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Efficacy of *Streptomyces* spp. from Syrian soils as biocontrol agents against tomato gray mold caused by *Botrytis cinerea*

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Abstract

Grey mould caused by the fungus *Botrytis cinere*a is an economically important disease in numerous crops. Biocontrol is a promising method to control the disease. Species of *Streptomyces* are potential biological control agents**.** In this study we evaluated the efficacy of One hundred twenty-four isolates of *Streptomyces* spp. against *B. cinerea in vitro***.** Strains Str98 of *S. hypolithicus* and Str112 of *S. albidoflavus* have shown the highest ability to inhibit the growth of *B. cinerea*, with inhibition of 86 % and 81% consecutively. These isolates showed efficiency in the production of hydrolytic enzymes that may have an important role in their antifungal activity. The results also showed that cell free supernatant of Str98 and Str112 had the ability to inhibit the growth of fungus. Treating tomato leaves that were infected with gray mold reduced the infection by 50% when treated with the strain *S. hypolithicus* Str98. The results of this study demonstrated that selected *Streptomyces* strains and their free cell crude extract could be used as biocontrol agent against gray mold fungus.

Keywords: *Streptomyces*; antifungal activity; *Botrytis cinerea*; tomato

1. Introduction

Botrytis cinerea, the causal agent of gray mold, has a wide range of hosts and may infect over 200 plant species in the field, greenhouse and warehouses [11]. It has been considered as one of the common diseases of important crops such as grape berries, legumes, bulb flowers, strawberries and many other fruits and vegetables [7, 20]. This fungus infects the plant at every stage of its development [2], and has been found in the entire parts of the plant, including leaves, fruits, flowers, petioles [17,18]. *B. cinerea* is also one of the most important pathogens causing postharvest decay of fresh fruits and vegetables [26]. *B. cinerea* is necrotroph, inducing host-cell death which leads to progressive decay of the infected plant tissues. This pathogen produces abundantly sporulating gray mycelium on infected tissues [24]. Economically, it causes annual losses of 10 to 100 billion $ [3, 25].

Prevention of pathogens is critically important in agricultural production systems. In the last decades, the available methods of prevention for the conservation of sustainable agriculture have been evaluated, with emphasis on the importance of using environment friendly and safe methods. In this context, biocontrol is a good and safe alternative of synthetic fungicides, and fulfills consumer requirements for more natural and healthy food (Martinez-Romero *et al*., 2008) [12]. The biocontrol has been shown to reduce *Botrytis* infections successfully on flowers and fruits in many crops using antagonistic microbes and has potential future [3,6].

In fact, many microorganisms have been recruited as biocontrol agents for various plant diseases, since they have the capability of synthesizing bioactive products that constitute a library of compounds with a large and privileged structural diversity, showing a variety of biological activities (Sihem *et al*., 2011). The search for new antifungal microorganisms with greater potency has progressed slowly [15]. In the purpose of screening for new antifungal microorganisms, several researches were oriented towards isolation of new microorganism species from different soils and ecosystems [9,16].

Among the microorganisms, *Streptomyces* spp. can be an alternative to synthetic biochemical fungicides and used as biocontrol agents. *Streptomycetes* are

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Gram-positive bacteria belonging to the order *Actinomycetales* and the family *Streptomycetaceae*; roughly, *Streptomyces* are represented by more than 570 different species. *Streptomycetes* are aerobic and filamentous bacteria able to produce vegetative hyphae that eventually form a complex mycelium and are able to grow and colonize different substrates. They are spore-forming bacteria and their spores may aid their dispersion and dissemination [27]. It has been widely demonstrated and used for plant growth promotion, nutrient assimilation, mineral availability, biofortification, and biological control of plant pathogens of agriculturally important crops (Gopalakrishnan *et al*., 2020).

The aim of the present study is screening and testing the effectiveness of some local *Streptomyces* isolates as biocontrol agents for *B. cinerea*, the causal agent of gray mold disease, *in vitro* and in tomato plants.

2. Materials and Method

2.1. Fungi

Botrytis cinerea was isolated from strawberry fruits [1]. The isolate was subcultured on potato dextrose agar (PDA) for 15 days at 25˚C in a 12hrs light period to stimulate conidia formation. Spore suspension was prepared by flooding the culture with sterile distilled water containing 0.1% (v/v) TritonX100, and dislodging spores from the hyphae by using a sterile glass spreader. The conidial suspension was then filtered through sterile absorbent cotton wool plugs to remove any hyphal fragments, and the number of spores in suspension was counted using a hemocytometer and adjusted to 10^5 spores/ml by dilution with sterilized distilled water.

2.2. Bacteria

Isolation and storage of *Streptomyces* spp. For each collected sample, 3 g soil were diluted in 100 ml of saline solution (0.85% NaCl) and allowed to stand for 15 min. Three different dilutions (1:10, 1:100 and 1:1000) were prepared using sterile saline solutions in a total volume of 10 ml. An aliquot of 0.1 ml of each dilution was plated on YM agar: (yeast extract, 10; malt extract, 10; glucose, 4; $CaCO_3$, 2; agar, 15 g/l). Plates were incubated at 28 °C, and monitored after 48, 72, and 96 h. Representative colonies were selected and streaked on new plates of YM agar. The isolated *Streptomyces* species were preserved on YM agar plates at 4°C until further use. Visual observation of both morphological and microscopic characteristics using light microscopy, and Gram-stain properties were performed for genus confirmation. The spore suspension was prepared from the growing and sporulated isolates after 3-7 days of incubation at 28°C spores were harvested using sterile saline solution containing: (3 g/L Pepton, 5 g/L NaCl, 1 mL/L $Triton_{x100}$), then filtered through a layer of cotton to get rid of the mycelium hyphae and the remnants of agar. Enumeration of spores in the spore suspension was performed using a Neubauer improved count plate. The inoculum suspension was adjusted at 10^9 spores/mL.

2.3. Antagonistic activity of Streptomyces isolates against B. cinerea

In vitro antagonism tests were performed on NA medium in 9cm Petri plates by applying a dual culture technique. Five mm diameter disc of fungal growth was cut from an actively growing culture by a sterile cork borer and placed onto the center of above NA, and 5ul of tested *Streptomyces* spore suspension were transferred from five-day old cultures and cultivated in 2.5cm from the center of plate, and that is on three sides of the plate, and keep the fourth side for the fungus to grow without the influence of *Streptomyces* as control. Secondary screening for isolates which have antagonistic efficiency was carried out, where 5ul of selected *Streptomyces* spore suspension adjusted at 2×10^9 spore/mL and pipetted in 2.5cm from the center of plate, and the pathogenic was cultivated in the center of control plate. The growth of the fungus was measured after a 7-day incubation period, and the antagonistic efficiency of isolates was estimated by applying the formula: GI=(C-T)/C×100, where; GI=Percent inhibition, C=Radial growth of the pathogen in control, and T=Radial growth of the pathogen in treatment. Growth inhibition was categorized on a scale from 1 to 3, where 1= 1 to 25%; 2= 26 to 50%, 3= 51 to 75%, and 4= more 75% growth inhibition [21].

2.4. Identification of selected Streptomyces strains

The primers Sm6 F (5’-GGTGGCGAAGGCGGA-3’) and Sm5 R (5’-GAACTGAGACCGGCTTTTTGA-3’) flanking a highly variable sequence region of 600 bp towards the 5’end of the 16S rDNA region were used in polymerase chain reaction PCR [19]. Genomic DNA was extracted and purified using DNA extraction DNeasy Plant Mini Kit according to the

manufacturer's recommendations (Qiagen, Cat. NO. 69104). PCR mixtures were prepared using 20ng of template DNA, 0.2µM of each primer, and hotstar taq master mix kit (Qiagen, Cat. NO. 203446). Amplification was done under the following conditions: 10 min denaturation step at 95°C, followed by 30 amplification cycles(45 sec at 94°C, 45 sec at 65°C and 105 sec at 72°C) and an extra extension step of 10min at 72°C. PCR products were separated on a 1.5% agarose gel to which ethidium bromide was added and photographed under UV light. Amplification products were purified using QIAqick Gel Extraction kit (QIAGEN, Cat. No.28704) and sequenced on both strands using an ABI 310 sequencer machine. The sequences were subjected to a BLAST search against the full GenBank database available at NCBI public database using Basic Local Alignment Search Tool for Nucleotides (BLASTN).

2.5. The inhibitory effect of cell free supernatant of Streptomyces strains (in vitro)

A liquid culture of selected isolates was performed on NB medium with 7% (w/v) wet mycelium fungi and incubated at 28°C with constant shaking at 200 rpm for 7 day. The culture was removed and centrifuged at 8000×*g* for 10min. Cell free supernatant was obtained by filter (0.45µm) sterilized and tested for antifungal activity. Four wells (5 mm in diameter) were made in NA plate on the side 2.5cm from the center of plate using a sterilized cork borer, subsequently wells filled with 200µl of filtered supernatant. Five mm diameter disc of *B. cinerea* growth was inoculated in the center of NA plate, and compared with control. Plates were incubated for 5 days at 28°C.

2.6. Detection of enzymatic hydrolysis activities by the selected Stryptomyces strains

The selected isolates of *Stryptomyces* spp. which exhibited antifungal activity against *B. cinerea* were cultured on a synthetic agar media for envestigating their production of hydrolyzing enzymes. The enzymes investigated include: chitinase, amylase, protease, glucanase, lipase, xylanase, pectinase, carboxymethyl cellulase (CMCase). Isolates that have hydrolyses enzymatic activity showed clearing zones on agar media, and the hydrolytic efficiency of isolates was estimated 5 days after incubation at 28°C by calculating the ratio of clear zone CZ diameter to growth zone GZ (CZ/GZ).

2.7. Evaluation of the antagonistic activity of selected Streptomyces strains against B. cinerea on tomato plant

Antagonism tests were carried out under growth cabinet conditions using a local variety of tomato. The seeds were superficially sterilized using 5% sodium hypochlorite solution for five minutes, and then washed three times with sterile distilled water. Then it was planted in pots containing sterilized peat moss. Pots were incubated in a growth room at a temperature of 25 °C, an illumination period of 16 hours, and a relative humidity of 90 - 80%. Fungal infection of the leaves was carried out by spore suspension (10^5 spore/mL) at the age of one month, then, the infection points were treated by spore suspension of two selected *Streptomyces* strains. After 5 days, the dimensions of the infested area were evaluated on the leaves by measuring the horizontal and longitudinal growth of the fungus and calculating the average area of the infestation.

2.8. Statistical analysis

The statistical analysis was done using the Statistica program version 6 (Statsoft, Inc. 2003) at 5% significance level (P = 0.05). Data were subjected to the analysis of variance for determining statistical significance of differences between means, according to ANOVA-Tukey HSD test.

3. Results and Discussion

3.1. Screening of Streptomyces isolates for antagonistic activity against B. cinerea

One hundred and twenty-four isolates of *Streptomyces* were tested for their efficacy in inhibiting growth of *B. cinerea*, and only 88 of them showed clear inhibition zones in varying proportions (Figure 1). This clearly suggested that these isolates possessed antagonism against *B. cinerea in vitro*, that may be attributed to the production of antifungal compounds which reduced the mycelial growth of *B. cinerea* by forming an inhibition zone. A secondary screening was carried out for the isolates, which inhibited the growth of the fungus. The results showed that 2 isolates were within category 4 (Table 1). The isolates: Str98, Str112, exhibited the highest growth inhibition of *B. cinerea*by 86 and 81%respectively. This clearly suggested that these isolates possess antagonism against the fungus *in vitro*, that may be attributed to the production of antifungal compounds which reduced the mycelial growth of fungi by forming an inhibition zone. Several studies have pointed to the

use of *Streptomyces* species to inhibition of *B. cinerea* growth. Several rhizospheric and endophytic microorganisms have been studied with regard to the development of new biopesticides, and currently, some of them are used for *Botrytis* control in crops where, owing to the impact, economic relevance, and intrinsic characteristics of this fungal phytopathogen, the use of biopesticides is a suitable tool for control that improves the sustainability of crop management [22, 23]. Boukaew *et al.* (2016) [4] recorded efficiency in varying proportions of *Streptomyces* in inhibiting the growth of *B. cinerea* in the laboratory and on tomato plants.

Table 1. Effect of *Streptomyces* spp. isolates from Syrian soils on *in vitro* growth of *B. cinerea* (secondary screening)

***Streptomyces* spp. isolates**	**GI category[1]**	**Streptomyces spp. isolates**	**GI category[1]**	***Streptomyces* spp. isolates**	**GI category[1]**
str98	4	str5	2	str99	2
str112	4	str6	2	str40	2
Str69	3	str29	2	str37	1
str74	3	str35	2	str60	1
str72	3	str114	2	str106	1
str50	3	str7	2	str107	1
str67	3	str38	2	str33	1
str113	3	str49	2	str36	1
str25	3	str22	2	str39	1
str10	3	str20	2	str46	1
str13	2	str92	2	str76	1
str86	2	str100	2	str109	1
str65	2	str101	2	str16	1
str111	2	str105	2	str27	1
str82	2	str4	2	str120	1
str104	2	str115	2	str18	1
str31	2	str47	2	str118	1
str8	2	str117	2	str34	1
str9	2	str44	2	str57	1
str119	2	str55	2	str58	1
str24	2	str14	2	str59	1
str110	2	str15	2	str61	1
str21	2	str121	2	str84	1
str30	2	str123	2	str3	1
str79	2	str124	2	str1	1
str97	2	str42	2	str17	1
str23	2	str43	2	str19	1
str122	2	str87	2	str41	1
str103	2	str11	2	str2	1
str85	2				

[1] Percent growth inhibition was determined **7** days after incubation using Whipps' (1987) formula. Values were categorized on a scale from 1 to 4, where 1: 1 to 25%; 2: 26 to 50%; 3: 51 to 75%; 4: > 75%.

Table 2. Tests of selected antagonistic isolates in the production of hydrolyzing enzymes on agar media

Enzymatic hydrolysis efficiency(CZ/GZ)								***Streptomyces* Isolates**
amylase	**chitinase**	**lipase**	**proteiase**	**xylanase**	**pectinase**	**glucanase**	**CMCase**	
1.4	1.9	1.4	0.0	1.5	4.3	4.6	2.4	Str98
2.3	1.2	1.2	2.9	2.8	3.7	3.9	2.9	Str112

3.2. Streptomyces strains identification

Identification of selected isolates was performed using 16S DNA gene sequences. PCR amplification with specific primers Sm yielded single DNA fragments of ~ 600 bp, present in all *Streptomyces* sp. isolates (Figure 2).

The nucleotide BLAST similarity search analysis, based on 16S DNA gene sequence revealed that these two isolates belong to the *streptomyces* genus.

The closest phylogenetic neighbor according to the 16S DNA gene sequence data for Str98 was *S. hypolithicus*, with 99% of homology (Accession number MW922870), and for Str112 was *S. albidoflavus* with 99% of homology (Accession number ON076879).

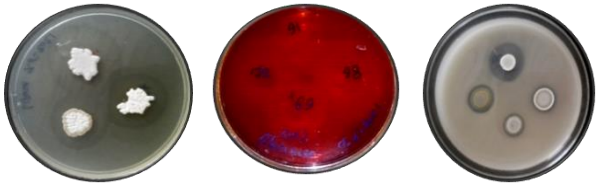
Antifungal activity of *Streptomyces albidoflavus* L131 was recorded by Chen *et al.* (2015) [5].

3.4. Detection of enzymatic hydrolysis activities

Another type of molecules that are highly involved in *Botrytis* biocontrol is the hydrolytic enzymes. These are able to cleave polymeric compounds, such as chitin, proteins, cellulose, hemicellulose [10]. The results of investigating the enzymatic activity of the selected isolates showed a different efficiency of the tested hydrolytic enzymes (Table 2), Where these isolates were able to hydrolyze substrates and form clearing zones around the growth zones (Figure 2), which could play an important role in their antifungal activity. Chitinase and glucanase are considered to be important hydrolytic enzymes in the lysis of fungal cell walls and could be related to the inhibition of fungi growth [8, 13, 14].

Str 69 against B. cinerea Str 98 against B. cinerea

Figure 1. Antagonism test between *Streptomyces* sp. and *Botrytis cinerea* by dual culture method on NA medium



a b c

Figure 2. Enzymatic activity on agar media showing clear zone around *Streptomyces* sp. growth zone. a: lipase, b: glucanase, h: chitinase

3.5. In vitro inhibitory effect of cell free supernatant of Streptomyces sp.

Current study results showed that the cell free supernatant of *S. hypolithicus* Str98, and *S. albidoflavus* Str112 strains were sufficient to inhibit fungal mycelium growth (Table 3). This antagonistic activity in the cell-free extract may be mainly due to the previously indicated hydrolytic enzymes, in particular chitinase and glucanase. Crude chitinase exhibited antifungal activity against a wide range of pathogenic organisms, where chitinase can degrade of the cell wall of many phytopathogenic fungi. Ekundayo *et al*. (2022) recorded that *Streptomyces albus* had the highest ability to produce chitinase, and its culture filtrate was able to inhibit the growth of *B. cinerea* and all studied pathogenic fungi.

Table 3. Effect of cell free supernatant of selected *streptomyces* strains in *Botrytis cinerea* growth inhibition.

fungus	treatment	Fungus growth diameter (mm)*
Botrytis cinerea	Control	59 ± 1 C
	Str. 112	29 ± 1 B
	Str. 98	3.5 ± 0.5 A

* The capital letter is used to compare treatments within each fungus. Values followed by the same letters do not have a significant difference at P=0.05.

3.6. The effect of treatment with Streptomyces in reducing the infestation of tomato leaves with gray mold

The results showed that the fungus *B. cinerea* caused infection on treatment leaves at different degrees (Table 4).The infection decreased when treated with *S. albidoflavus* Str112 by 35%, while it decreased by about 50% when treated with *S. hypolithicus* Str98 and the spore suspension mixture of the two strains. These results showed the efficiency of the *S. hypolithicus* Str98 strain in inhibiting the growth of gray mold fungus on infected tomato leaves.

Table 4. Effect of treatment with strains Str98 and Str112 in reducing gray mold infestation on tomato leaves.

Treatment	infection diameter (mm)
Control	15.6±1.11 A
Str 112	10.2±1.03 B
Str 98	8.04±0.95 C
Mix. Str112, Str98	7.93±1 C

* The capital letter is used to compare treatments within each fungus. Values followed by the same letters do not have a significant difference at P=0.05.

4. Conclusion

The results of this study indicate Str98 and str112 strains possess antifungal activity to plant pathogenic *B. cinerea in vitro* and in tomato plant. Both *streptomyces* sp. spore suspension and filtered culture extract were effective in inhibiting the growth of *B. cinerea*. This antifungal activity may be due to the efficiency of the two selected *Streptomyces* strains Str98 and Str112 to produce a number of hydrolytic enzymes that have the role of degrading the cell wall and limiting the growth of the fungus.

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Conflict of Interest. Author has declared that no competing interests exist.

Compliance with Ethics Requirements. Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human / or animal subjects (if exist) respect the specific regulation and standards.

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