Antifungal activity of *Streptomyces Bungoensis* (BF26) Against *Alternaria Sesame*, *Fusarium Oxysporum* and *Rhizoctonia Solani* in *Vitro*.

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ABSTRACT

Many *Streptomyces* species are effective as biological controls for many pathogenic fungi. The research on new biocontrol principles for some phytopathogenic species that aren't as effective as traditional fungicides have sparked global interest. The *Streptomyces* genus is responsible for most of all known antibiotics. These are used in various fields such as medical and agricultural settings. *Streptomyces* was isolated from soils obtained from different sites in Egypt. Antifungal activity against phytopathogenic fungi was tested on 65 isolates, i.e., *Alternaria sesame*, *Fusarium oxysporum*, and *Rhizoctonia solani*. The *Streptomyces* isolate BF26 showed the highest antifungal activity against the investigated fungi by dual culture bioassay and paper disc diffusion techniques. Biochemical, microscopy, and morphological procedures were used to characterise them better. The results showed that the isolate BF26 was gram-positive and belonged to members of the *Streptomyces* genus. Based on DNA sequences using 16S rRNA, the data showed that the isolate BF26 belonged to *Streptomyces bungoensis*. The isolate BF26 was saved in the NCBI Gene nucleotide sequence database with the accession numbers ON130197.

Keywords: Phytopathogenic fungi, Antifungal activity, *Streptomyces bungoensis*.

INTRODUCTION:

In numerous countries around the world, plant diseases have caused epidemics, resulting in famines and economic losses. Phyto diseases are caused by some microorganisms such as fungi, bacteria, viruses and viroids. However, fungi are responsible for the majority of this diseases. Chemical fungicides are used to control phytopathogenic fungi. (Njoroge *et al*., 2018). However, due to overuse and misuse of chemical pesticides, these plant diseases have evolved resistance to fungicides. Agrochemical pollution of the environment, and disease resistance to pesticides have all caused concerns. As a result, there is a pressing need to research and develop novel techniques that are both safe and effective. Secondary metabolites with antibacterial activity produced by some microbes have shown promise in the treatment of plant diseases. (Davila *et al*., 2016). *Streptomyces* is a member of the Streptomycetales order of the actinobacteria class. (Whitman *et al*., 2012). This gram-positive bacterium is a filamentous and has a DNA G+C content of 63–78% mol (Kampfer, 2006). *Streptomyces* has been used for biotechnological and commercial purposes. Antibiotics, anticancer agents, immunosuppressive agents, and enzymes are all produced by this class of prokaryotes (De Lima Procopio *et al*., 2012; Nagpure *et al*., 2014; Aftab *et al*., 2015). *Streptomyces* is a particularly prolific genus accounting most of total antibiotic production (De Lima Procopio *et al*., 2012; Madigan and Martinko, 2007). In this study, *Streptomycetes* isolated from various Egyptian soils were tested for antifungal activity against phytopathogenic fungi.

MATERIALS AND METHODS:

Soil sampling:

Egypt's soil samples were obtained from various governorates. Different crops' rhizosphere soil was sampled such as *Beta vulgaris*, *Vicia faba*, *Triticum aestivum* and *Citrus aurantifolia*. The samples were air-dried at room temperature (Njoroge *et al*., 2018).

Isolation of the Streptomycetes:

Streptomyces isolates were isolated by the serial dilution plate technique according to (Yekkour *et al*., 2012; Njoroge *et al*. 2018) on ISSA medium plates . The apparent colonies produced on the plates were counted and peaked up to purification by more streaking on the same medium after incubation for 4–7 days at 28°C. (Hossain and Rahman, 2014; Shirling and Gottlieb, 1966). The purified *Streptomyces* isolates were streaked on differential agar slants and stored at 4°C for three months before being subcultured again (Njoroge *et al* ., 2018).

Cell Morphology and Cultural Characteristics:

At 30°C, the *Streptomyces* isolates were tested on yeast extract malt extract agar (ISP-

2), oatmeal agar (ISP-3), inorganic salt starch agar (ISP-4), and glycerol asparagine agar (ISP-5) media (Shirling and Gottlieb, 1966; Yekkour *et al*., 2012). After 7, 14, and 21 days after the inoculation, observations were made. According to the aerial mycelium morphology, color, presence of soluble pigment, and reverse color (Shirling and Gottlieb,1966; Pridham, 1965; Szabo and Marton, 1964; Sharma *et al*., 2014). The cover slip method was used for microscopic inspection, as detailed in (Radhakrishnan and Varadharajan, 2022; Daigham and Mahfouz, 2021; Sharma *et al*., 2014). Under a high power objective in the light, the arrangement of spores on mycelium was noticed. Observed by a transmission electron microscope (TEM) for direct examination of whole spores, carbon-coated grids were touched to the surface of mature aerial growth. Large numbers of spores adhered to the carbon film and could be observed with a JEOL-JEM 1010 transmission electron microscope (TEM) at 80 kV at the Regional Centre for Mycology and Biotechnology (RCMB), Al-Azhar University.

Physiological and biochemical characteristics:

A Gram test was finished by spreading the stock culture on a glass slide followed by heat drying. The smear was covered with crystal violet for 30-60 seconds and flushed off with water. From that point onward, it was covered with Gram's iodine for 30-60 seconds, then, at that point, decolorized with liquor and washed with water. At long last, the smear was stained with safranin counter stain for 2 minutes. It was flushed off with water and left to dry. The slides were seen at x100 oil drenching under the light magnifying lens. Carbon usage was accomplished by becoming the confines in a basal mode of the ISP9 medium. The wellspring of carbon was added by adding 1% of one or the other glucose, sucrose, soluble starch, maltose, or fructose. Nitrogen source use was finished by becoming the confines in a basal mode of ISP9 medium as per (Shoukry *et al*., 2019; Bhosale *et al*., 2015). The source of nitrogen was achieved by adding 0.2% of either $(NH_4)_2S0_4$-$NaNO_3$-KNO_3–Peptone-Yeast extract. And NaCl concentration was experimented by adding (0%-0.5%-1%-2%-2.5%) individually, and the PH value (6-7-8 and 9), different temperatures (25-30-35-40 °C). In addition, different incubation periods (7, 8, 9, 10, and 11 days) were tested. Growth was estimated as turbidity by utilizing a spectrophotometer to see how much growth is there (Bharti and Arora, 2007).

Melanin pigment formation:

The melanin pigment production was tested on agar slants of medium (ISP 6) Peptone-yeast extract iron agar and medium (ISP 7) Tyrosine agar medium using **a** heavy inoculum by standard wire loop and streaked on the agar slant medium. Each experiment was replicated as two slants of each medium. Melanin pigment was observed after two and four days compared with control. According to (Shoukry *et al*., 2019 ; Shirling and Gottlieb,1966), cultures forming a greenish brown, brown, black diffusible, or a distinct brown changed through different color will be recorded as positive (+) or negative (-) as absence of pigment.

***Streptomyces* Screening for Antimicrobial Activity:**

Antifungal activity was tested using three plant pathogenic fungi by the dual inoculation method. These are: *Alternaria sesame*, *Fusarium oxysporum*, and *Rhizoctonia solani*. For seven days, the fungal test pathogens were cultured in a Potato Dextrose Agar (PDA) medium; after that, an 8 mm disc plug was picked and positioned at the center of the PDA plate. At the same time, *Streptomyces* growth was obtained as a disc of 8 mm in diameter and placed opposite in the PDA medium containing fungal test pathogens. According to (Njoroge *et al*., 2018) antifungal activity around the *Streptomyces* agar discs was evaluated.

Submerged Cultures and Preparation of Crude Extract:

The active isolates were grown for 7 days at 28°C on a casein glycerol agar medium. This culture was transferred aseptically into 250ml Erlenmeyer flasks containing 50 ml Casein Glycerol Broth (CGB) in 0.6 cm diameter discs. For 12 days, the inoculation flasks were shaken at 130 rpm at 30°C using a rotary shaker (Shahid *et al*., 2021). The cells were extracted by centrifuging the broth culture for 20 minutes at 5000 rpm. A 0.2m pore size membrane filter (Millipore) was used to separate the free supernatant of the cells, and the filtrate obtained contained antibiotic secretion (Njoroge *et al*., 2018).

Antifungal Activity of the Culture Filtrate:

The paper disc diffusion method was used to test the antifungal activity of the culture filtrate. A 10^6-spore suspension of the fungus (*Fusarium oxysporum* and *Alternaria sesame*) was produced and distributed on PDA plates to determine antifungal activity (Njoroge *et al*., 2018). While *Rhizoctonia solani* was collected

and placed on the surface of a PDA plate for disc 8mm and left to grow, 3 ml of sterile distilled water was added to the plate growth surface, swapped prepared and spread plated on PDA plates. According to (Maiti *et al*., 2020), the paper disc diffusion method was used to test for antifungal activity. (Shoukry *et al*., 2019).

Separation of Antimicrobial bioactive Metabolites:

Solvent extraction was used to recover antimicrobial compounds from the filtrate, as reported by (Njoroge *et al*., 2018; Yang *et al*., 2019; Sapkota *et al*., 2020; Maiti *et al*., 2020; Qi *et al*., 2022; Mothana *et al*., 2022). Ethyl acetate was added to the filtrate in a 1:1 (v/v) ratio for full extraction and vigorously agitated on the rotary shaker at 130 rpm for 1 hour. From the aqueous phase, the antibiotic-containing ethyl acetate phase was separated. It was evaporated to dryness in a water bath at 80-90°C, according to (Njoroge *et al*., 2018). According to the protocol, the residue was dissolved in dimethyl sulfoxide (DMSO) (Valan Arasu *et al*., 2009). Then, after dissolving in DMSO, 6mm paper discs were placed in the residual and used to analyze the zone of inhibition that appeared around the spread plate of the fungal spore solution on the PDA plates, with DMSO serving alone as a control.

Molecular Identification of high antifungal activity *Streptomyes* isolates:

16S rRNA genes, which are around 1500 bp in length, are found in all bacteria. RNA genes have varied DNA sequence portions that are unique to the species that carries them. An unknown bacterium's species identity can thus be determined from its unique rRNA gene sequence.

DNA Extraction:

DNA was extracted from the actinomycetes according to (Shoukry *et al*., 2019). This method is improved by standard phenol/chloroform method described by (Anwar *et al*., 2016).

16S analysis:

PCR Reactions:

The PCR amplification was performed in a total volume of 50 ul, containing 1X reaction buffer, 1.5 mM $MgCl_2$, 1U *Taq* DNA polymerase (promega), 2.5mM dNTPs, 30 poml of each primer and 30 ng genomic DNA.

Thermo-cycling PCR program:

After a 5-minute denaturation cycle at 94oC, PCR amplification was carried out in a Perkin-Elmer/Gene Amp® PCR System 9700 (PE Applied Biosystems) designed to complete 40 cycles. Each cycle included a 30-second denaturation phase at 94°C, a 30-second annealing step at 45°C, and a 1-minute elongation stage at 72°C. In the last cycle, the primer extension section was extended to 7 minutes at 72°C.

Detection of the PCR Products:

Electrophoresis of the amplification products in a 1.5 percent agarose gel containing ethidium bromide (0.5 ug/ml) in 1X TBE buffer at 95 volts resolved the amplification products. As a molecular size standard, a 100 bp DNA ladder was used. Under UV light, PCR products were seen and photographed using a Gel Documentation System (BIO-RAD 2000).

Purification of PCR Products:

The EZ-10 spin column PCR product purification device was used to purify amplified products for all PCRs. Three volumes of binding buffer were added to the PCR reaction mixture in a 1.5 ml microfuge tube. After that, the combination solution was transferred to the EZ-10 column and left to sit for 2 minutes at room temperature. The column was then filled with 750 ul of wash solution and centrifuged for two minutes at 10.000 rpm. To eliminate any remaining wash solution, one extra minute of washing at 10.000 rpm was undertaken. The column was placed into a clean 1.5 ml microfuge tube with 50 ul of elution buffer, incubated for 2 minutes at room temperature, and pure DNA was kept at -20 °C.

16S sequencing analysis:

The resultant PCR was sequenced in an automated sequencer, the ABI PRISM 3730XL Analyzer, using Big Dye TM Terminator Cycle Sequencing Kits and following the manufacturer's instructions. The 16S Forward primer was used to perform single-pass sequencing on each template. Using an ethanol precipitation technique, the fluorescent-labeled fragments were separated from the unincorporated terminators. The samples were resuspended in distilled water and run through an ABI 3730xl sequencer for electrophoresis (Microgen Company).

Computational analysis (BLAST n) 16S:

The sequences were analysed using the BLAST programme (http://www.ncbi.nlm.nih.gov/BLAST). Sequences were aligned using Align Sequences Nucleotide BLAST.

RESULTS AND DISCUSSION:

Isolation of *Streptomyces* isolates:

The Egyptian soils were ideal for research because they contain a diverse spectrum of soil types, each with a unique distribution of *Streptomycetes*. In comparison to *Triticum aestivum*, *Vicia faba*, and *Citrus aurantifolia*, soil grown with *Beta vulgaris* plants exhibited greater CFUs/g^{-1} of the S*treptomycetes* population (Table 1).

Antifungal Activity of *Streptomyces* Isolates:

Antifungal activity was investigated on 65 *Streptomyces* isolates that were identified. It was decided to apply the original screening process. Antifungal activity was observed in 83.0769 % of the isolates, of the 83.0769% isolates that demonstrated antifungal activity, isolate BF26 demonstrated the greatest antifungal activity against the fungal tester. The dual culture bioassay was used to test the antifungal activity of various *Streptomyces* isolates. (Figures 1 and 2).

By growing the *Streptomyces* isolate in broth, we were able to learn as much about them and separating the antifungal substances from the culture filtrate by ethyl acetate. After being tested on fungal pathogens, the data showed that the isolate BF26 had a broad inhibitory effect on the fungal tester (Table 2) and (Fig. 3).

The results showed that the zone of inhibition against plant pathogenic fungi was greater than 15 mm and up to 22 mm, and this showed that the isolate BF26 had highest antifungal activity in the control of phytopathogenic fungi as a tester. Similar results were followed by (Hong-Thao *et al* ., 2016) isolating a *Streptomyces* isolate that showed antifungal activity against *Fusarium oxysporum* and (Kunova *et al* ., 2016) isolated a *Streptomyces* isolate that showed antifungal activity against *Rhizoctonia solani* , *Fusarium oxysporum* f.sp. lactucae , and (Patil *et al*., 2010) isolated a *Streptomyces* isolate that showed antifungal activity against *Rhizoctonia solani* , and (Kanini *et al*.,2013) isolated a *Streptomyces* spp. that had antifungal activity against *Fusarium oxysporum* f. sp. lycopersici.

(Aghighi *et al*., 2004) isolated a *Streptomyces* isolate that showed antifungal activity against *Alternaria solani* and *Alternaria alternata*. Studies showed that the production of antibiotic compounds is more efficient in solid culture media, compared with submerged media, where activity may decrease or even cease completely. For example, according to research by (Sapkota *et al*., 2020), it was founded that 19 among 41 isolates demonstrated antimicrobial activity against the test pathogens during primary screening.against both gram-positive and gram-negative test organisms that were found to be active in 12.19% of total isolates. In secondary screening, 13 of the 19 active isolates showed a zone of inhibition against the test organisms. (Njoroge *et al*., 2018) discovered that eight out of the 39 isolates in the culture filtrate had wide inhibitory effects on bacterial and fungal pathogens. According to (Thakur *et al*., 2007), 15 among of 65 isolates that recorded antimicrobial activity in solid media did not demonstrate antibacterial activity in liquid medium. Other authors reported similar findings (Salamoni *et al*., 2010; Anibou *et al*., 2008). This, according to (Oliveira *et al*., 2010), is because of the constrained synthesis of antibiotic compounds in liquid media, and the detection of bioactive compounds that requires excessive concentrations of the compounds.

Morphological and Cultural Characterization of *Streptomyes* Isolates:

The colonies of *Streptomyces* isolates on the media were distinct with a powdery surface, and all isolates grew abundantly on the media, indicating sporulation. The aerial mycelium was examined using the cover slip method, and data revealed that the sporophore of the isolate BF26 was the spirales type. Direct analysis of entire spores using an electron microscope (TEM) at 80 kV at Al-Azhar University's Regional Centre for Mycology and Biotechnology (RCMB) revealed that the spore surface was spiny or spinous (Figure 4).

The isolates' color on the different media is depicted in Table (3). The consequences of morphological properties were subjective in nature and showed common attributes of *Streptomyces* in Bergey's Manual of Systematic Bacteriology (Whitman *et al.,* 2012). These findings showed that the isolates were presumptive positive and identified as Streptomycetes.

Biochemical and Physiological characteristics:

The isolate BF26 was gram stained and found to be positive. They were then grown on different agar media. The isolate BF 26 showed that production of melanin pigmented on ISP6 media is only compared to ISP7 media. The data are listed in (Table 4 and Figure 5). The results indicated that the isolate BF26 belong to *Streptomycetes* according to Bergey's Manual Sytematic Bacteriology (Whitman *et al.,* 2012).

The physiological tests showed that the best carbon source for the *Streptomyces* isolate BF 26 growth was Glucose , while the best nitrogen source for the isolate's growth was yeast extract. The results agreed with (Bhosale *et al*., 2015) and it was the best NaCl concentration for the growth of isolate 1% (w/v), and the best pH value for the growth of isolate was pH 9, and the best temperature for growth of isolate was 30^{o}C, and the best incubation period for the growth of isolate BF26 was 10 days.

Molecular analysis of superior *Streptomyces* isolates:

Molecular characterization of *Streptomyces* isolate was done by 16S rRNA gene amplification; this exhibited a molecular weight of 1.5 kb and sequenced the 1.5 kb of DNA fragment after purification from agarose gel. Further analysis, including BLAST search and phylogenetic tree, was accomplished to correlate the selected *Streptomyces* sp. isolates with other species of the genera in the database conserved library. The results as described in the phylogenetic tree showed that the isolate BF26 belonges to *Streptomyces bungoensis* (Figure 6). The 16S rDNA sequences of the *Streptomyces* isolate BF26 was saved in the NCBI Gene nucleotide sequence database with the accession numbers ON130197.

CONCLUSION:

Streptomycetes with antifungal activity have been isolated from Egyptian soils. The soil cultivated with the *Beta vulgaris* plant had higher CFUs. g^{-1}. The *Streptomyces bungoensis* BF26 inhibited the growth of phytopathogenic fungi with zones of inhibition that had been greater than 15 mm up to 22 mm. This work showed that isolation and examination of *Streptomyces* from Egyptian soils could serve as a potential renewable source of antifungal important strains with different spectra of activity against phytopathogenic fungi. The precise characterization of the active substances of the antibacterial and fungal extracts is the subject of continuous investigation in our work.

RECOMMENDATION:

This study revealed that (*Streptomyces bungoensis* ON130197) had antifungal activity against phytopathogenic fungi as tested by dual culture bioassay and paper disc diffusion methods. We recommend using these isolates *in vivo* or in a greenhouse set-up as a biological control to save the environment from pollution and to produce healthy food.

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Primer Code	Sequence	Product Size
27F	5′- AGAGTTTGATCCTGGCTAG -3′	1500bp
1492R	5′- GGTTACCTTGTTACGACTT -3′	

Table 1: Total viable count of *Streptomyces* in the different soil samples (CFUs/ g^{-1})

Soil samples from different locations	Total count of *Streptomyces* (CFU $x10^{-4}$ /g^{-1}).
Soil cultivated with *Triticum aestivum* plant Governorate Kafr El-Sheikh	6
Soil cultivated with *Beta vulgaris* plant Governorate EL-Gharbeia	29
Soil cultivated with *Vicia faba* plant Governorate Albuhayrah	28
Soil cultivated with *Citrus aurantifolia* plant Governorate Albuhayrah	15

Table 2: Antifungal activity of the culture filtrate by paper disk diffusion method.

Fungal tester *Streptomyces* Isolates	Inhibition zones (mm)		
	Alternaria sesame	*Rhizoctonia solani*	*Fusarium oxysporum*
BF26	15.67	17.33	18.00

Table 3: *Streptomyces* isolate BF 26 characteristics on different growth media:

Isolates No	BF 26		
Characteristics / Media are used	Color of aerial mycelium	Color of substrate mycelium	Color of soluble pigment
ISP2 Yeast extract malt extract agar	Gray	Yellow-brown	-
ISP3 Oatmeal agar	Gray	Creamy Colored	-
ISP4 Inorganic salt starch agar	Gray	Yellow-brown	-
ISP5 Glycerol asparagine agar	Gray	Creamy colored	-

ISP: International *Streptomyces* Project

Table 4: Melanin pigment production by *Streptomyces* isolate BF 26 on ISP6 and ISP7 media.

Isolates No	Melanin pigment production on media	
	ISP6	ISP7
BF26	+	-

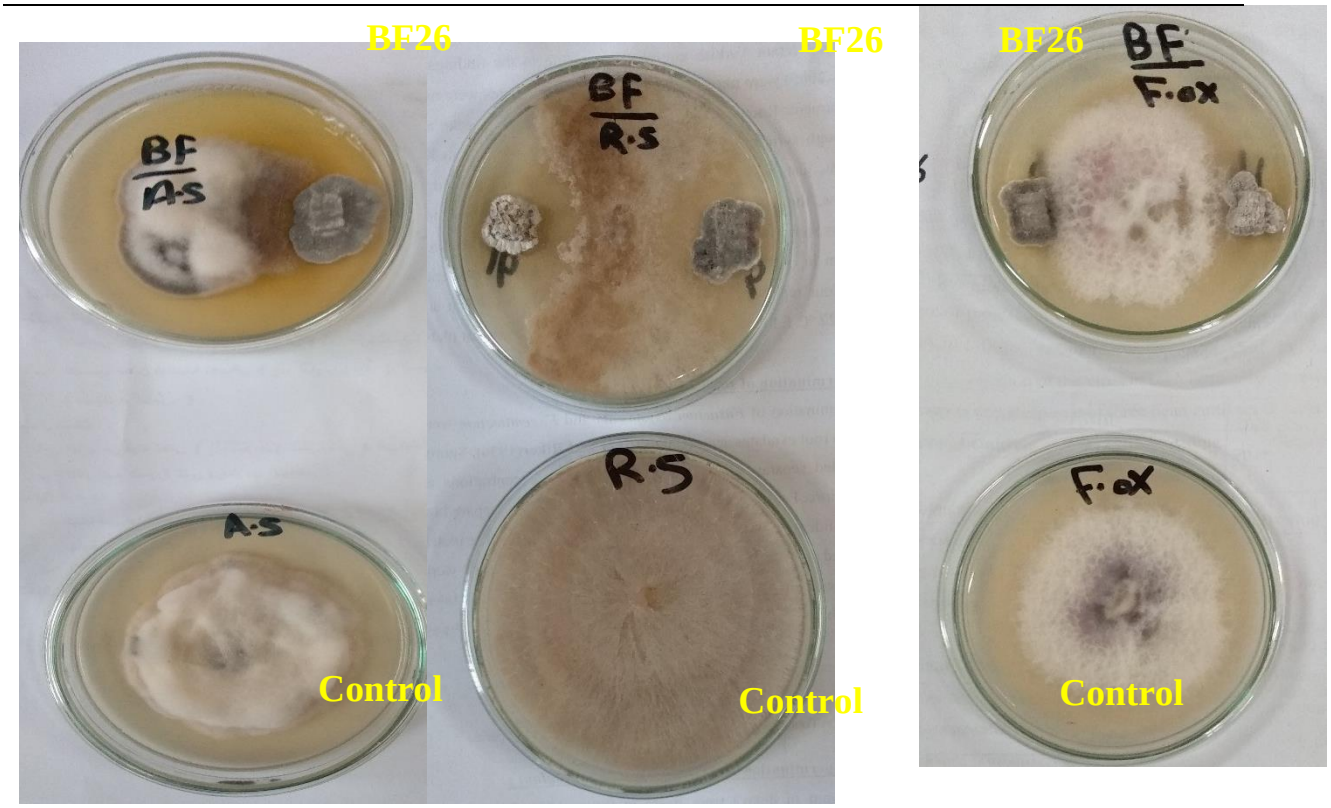

Figure 1: Antifungal activity of *Streptomyces* isolate (BF26) by Dual culture method against phytopathogenic fungi (*Rhizoctonia solani (Rs), Alternaria sesame (As)* and *Fusarium oxysporum (FOX).*

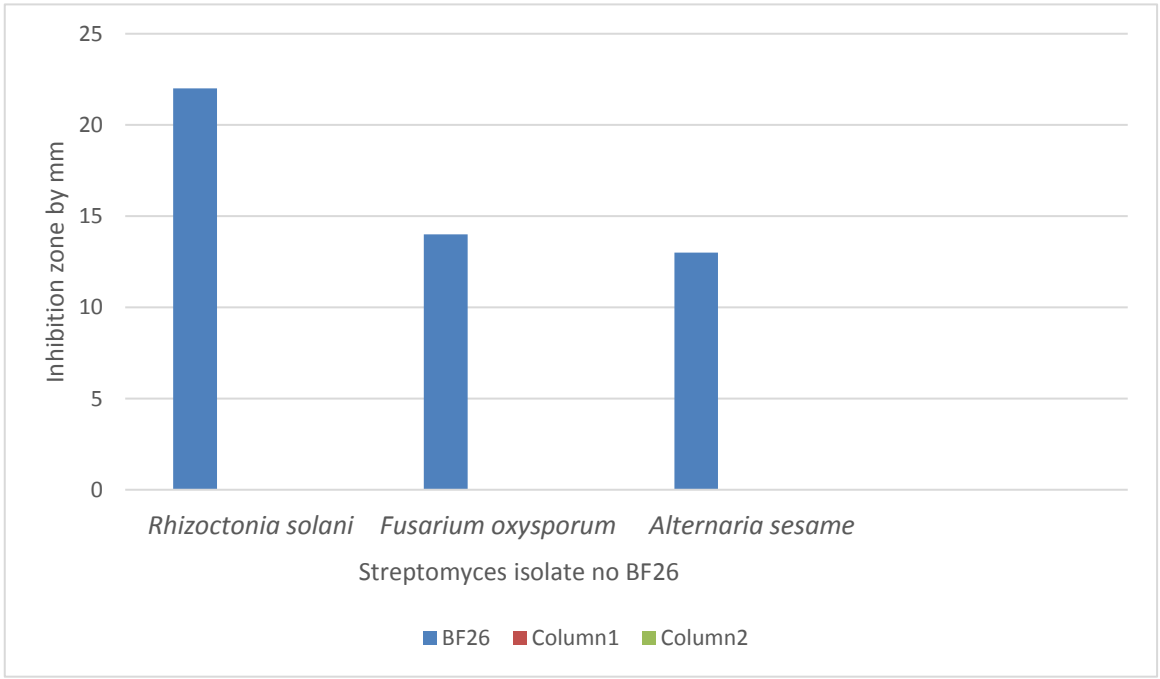

Figure 2: Antifungal activity of *Streptomyces* isolates (BF26) by Dual culture method against *Rhizoctonia solani, Alternaria sesame* and *Fusarium oxysporum*

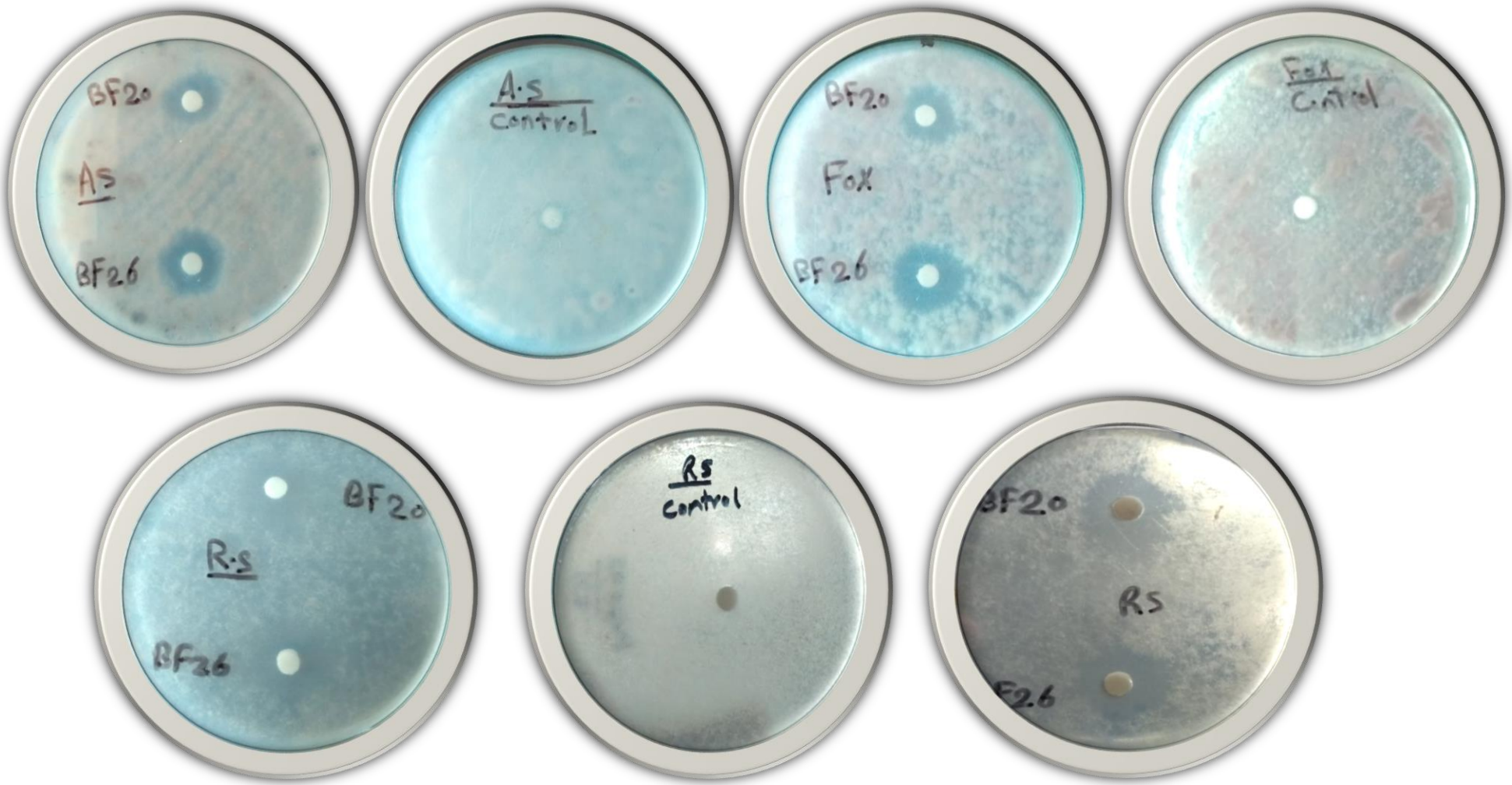

Figure 3: Antifungal activity of the culture filtrate against phytopathogenic fungi [A] Antifungal activity of isolate BF26 against *Alternaria sesame;* [B] antifungal activity against *Fusarium oxysporum*; and [C] antifungal activity against *Rhizoctonia solani*.

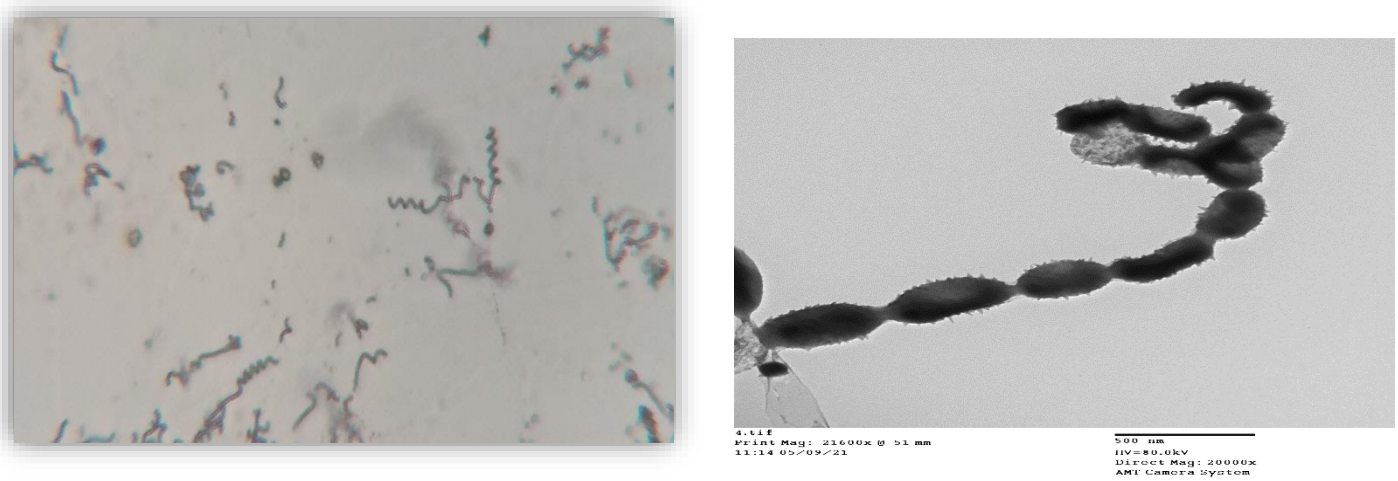

Figure 4: *Streptomyces* isolate BF26 (A) sporophore shape and (B) spore surface shape.

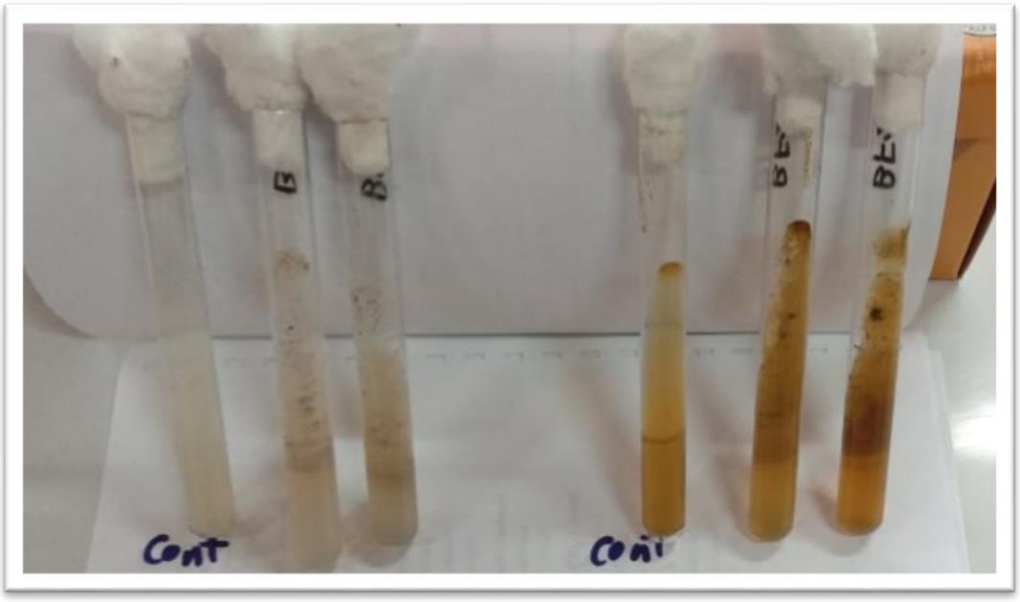

Figure 5: The isolate BF26 showed melanin pigment production on ISP6 media compared with control.

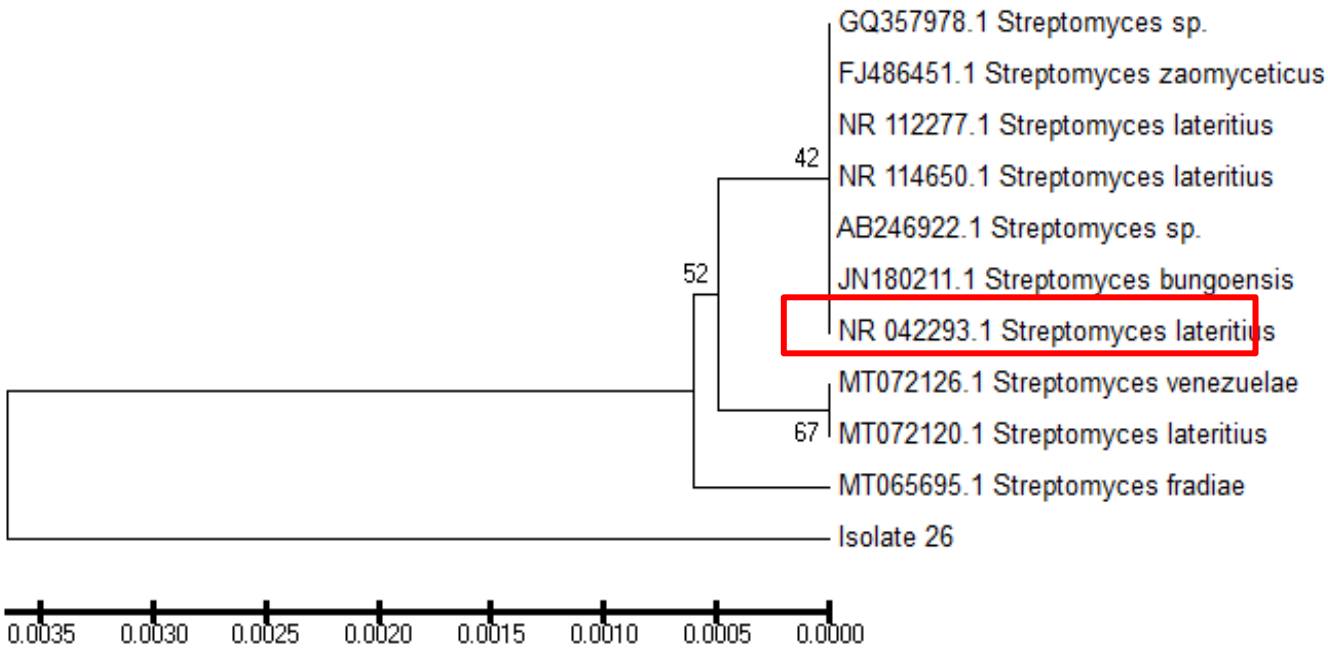

FigURE 6: Phylogenetic tree of the nucleotide sequences of the PCR product of 16S rRNA gene amplified from the DNA of *Streptomyces* strain BF26 and universal bacterial strains from BLAST.

النشاط التضادي للفطريات من *Streptomyces bungoensis* (BF26) ضد Alternaria *sesame*, *Fusarium oxysporum* and *Rhizoctonia solani.*

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الملخص العـربي

يهدف البحث إلى الحصول على عزلات من الإستربتوميسيس لها نشاط تضادي ضد الفطريات الممرضة للنبات محل الدراسة *Rhizoctonia solani* , *Fusarium oxysporum* , *Alternaria sesame* حيث إن الممرضات النباتية وخاصة الفطريات تتسبب في خسائر اقتصادية كبيرة جدا في جميع أنحاء العالم وحيث إن الفطريات الممرضة للنبات نتيجة للاستخدام المفرط للمبيدات الكيماوية اكتسبت المقاومة لها وكذلك أيضا أدى الاستخدام المفرط لهذة المبيدات الكيماوية الى تلوث البيئة لذلك فإن الاتجاهات الحديثة هي الحصول على النواتج الأيضية الثانوية من الميكروبات لاستخدامها في مكافحة هذه المسببات المرضية الفطرية . وأظهرت النتائج أن العزلة Streptomyces *bungoensis* (BF26) ذات نشاط تضادي ضد الفطريات محل الدراسة حيث بلغ قطر منطقة التثبيط ٢٢ ملليمتر مع فطر *Rhizoctonia solani* و١٤ ملليمتر مع فطر *Fusarium oxysporum* و١٣ ملليمتر مع فطر *Alternaria sesame* وذلك بطريقة Dual culture bioassay. وتم أيضا استخلاص المواد ذات النشاط التضادي للفطريات المختبرة والتي أنتجتها العزلة BF26 في البيئات السائلة وذلك باستخدام ال Ethyl acetate وتم اختبار هذه المواد لمعرفه قدرتها في تثبيط الفطريات المختبرة بطريقة paper disc diffusion حيث أظهرت النتائج أن هذه المواد لها القدرة على تثبيط نمو الفطريات المختبرة حيث بلغت قطر منطقة التثبيط ١٧,٣٣ ملليمتر مع فطر *Rhizoctonia solani* بينما بلغت ١٥,٦٧ ملليمتر مع فطر *Alternaria sesame* بينما بلغت ١٨ ملليمتر مع فطر *Fusarium oxysporum* . وتم تعريف هذه العزلة بإستخدام 16S rRNA حيث أظهرت النتائج أن هذه العزلة تنتمى إلى *Streptomyces bungoensis* وتم تسجيل هذه العزلة في بنك الجينات تحت رقم إيداع ON130197.

الكلمات الاسترشادية : الفطريات الممرضة للنبات, النشاط التضادى للفطريات, *Streptomyces bungoensis*