and can also gave useful phylogenic data. At the level of molecular many studies identify some isolates of *Streptomyces* using many techniques (Mohamed *et al.,* 2012). Recently, the random amplified polymorphic DNA (RAPD) and sequence related amplified polymorphism (SRAP) were used in identification of most of living organisms, SRAP system was developed by Li and Quiros (2001) to anneal with overlapping non-coding and coding regions of the different genomic materials. It recognized as a powerful marker system used in genetic map construction and genealogical classification. RAPD fingerprints allowed us to successfully discriminate the genetically distinct bacterial isolates (Oliveira, *et al.,* 2012).Moreover, Al-Kahtani *et al.,* (2008) reported that RAPD procedure can be a useful method to distinguish *Streptomyces* species, and appears to be a simple, quick and sensitive technique for the characterization of other local *Streptomyces*.

Classification and identification of *Streptomyces* have been elucidated by genotypic and phenotypic approaches (Anderson and Wellington 2001). Moreover, Ribosomal RNAs are the molecules most widely employed in phylogenetic studies, with 16S rDNA used in prokaryotes and 18S rDNA used in eukaryotes (Weisburg *et al.,* 1989). Over several years, the 16S rRNA gene (16S rDNA) has been sequenced in many prokaryotes, and the importance of this molecule has increased since its use in the current phylogenetic classification. 16S rDNA nucleotide sequence is a highly conserved and well-constructed accurate tool for characterization and taxonomic of unknown or newly isolated microbes up to the species level and used as a source for determining phylogenetic and evolutionary relationships among microorganisms (Mehling, *et al.,* 1995). This study aim was to isolate and identify some antimicrobial producing by *Streptomyces* sp. isolates from soil rhizosphere of Egypt against *Fusarium verticillioides* and *Fusarium solni* as plant pathogens. In addition, morphological, cultural, physiological, microscopically identification and molecular PCR and 16S rDNA sequence analysis of highly antibiotic producer isolates.

MATERIALS AND METHODS

Soil sample: The soil was collected from root rhizosphere of tomato plants cultivated in Klein district Kafr El-Sheikh Governorate Egypt.

Microorganisms used: The typical reference strains were as follows: The *Bacillus subtilis* NRRL 543, *Micrococcus letueus* NRRL 287, *Saccharomyces cerevisiae* NRRL139, *Candida utili*s NRRL1084 and *Asergillus niger* NRRL326 were obtained from Northern Regional Research Laboratories (NRRL), Peoria, IL, USA. *Fusarium verticillioides* Z-Kh-F4 (GenBank accession number MF373436) and *Fusarium solni* Z-Kh-F11(GenBank accession number MF373443) as a plant pathogens were obtained from Applied Microbial Genetics Lab., Genetics and Cytology Dept., National Research Centre, Cairo, Egypt. Luria-Bertani (LB) medium was used for cultivation and sub-culturing of bacterial testers. Yeast extract-peptone-dextrose growth medium (YEPD) was applied cultivation and sub- culturing of *Saccharomyces cerevisiae* and *Candida utili*s. Potato Dextrose (PD) was used for cultivation and sub- culturing of the molds fungal strains.

Isolation and screening of the antibiotic producing *Streptomyces* isolates:

The media used to isolate of antibiotic producing *Streptomyces* isolates were inorganic salt starch agar (ISSA), oatmeal agar and starch casein media (Kuster and Williams, 1964). Three soil grams was collected from root rhizosphere of tomato plants; and mixed with sterile water (100 ml) in a conical flask, shaken well then leave without shaking for 30 minutes to precipitation. The necessary dilution (up to 10^{-5}) was made with this original solution. The dilution (0.1 ml) were used for inoculating 15 ml of ISSA medium at 45-50°C in Petri plates and the plates were incubated at 30°C. Observation was done at 5-7 days intervals to detect any colony surrounded by a clear zone of inhibition. The isolated *Streptomyces* isolates were purified through repeated plating. Streaking on ISSA and oat meal agar was used for these purposes. For further analysis, the purified isolates were re-cultured onto starch casein agar.

Fermentation media for antibiotic production:

Three fermentation media (FM) according to Waksman, 1959, Singh *et al.,* 2009 and Pandey *et al.,* 2005, FM1, FM2 and FM3 respectively. The flasks containing production medium were examined for antibiotics by a paper disk diffusion assay.

Disk-diffusion assay:

The collected culture filtrate for each isolate of *Streptomyces* was used to prepare antibiotic disks by adding 30 µl to filter paper disc (6-mm) then left to dry under aseptic conditions. The bacterial and fungal tester strains were grown in conical flask containing 50 ml of broth medium. The flasks were incubated at appreciate temperature for 24 h and 3days according the tester strain. On surface plates containing solid medium, 2 ml of suspension was placed and spread carefully with swab. Three antibiotic disks of each *Streptomyces* isolate were placed onto agar plates and used for each treatment. Plates were incubated as above condition. The diameters of inhibition zones were measured after 48 h.

Cultural and Morphological characteristic of *Streptomyces* isolates:

The taxonomic properties of promising *Streptomyces* isolates were evaluated following light, electron microscope; Jeol model, 2100 and methods given in the International *Streptomyces* Project (ISP) according Shirling and Gottlieb,(1966).Cultural and morphological characteristics of the selected isolates of *Streptomyces* (isolates; C1, 8SW, 30SW and 11SA) were detected by naked eyes examination for 14 day old cultures cultured on various media and methods the ISP by Shirling and Gottlieb,(1966). The mature determination of aerial mycelium and the soluble pigment production were recorded on ISP 5, aerial mass color on ISP 3 and ISP 4 following incubation at 30°C. The properties of the spore bearing hyphae were determined by direct examination of the culture surface (21 days) on opened dishes of the cross hatched strains by light microscope using 100 x magnifications. Moreover, ultra structural features as spore surface topology were evaluated by use of Transmission Electron Microscopic (TEM) (Arai, 1997; Keiser, *et al.,* 2000). The spore surface topology was examined under TEM; Jeol model, 2100 at magnification of 8000 to 100000 x.

Physiological and biochemical characteristics:

The criteria of physiological properties such as the ability to degrade starch, casein, tyrosine, coagulation of skim milk and gelatin liquefaction as substrates were studied for genus confirmation. The different carbon utilization, nitrogen sources and urea utilization were examined in order to reach a possible classification to species (Cappuccino and Sherman, 2005; William *et al.,* 2012). The carbon utilization test was carried out in carbon utilizing medium (ISP 9) as described in the ISP, with the addition of one of the following sugars: glucose (control), sucrose, arabinose, xylose, sorbose, fructose and lactose in addition other compounds as a sole carbon and ammonium sulfate (control), ammonium carbonate, ammonium chloride, potassium nitrate and ammonium nitrate compounds as a sole nitrogen were examined (Gordon *et al.,* 1974). The ability to utilize nitrogen sources was determined in a basal medium containing glucose 10 g, $MgSO4.7H_2O$ 0.5 g, $FeSO4.7H_2O$ 0.01 g, K_2HPO4 1.0 g, NaCI 0.5 g, Agar 3.0 g and distilled water 200 ml; after 15 days. The production of the melanin pigment tyrosine yeast extract agar (ISP 7) for the color examination of brown to black pigment (+ positive or - negative).

DNA extraction and PCR assay:

Total DNA was extracted using i-genomic BYF DNA extraction Mini Kit, iNtRON Biotechnology Inc., Concentration of the obtained DNA (ng/µl) and purity were calculated by Nanodrop-photometer. Eighteen primers (ten for RAPD and eight for SRAP) were used in this study (Table 1). Polymerase chain reaction (PCR) amplification was done according El Fadly *et al.,* (2016). The SRAP and RAPD products were separated by 1.5% agarose gels electrophoresis, which run with 1X TAE buffer. The bands weights were recorded by 100bp DNA ladder which was also run on each gel as a molecular weight standard marker.

Photo documentation was performed by Gel Documentation System under UV light.

The amplification of 16S rDNA gene was done from bacterial genomic DNA by PCR with forward primer FP1 (5'-AGA GTT TGA TCC TGG CTC AG-3') and reverse primer RP2 (5'-ACG GCT ACC TTG TTA CGA CTT-3') designed by Weisburg *et al.,* (1991). PCR was run in a total volume of 50 µl containing 40-80 ng DNA according El Fadly *et al.,* (2016). The PCR products were electrophoresis in 1.2% (w/v) agarose gel mixed with Red safe dye. The 16S-PCR purified products of the tested strains were assayed for nucleotide sequence determination by using ABI PRISM® 3500XL DNA Sequencer (Applied Biosystems). The final step only was done through Sigma Scientific Services Co., 6th October City, Giza, Egypt.

Table 1. The primers of RAPD and SRAP used and their nucleotide sequences:

RAPD 5′--3′			
Opa-04	AAT CGG GCT G	Opa-12	TCG GCG ATA G
Opa-5	AGG GGT CTT G	Opa-13	CAG CAC CCA C
Opa-06	GGT CCC TGA C	Opa--14	TCT GTG CTG G
Opa-10	GTG ATC GCA G	Opb-01	GTT TCG CTC C
Opa-11	CAA TCG CCG T	Ope-02	GGT GCG GGA A
SRAP 5′--3′			
me1+em1	TGA GTC CAA ACC GGA TA	GAC TGC GTA CGA ATT AAT	
me1+em2	TGA GTC CAA ACC GGA TA	GAC TGC GTA CGA ATT TGC	
me1+em3	TGA GTC CAA ACC GGA TA	GAC TGC GTA CGA ATT GAC	
me1+em4	TGA GTC CAA ACC GGA TA	GAC TGC GTA CGA ATT TGA	
me2+em1	TGA GTC CAA ACC GGA GC	GAC TGC GTA CGA ATT AAT	
me2+em2	TGA GTC CAA ACC GGA GC	GAC TGC GTA CGA ATT TGC	
me2+em3	TGA GTC CAA ACC GGA GC	GAC TGC GTA CGA ATT GAC	
me2+em4	TGA GTC CAA ACC GGA GC	GAC TGC GTA CGA ATT TGA	

Data analysis and phylogenetic analysis:

The RAPD and SRAP profiles obtained after PCR amplification for tested isolates were compared with each other and DNA bands were recorded as a binary data. Each band was recorded as absent (0) or, present (1) and pairwise comparisons between individuals were made to calculated according Jukes-Cantor coefficient using PAST program adapted by Hammer *et al.,* (2001). To produce a dendrogram using neighbor-joining (NJ) trees, the cluster analysis was used (Saitou and Nei, 1987). DNA sequences for selected closely related four isolates partial DNA were checked and assembled using the ATGC program ver. 4 (GENETYX CORPORATION). Sequence adjusted manually using BioEdit program (Hall, 1999), and alignments with CLUSTAL X program (Thomson et al., 1997). 16S rDNA Partial sequences were used to GenBank database search with the Blast N algorithm to exhibit the relative phylogenetic bands to infer relationships among four isolates. The alignments of the sequences were done against corresponding nucleotide sequences retrieved from GenBank. The editing of the obtained tree was done using Tree View (Page, 1996).

RESULTS AND DISCUSSION

Antibiotic production by selected *Streptomyces* isolates:

A total of sixteen isolates of *Streptomyces* were obtained from rhizosphere of tomato plants. The most colonies that detected on ISSA plates were belonged to the genus *Streptomyces* since the colonies were slowly grown. The growth was observed after 48 hrs of incubation to produce the antibiotics.

In Table (2) data clearly exhibited that the four selected isolates as the highest records of the antibiotic productivity. The productivity on FM 1 was 18 and 22 mm which were obtained from the original isolates 8SW and 11SA for 5 days when used E. *coli* DH-5α as a tester. Also, isolate C1 produced antibiotic on all fermentation media when used *Micrococcus luteus* as a tester. Moreover, isolate 30SW produced antibiotic on the FM 2 and FM 3 but this isolates did not produce any antibiotic on FM1. On the other hand, all of the tested strains did not produce any antibiotic when used the fungal strains as testers except for isolate C1. In general, the FM1 proved to be the best one since the most of the tested isolate produced higher amounts of the antibiotic on this medium. The following medium for the high antibiotic productivity of most isolates was FM2.

Table (3) and Figures (1, 2 and 3) clearly showed that the same trend which recorded in the Table (2) but the antibacterial productivity was slightly increased. On the other hand, the antifungal production was increased (with 10.23% more than the production after 5 days).

Table 2. Effect of different fermentation media on antibiotic production by four *Streptomyces* isolates cultured in shake flasks as a batch fermentation after 5 days of incubation.

Code of *Streptomyces* isolates	F M1					F M2					F M3				
	DH	M	A	C	Y	DH	M	A	C	Y	DH	M	A	C	Y
C1	15	13	8	7	7	15	12	0	7	7	13	14	0	0	0
8SW	18	0	0	0	0	0	0	0	0	0	13	0	0	0	0
30SW	0	0	0	0	0	14	0	0	0	0	12	0	0	0	0
11SA	22	0	0	0	0	12	0	0	0	0	12	0	0	0	0

DH=*E. coli* DH-5α; M= *Micrococcus luteus*; A= *Aspergillusniger*; C=*Candida utilis*; Y= *Saccharomyces cervisiae.*

Table 3.Effect of different fermentation media on antibiotic production by four *Streptomyces* isolates cultured in shake flasks as a batch fermentation after 10 days of incubation.

Code of *Streptomyces* isolates	F M1					F M2					F M3				
	DH	M	A	C	Y	DH	M	A	C	Y	DH	M	A	C	Y
C1	18	18	12	9	11	15	13	0	7	8	16	14	0	0	0
8SW	21	0	10	0	0	0	0	0	0	0	13	0	0	0	0
30SW	0	0	0	7	7	14	0	0	0	0	12	0	0	0	0
11SA	25	0	0	0	0	13	0	0	0	0	14	0	0	0	0

DH=*E. coli DH 5α*; M= *Micrococcus luteus*; A= *Aspergillusniger*; C=*Candida utilis*; Y= *Saccharomyces cervisiae*.

Table 4.Effect of different fermentation media on antibiotic production by four *Streptomyces* isolates cultured in shake flasks as a batch fermentation after 15 days of incubation

Code of *Streptomyces* isolates	F M1					F M2					F M3				
	DH	M	A	C	Y	DH	M	A	C	Y	DH	M	A	C	Y
C1	18	16	11	8	10	16	16	10	10	11	16	15	0	0	0
8SW	23	0	10	0	0	0	0	0	0	0	13	0	0	0	0
30SW	0	0	0	8	9	14	0	0	0	0	16	0	0	0	0
11SA	24	0	0	0	0	13	0	0	0	0	16	0	0	0	0

DH=*E. coli DH 5α*; M= *Micrococcus luteus*; A= *Aspergillus niger*; C=*Candida utilis*; Y= *Saccharomyces cervisiae.*

The obtained results confirmed that the productivity was increased and the incubation of 10 days led to the high production of both antifungal and antibacterial agents. Table (4) presents the antibacterial and antifungal productivities of *Streptomyces* isolates after 15 days of incubation. The obtained results showed since the majority of the tested isolates produced antibiotic at the same of the production in Table (3) or less. The obtained results confirmed that the productivity was fixed and the incubation of 10 days was optimized for the high production of both antifungal and antibacterial agents.Using *Streptomyces* isolates from nature has been recommended as the method of choice for increasing the antibiotic productivity (Don, *et al.,* 1993; Cao, *et al.,* 2004 and Gebreselema, *et al.,* 2013). The obtained data were in agreement with those by Don, *et al.,* (1993). They reported that, five isolates of actinomycetes were very strong antagonists of the *Pythium ultimum*, four isolates were strong antagonists, and others ten isolates were weakly antagonistic. The remaining isolates showed no antagonism by this assay. Moreover, Cao, *et al.,* (2004) exhibited that, 20% of endophytic *Streptomyces* isolates produced antibacterial materials and 41% produced antifungal agents. Also, 60% of most isolated strains inhibit the growth of *Rhizoctonia solani* by antimicrobial testing, but only 32% produce active substances against *R. solani.*

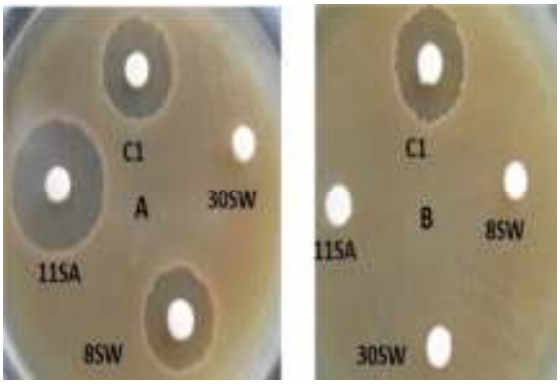

Fig. 1. The antibacterial production on FM1 by four *Streptomyces* isolates against *E. coli* DH-5α(A) and *Micrococcus luteus* (B).

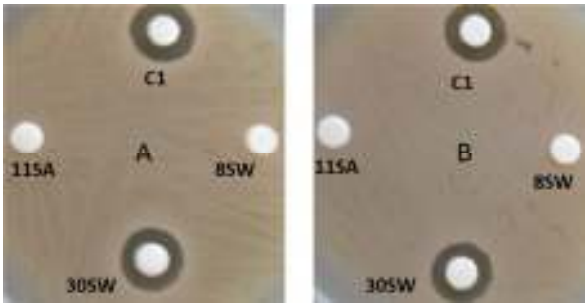

Fig. 2, The antifungal production on FM1 by four *Streptomyces* isolates against *S. cerevisiae* (A) and *Candida utilis* (B)

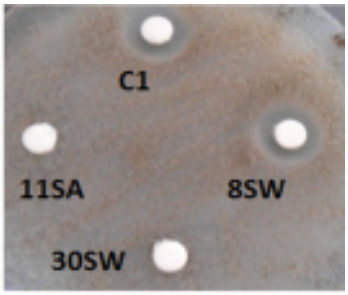

Fig. 3. The antibacterial production on FM1 by four *Streptomyces* isolates against *A. niger*

Activity of *Streptomyces* isolates antagonistic against some plant pathogens:

Studying antagonism against fungal pathogens of various plant diseases is very important for identification of antibiotic spectrum secreted by selected isolates. So, four *Streptomyces* isolates were showed antagonistic activity against *Fusarium verticillioides* and *Fusarium solni* as plant pathogens (Fig. 4). The obtained results showed that, the three of the four tested *Streptomyces* isolates were exhibited highly inhibited (31 mm) of the two fungal plant pathogens used. Similar results have been reported previously in *Streptomyces* screening studies (Hyo *et al.,* 2006; Valois *et al.,* 1996), they isolated, the *Streptomyces* strain VC-A46 was produced antifungal activity against the plant-pathogenic fungi *Alternaria mali*, *Colletotric humorbiculare*, *Magnaporthe grisea*, *Fusarium oxysporum* f. sp. *lycopersici* and *Rhizoctonia solani* and antioomycete activity against *Phytophthora capsici*.

It is proposed that strain VC-A46 represents the type strain of a novel species, named *Streptomyce scheonanensis* after molecular identification process. Furthermore, Houssam (2009) isolated the active metabolites produced by actinomycete culture*;* AZ-SH514 was exhibited various degrees of activities against unicellular and filamentous fungi. The antifungal activity was produced by AZ-SH514 strain exhibited maximum inhibitory activity against *Saccharomyces cervisiae* ATCC 9763 (23.4 mm) *Candida albicans,* IMRU 3669 (31.25 mm), *Aspergillus niger* IMI 31276 (31.25 mm) and *Fusarium oxysporum* (31.25 mm) and minimum inhibitory activity was observed with *Rhizoctonia solani* (41.6 mm),*Aspergillus fumigates* ATCC 16424 (46.9 mm), *Alternaria alternate* (46.9 mm), *Aspergillus flavus*(52.7 mm) and *Botrytis fabae*(52.7 mm).

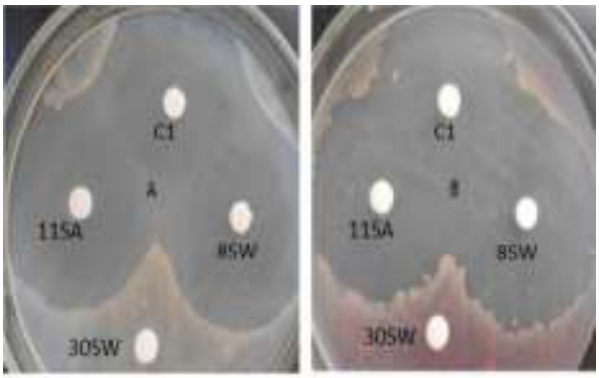

Fig. 4. Antagonistic activity on FM1 by four *Streptomyces* isolates against *Fusarium verticillioides* (A) and *Fusarium solni*(B) as a plant pathogens.

Identification of the *Streptomyces* isolates:

Morphological and cultural characters of the selected *Streptomyces* isolates (C1, 8SW, 30SW and 11SA) based on produced antibiotics as reflected by zones of growth inhibition among other inhabitants of soil samples. The cultural and morphological characteristics of the isolates were examined after incubation for 7-14 days at 30°C on various media are presented in Table (5). The isolates grow well on most of the starch inorganic salt and ISP tested media, the aerial mycelium grown well on most of the ISP media. The aerial mycelium color, color of the substrate mycelium, and diffusible pigment were very useful (especially with the genus *Streptomyces*). Hence, the conventional methods described by Korn-Wendisch and Kutzner (1992) were highly recommended. Particularly the microscopic characterization of aerial mycelium morphologies, arthrospores and vegetative mycelium were high valuable. The Spore chains were Rectiflexibiles for isolates; C1 and 8SW while, the spores chain for the isolates 30SW and 11SA were Spirales. Moreover, the mature spore chains were short for isolates; C1 and 8SW while long for the isolates 30SW and 11SA. The spore surface was examined by electron microscopy was smooth for all the isolates of *Streptomyces.*

Based on the activity of *Streptomyces* isolates antagonistic against some plant pathogens, the four isolates were identified as *Streptomyces* sp. The isolates were Gram positive with aerial mycelium and most of them were filamentous with short and long chain of spores (Fig. 5). The detailed results of biochemical and physiological assays of the isolates are shown in Tables 6. Similar results were followed by Rahman, *et al.*, (2011); Shirling and Gottlieb (1966) for the *Streptomyces* sp identification from soil. The most important physiological criteria used for taxonomical characterizations were: Carbohydrates utilization of were investigated on ISP 9 medium using glucose as positive control. The nitrogen sources utilization was determined in a basal medium after 15 days.

The biochemical and physiological characteristics were determined using the study described by Williams *et al.,* (1983 a&b). All tests were done at 30°C. (Shirling& Gottlieb 1966) and the Bergey's Manual of Systematic Bacteriology (Williams *et al.,* 1983a).The observed data of physiological and biochemical tests of the isolates are recorded in Table 6.

Molecular analysis of superior *Streptomyces* isolates;

Molecular characterization of *Streptomyces* sp. isolates was done by 16S rRNA gene amplification; this exhibited the molecular weight of 1.5 kb and sequenced the1.5 kb of DNA fragment after purification from agarose gel. Further analysis including BLAST search and phylogenetic tree were accomplished to correlate the selected *Streptomyces* sp. isolates with other species of the genera in the database conserved library. The 16S rDNA sequences of the four *Streptomyces* isolates C1, 8SW, 30SW and 11SA were saved in the NCBI Gene nucleotide sequence database with the accession numbers MK256478, MK256479, MK256480 and MK256481.

Data in Figures 6 and 7 shows that the amplified fragments number differed with used primers, the sizes and number of amplified fragments differed from one strain to another with the same primer. A total number of 75 amplified fragments were obtained using the ten RAPD-PCR with36 bands and eight SRAP primers, which showed an obvious amplification for each isolates. The identification of bacteria according to phenotypic characteristics is generally not as accurate like the identification based on genotypic protocols.

The sequences comparison of the obtained bacterial 16S rRNA gene has approved as a preferred genetic method (Clarridge, 2004). More PCR assays like RAPD and SRAP were used in some studies to detect differentiation and fingerprinting of actinomycetes (Mohamed *et al.,* 2012).

Using RAPD marker the isolates were differ and RAPD grouped isolates C1 and 8SW), (30SW and 11SA) to two clusters as shown in Fig. 8 with different similarities as shown in Table 7. However, SRAP grouped isolates (C1, 8SW and 30SW) in one cluster; however isolate 11SA clustered separately as shown in Fig. 9 with different similarities as shown in Table 7. Based on DNA sequences using 16 S rDNA fragments of ~ 1500 bp were amplified using the forward and reverse primers as shown in Fig 10. Partial sequences with 1009 bp were aligned and compared with *Streptomyces and Actinomycetes* strains and four bacterial strains (Fig 11), results show that the isolates C1, 8SW and 30SW and 11SA. Moreover, C1 and 8SW isolates belonged to *S. albidoflavus*, however isolates 30SW and 11SA belonged to *S. bobbili*. For more accuracy the isolate C1 aligned with all *Streptomyces* in gene bank using BLAST and confirmed in belonged to *S.*

albidoflavus as shown in Fig. 12. Based on the phenotypic (Shori *et al.,* 2012) and genotypic (16S rRNA gene) characteristics, the strain could be identified as a new strain of *S. albidoflavus*. Similar result was found by Al-Askar *et al., (*2011) who identified *Streptomyces* isolate from Saudi Arabia as *Streptomyces* according to analysis of 16S rRNA gene sequence.

Table 5. Morphological characteristics of the four isolates of *Streptomyces* spp.

Characteristics / Media	C1		8SW		30SW		11SA	
	Color of aerial mycelium	Color of compact mycelium	Color of aerial mycelium	Color of compact mycelium	Color of aerial mycelium	Color of compact mycelium	Color of aerial mycelium	Color of compact mycelium
Malt Yeast extract ISP* 2	Grey	Y-b	Grey	Greenish	-	Y -b	Grey	Y–b
Oatmeal agar (ISP 3)	Grey	Greenish	Green	Y –b +green	-	Grey	Grey	Y -b
Inorganic salt-starch agar (ISP 4)	Grey	Y –b	Grey	Green	Green	Yb	Grey	Y-b
Glycerol-asparagine agar (ISP 5)	White	Yellowish	White	Yellowish	-	Yellowish	Whitish	Yellowish
GYM (Glucose-yeast extract-malt extract) (TYA)	White	Y–b	White	Yellowish	Yellowish	Yellow	red	Brawn
Tyrosine yeast extract agar	Whitish	Y-b	Whitish	Brown red	Grey	Y - b	Yellowish	Yellow b
Potato dextrose agar(PDA)	Grey	Y –b	Grey	Y –b	Grey	Y –b	Grey	Y -b
Nutrient agar	White	Yellowish	White	Yellowish	-	Y-b	Whitish	Yellowish

*ISP: International *Streptomyces* Project **Y: Yellow ***b: brown **** - :no formation aerial mycelium

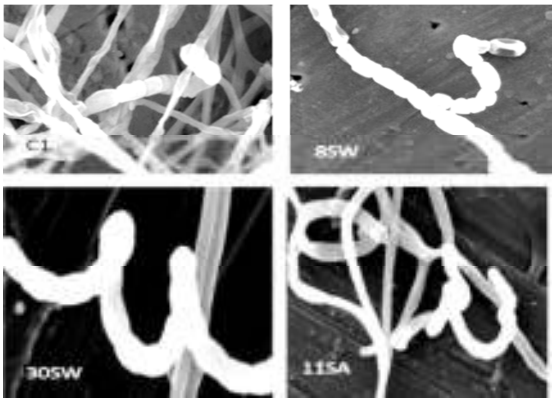

Fig. 5. Transmission electron microscopic (TEM) for electron micrographs of *Streptomyces* isolates C1; 8SW, 30SW and 11SA.

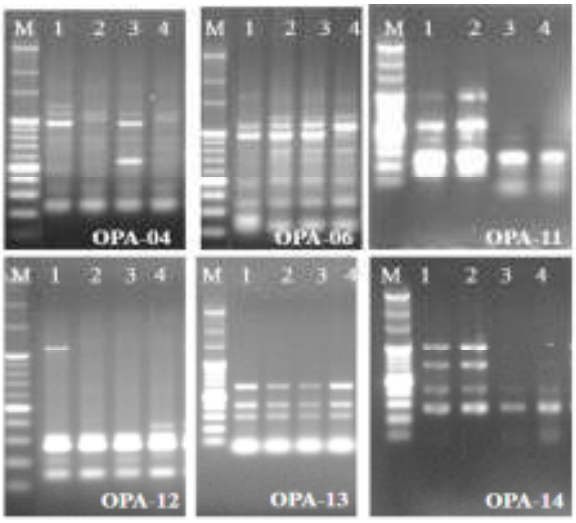

Fig.6. Banding pattern of RAPD (1=C1, 2=8SW, 3=30SW and 4=11SA), M marker,

Table 6. Physiological properties four isolates of the *Streptomyces* sp.:

Characteristics	Code of *Streptomyces is*olates			
	C1	8SW	30SW	11SA
Growth on sole carbon (1%, w/v)				
Glucose	+	+	+	+
Sucrose	-	-	+	+
Sorbose	+	+	+	+
Arabinose	+	+	+	+
Fructose	+	+	-	+
Xylose	+	+	+	+
Lactose	+	+	+	+
Raffinose	-	-	±	+
Mannitol	+	+	-	-
Glycerol	+	+	+	+
Inositol	-	±	+	+
Rhamnose	-	-	+	±
Growth on sole energy (0.1%, w/v)				
Sodium acetate	+	+	+	+
Sodium benzoate	-	+	-	-
Sodium citrate	+	+	+	+
Sodium succinate	+	+	+	+
Growth on sole nitrogen (0.1%, w/v)				
Ammonium sulfate	+	+	+	+
Ammonium carbonate	+	+	+	+
Ammonium chloride	+	+	+	+
Potassium nitrate	+	+	+	+
Ammonium nitrate	+	+	+	+
Enzymatic activity				
Hydrolysis of starch	+	+	+	+
Carboxy methyl cellulose	+	+	+	+
Hydrolysis of colloidal chitin	+	+	+	+
Liquefaction of gelatin	+	+	+	+
Coagulation of milk	+	+	+	+
Peptonization	+	+	-	-

+; utilized; ± poorly utilized; - not utilized

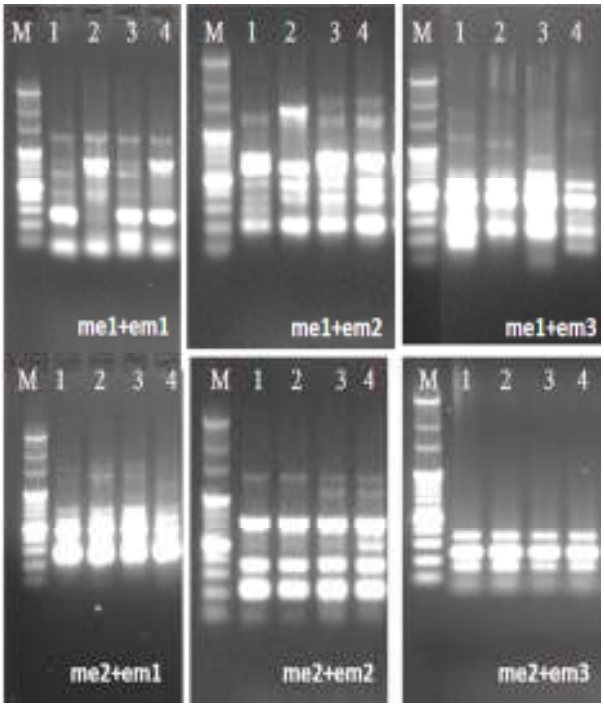

Fig. 7. Banding pattern of SRAP (1=C1, 2=8SW, 3=30Sw and 4=11SA), M marker,

Table 7. Distance matrix between the four *Streptomyces* strains (C1, 8SW, 30SW and 11SA) using RAPD (above diagonal) and SRAP (below diagonal)

0	11SA	30SW	8SW	C1
11SA	0	0.17	0.25	0.52
30SW	0.35	0	0.30	0.35
8SW	0.48	0.27	0	0.25
C1	0.39	0.27	0.31	0

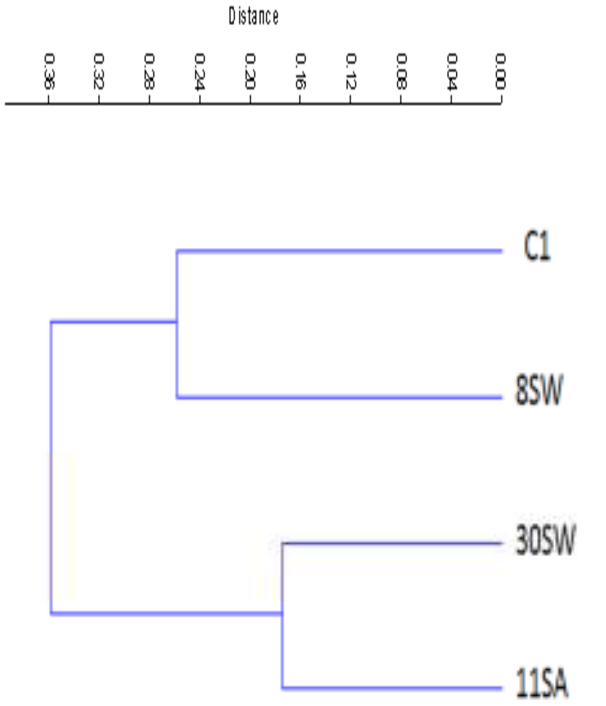

Figure 8. Dendrogram based on RAPD-PCR analysis of the four *Streptomyces* strains (C1, 8SW, 30SW and 11SA) using six RAPD-PCR primers.

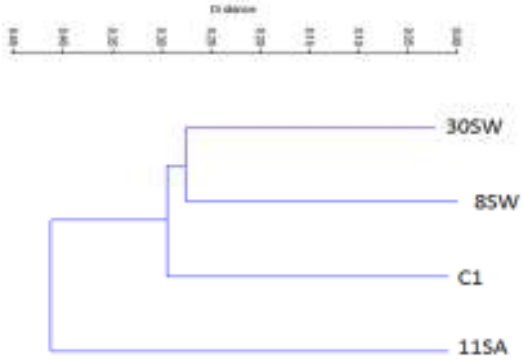

Fig. 9. Dendrogram based on SRAP-PCR analysis of four *Streptomyces* strains (1=C1, 2=8SW, 3=30SW and 4=11SA) using six SRAP primers.

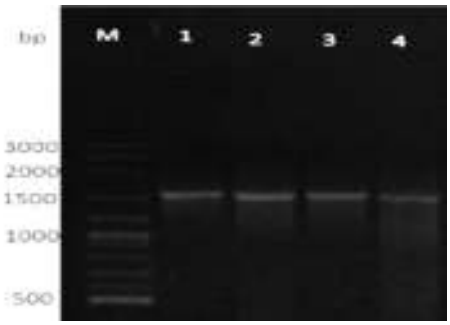

Fig. 10. Photograph of 16S-DNA amplified band for four *Streptomyces* strains (1= C1, 2=8SW, 3=30SW and 4=11SA) against 100 bp ladder DNA marker (lane M)

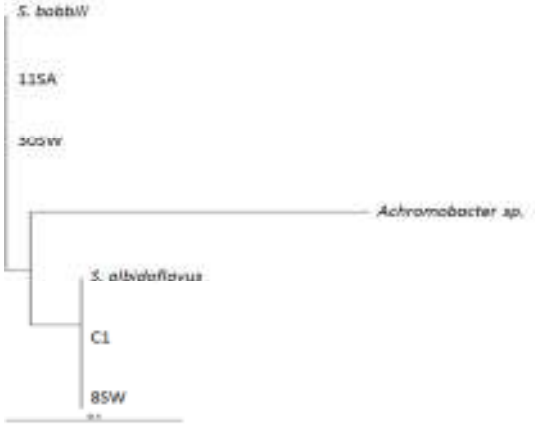

Fig 11. NJ Phylogenetic tree for four Streptomyces strains (C1, 8SW, 30SW and 11SA)using BiEdt compared with three strains.

Thenmozhi and Kannabiran (2010) reported that sequences of 16S rRNA gene have been helps to elucidate the evolutionary relationship of Streptomyces sp., and powerful tools for phylogenetic characterization. Moreover, selected Streptomyces isolates were grouped into divergent groups; *Streptomyces* isolates which were grouped in different clustered could be produce different antimicrobial agents (Intra *et al.,* 2011). These data suggested that selected Streptomyces isolates were diverse and suitable for antimicrobial production. Molecular characterization appears to be a useful method in identifying the isolates of bacteria according to the sequence of RNA genes. We can conclude that the application of the different molecular characterization protocols gives a better evolutionary relationship picture between species under the same genus

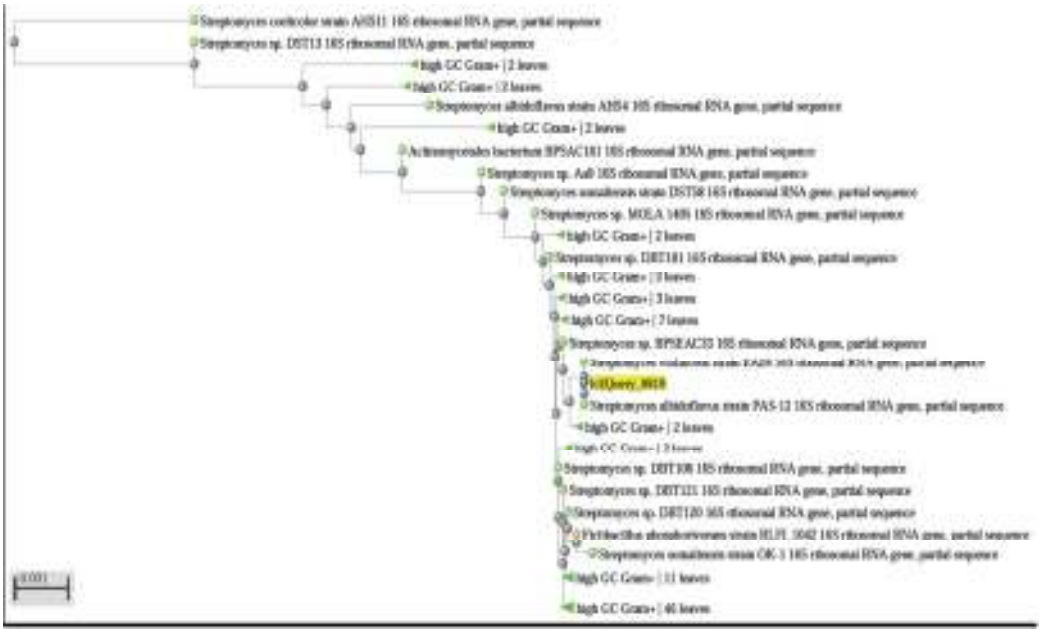

Fig.12. Phylogenetic tree of the nucleotide sequences of the PCR product of 16S rRNA gene amplified from the DNA of *Streptomyces* strain C1 and universal bacterial strains from BLAST.

CONCLUSION

This work showed that isolation and examination of cluster bacteria from Egyptian soil could serve as a potential renewable source of antimicrobial importance strains with different spectra of activity against different microorganisms. The precise characterization of the active substances of the antibacterial and fungal extracts is the subject of continuous investigation into our work.

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عزل وتقييم والتعريف الجزيئى لعزلات الاستربتوميسس النشطة فى انتاج المضادات الميكروبية

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مابين أكثر من 16 عزلة من الاستربتوميسس تم عزلها من منطقة المجموع الجذرى لنبات الطماطم المنزرعة بمحافظة كفر الشيخ– مصر، أربعة عزلات من الاستربتوميسس أعطت كودا (C1,8SW,30SW,11SA) كانت الاعلى فى انتاج المضادات الحيوية حيث كانت العزلات 11SA و8SW أعلى نشاطا فى انتاجية المضادات الحيوية وسجلت المنطقة الرائقة 21 و 25 ملم مع العزلة *E.coli* DH-5α وأيضا أظهرت أعلى نشاطا فى انتاجية المضادات الحيوية مع *Aspergillus niger* and *Saccharomyces cerevisiae* حيث بلغت المنطقة الرائقة11 و12ملم على الترتيب. وأظهرت ثلاثة عزلات من الاستربتوميسس نشاطا ملحوظا فى انتاجية المضادات الحيوية على سلالاتين الفطريات الممرضة للنبات حيث بلغت المنطقة الرائقة 31 ملم. *باستخدام طريقة RAPD و*SRAP لتعريف والتفريق بين العزلات الاربعة حيث أمكن وضع العزلة C1 و 8SW فى مجموعة واحدة وايضا وضع العزلة 11SA و 30SW فى مجموعة اخرى باستخدام RAPD بينما باستخدام SRAP تبين وجود تقارب بين ثلاث من عزلات الاستربتوميسس 30SW,C1,8SW وتنمى الى مجموعة واحدة فى حين أن العزلة 11SA تنمى الى مجموعه اخرى. وبمعرفة التتابع الجزيىء للمادة الوائية DNA لمنطقة 16S DNA أمكن تعريف العزلة C1 والعزلة 8SW حيث تنتمى للنوع *Streptomyces albidoflavus* بينما العزلة 30SW,11SA تنتمى للنوع *Streptomyces bobbili* ولتحديد دقة التعريف للعزلات تم ايفاد التتابع للعزلة C1 لبنك الجينات واكد على ان العزلة تنمى للنوع *Streptomyces albidoflavus*.