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RESEARCH

Identification and *in vitro* antifungal evaluation of *Streptomyces* sp. of desert soil against *Colletotrichum* sp.

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ABSTRACT

Streptomyces sp. has the potential to inhibit the incidence of fungal phytopathogens in crops due to their biochemical activity and the production of secondary metabolites. This work was aimed to identify and evaluate in vitro the antifungal potential of five strains of Streptomyces sp., isolated from desert soil, against *Colletotrichum* sp. The isolated strains were morphologically characterized by macroscopic and microscopic analysis, and their inhibition percentage against *Colletotrichum* sp. were determined by the dual challenge and well diffusion tests, applying a completely randomized design with three replicates. The inhibitory capacities of the five strains versus the phytopathogenic fungus varied considerably. Strains Q-84 and Q-60 showed the highest percentages of inhibition in the dual challenge test, with 92 and 88 %, respectively. In the well diffusion test, the crude bioactive extract of strain Q-84 showed the highest inhibition (56 %) statistically similar to that of commercial chemical fungicides AmistarTop® and Opera® (84 and 100 %, respectively). All the strains displayed lipase activity and three of them (Q-84 included) also showed protease activity. The genetic characterization by 16S rDNA sequencing evidenced the homology of the strain Q-84 DNA to that of *S. maritimus*, further coincident with the lipase activity profile. Overall, the Q-84 showed potential to be used as biocontrol against *Colletotrichum* sp.

Keywords: Streptomyces sp., inhibition, *Colletotrichum* sp., lipase activity, protease activity

RESUMEN

Identificación y evaluación antifúngica *in vitro* de cepas de *Streptomyces* sp. de suelo desértico contra *Colletotrichum* sp. *Streptomyces* sp., tienen potencial para inhibir la incidencia de fitopatógenos fúngicos en los cultivos debido a su actividad bioquímica y la producción de metabolitos secundarios. La presente investigación buscó identificar y evaluar in vitro el potencial antifúngico de cinco cepas de *Streptomyces* sp., aisladas de suelo desértico, contra *Colletotrichum* sp. Se caracterizó morfológicamente a las cinco cepas mediante análisis macroscópico y microscópico, y se determinó el porcentaje de inhibición, mediante las pruebas de enfrentamiento dual y difusión en pozo, se aplicó un diseño completamente aleatorizado con tres réplicas. La capacidad inhibidora sobre *Colletotrichum* sp. vario considerablemente entre las cinco cepas. Las cepas Q-84 y Q-60 mostraron los porcentajes de inhibición más altos en el enfrentamiento dual, con 92 y 88 %, respectivamente. En la prueba de difusión en pozo el compuesto bioactivo crudo de la cepa Q-84 mostró el porcentaje de inhibición más alto, con un 56 %, en comparación con los fungicidas químicos comerciales AmistarTop® y Opera® (84 y 100 %, respectivamente). Todas las cepas mostraron actividad lipasa, y tres de ellas, incluida la Q-84, mostraron actividad proteasa. Tras la caracterización genética mediante secuenciación de ADNr 16S, la cepa Q-84 mostró homología con el ADN de *S. maritimus*, complementario al comportamiento enzimático similar al reportado para dicha especie (fenotipo productor de lipasas). En resumen, la cepa Q-84 mostró potencial para convertirse en una alternativa para el biocontrol de *Colletotrichum* sp.

Palabras clave: Streptomyces sp., inhibición, *Colletotrichum* sp., actividad lipasa, actividad proteasa

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Introduction

Microorganisms antagonistic of phytopathogenic fungi are an ecologically attractive alternative to the use of chemically synthesized pesticides [1]. Among them, *Streptomyces* spp. isolated from different geographic regions have been characterized and are gaining attention as a source of compounds and enzymes with antifungal and insecticidal properties [2].

One strategy implemented to obtain metabolites comprises the finding of new isolates of microorganisms with potential industrial application, particularly those from land or sea extreme and inhospitable environments [3]. In this regard, a recent *Streptomyces* sp. isolate was found in an extremely arid soil, capable of synthesizing sixteen specialized metabolites belonging to different chemical classes, indicating the unique and diverse actinobacterial activity provided by extreme environmental conditions which led to the discovery of new chemical compounds [4].

Colletotrichum spp. is a genera ranked eight among scientific and economic relevant phytopathogenic

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fungi for agriculture in almost all crops (e.g., mango, avocado, banana, others) [5]. Commonly, triazole fungicides has shown good in vitro inhibitory activity on *Colletotrichum* sp. control, but its use has been limited due to the high risk of toxicity [6]. Therefore, safe and effective compounds are required to an adequate management of economically relevant crops against the damage caused by *Colletotrichum* sp [7]. This is remarked by the need for disease control methods other than chemical compounds, in order to reduce the agricultural application of pesticides for environmental preservation, one of which comprises biological control agents [8]. Therefore, this work was aimed to identify and evaluate *in vitro* the antifungal activity of five strains of *Streptomyces* sp. isolated from desert soil against *Colletotrichum* sp.

Materials and methods

Soil sample characterization

Bacteria were isolated from dessert soil at the Reque District, in the Chiclayo region, Peru. A soil sample was collected and its physical-chemical properties were determined at the National Institute of Agrarian Innovation: electric conductivity (mhos/cm), pH, microorganisms' concentration (%), $CaCO_3$ concentration (%) and soil type.

Isolation of bacteria from the *Streptomyces* sp.genera

Bacteria were isolated from dessert soil samples, which were screened in 5 g subsamples, serially diluted (10-3) in sterile distilled water. Then, 100-µL aliquots were collected and individually striated on solid medium glucose, yeast extract and malt extract agar (GYM Agar), further incubated at 25 °C for 7 days. Then, *Streptomyces* spp. colonies were isolated and purified in GYM Agar supplemented with 1 % Nystatin, and stored in glycerol at the ceparium of the Agrobiotechnology laboratory until use. Five bacterial strains denominated Q32, Q-45, Q-56, Q-60 and Q-84 were selected for this study. Their cultures were reactivated in Agar GYM and cultures were incubated at 28 ± 2 ºC for 7 days.

Isolation of the phytopatogenic fungus *Colletotrichum* sp.

The phytopatogenic fungus *Colletotrichum* sp. was isolated from spores collected from rotten fruits. Briefly, fruits were incubated in a humid chamber, and five days later, slices were cut off with a sterile scalpel from diseased fruit areas and put into an aqueous solution of 1 % hypochloride. Then, slices were washed thrice with sterile water and seeded on Potato Dextrose Agar at 25 ºC for 7 days [9]. Afterwards, isolated were identified attending to the morphologic characteristics typical of the genera *Colletotrichum* [10].

Dual challenge of *Streptomyces* sp. and *Colletotrichum* sp. strains

The five *Streptomyces* sp. Isolates were evaluated for its capacity to inhibit mycelial growth of *Colletotrichum* sp. in vitro, through the dual challenge test. Briefly, *Colletotrichum* sp spores of were lined-up in one side of PDA plates and were incubated at 28 ± 2 °C for 7 days. Subsequently, a 5-mm ø mycelial disc containing a 5 days-old *Colletotrichum* sp. colony was cut and transferred to the other side on each of the PDA-containing plates, each separated 5 mm from the *Streptomyces* sp. Additionally, a 5-mm ø mycelial disc containing a 5 days-old *Colletotrichum* sp. colony was transferred to a non-inoculated PDA plate as control. The double culture plates and the control were incubated at 28 ± 2° C for 5 days.

The radial mycelial growth inhibition percentage (RI) was calculated by using the following formula:

$$IR\ (\%) = \frac{Dc - Dt}{Dc}$$

Where:
IR = Inhibition range (%);
Dc = Negative control diameter (mm);
Dt = Treatment diameter

Macroscopic and microscopic characterization of the five *Streptomyces* sp. isolated strains

All the five *Streptomyces* sp. strains were macroscopically and microscopically characterized after seeding them by striation in Oat meal agar supplemented with 1 % Nistatin followed by incubation at 26 °C for 8 days. Then, they were characterized for macroscopic properties: of colony texture, anverse color, occurrence and shape of the substrate immerse and aerial mycelia, and the release into the medium of diffusible pigments [11].

For microscopic characterization, the septation pattern was analyzed for each *Streptomyces* sp. strain seeded on Oatmeal agar supplemented with 0.1 % Nistatin. Briefly, sterile coverslips were introduced into the agar in a 45° angle and further incubated at 22 °C for 15 days. Then, the coverslips were placed on microscopy slides containing Crystal violet for the observation of aerial and substrate-immerse mycelia and the propagles formed [11].

Characterization of the *Streptomyces* sp. strain by 16S rRNA PCR amplification

DNA extraction

Bacterial DNA was extracted by the CTAB method [12] from the bacterial isolate showing the highest inhibition percentage against *Colletotrichum* sp. The purified DNA concentration was quantitated by absorbance at 260 nm (A_{260} nm) and purity evaluated by spectrophotometry with the A_{260}/A_{280} ratio (range 1.8-2.0) in a Eppendorf biophotometer model AG spectrophotometer.

Obtention and sequencing of 16S rRNA amplicons of the Streptomyces sp. strain

The 16S rRNA region was PCR-amplified with universal primers 27F (forward, 5′-AGAGTTTAGTCMTGGCTCAG-3′) and 1492R (reverse, 5′-GGYTACCTTGTTACGACTT-3′), which generate an amplicon of approximately 1500 bp. A 25-µL PCR mix was prepared by adding 2.5 µL of PCR buffer 10×, 2.5 µL of 25 mM $MgCl_2$, 0.6 µL of

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10 mM dNTPs and 2U of high fidelity Taq DNA polymerase (Platinium; Thermo Scientific, USA). PCR conditions were set as follows: initial cycle of denaturing at 95 ºC for 5 min; 35 cycles of denaturing at 95 ºC for 30 s, annealing at 55 ºC for 45 s and elongation at 72 ºC for 1 min; and a final elongation step at 72 ºC for 10 min.

Amplification products were subjected to electrophoresis in 1.5 % agarose gels and further visualized by staining with 0.2 µg/mL ethidium bromide under UV. Amplicons of the expected size were further sequenced for confirmation in a DNA sequencer (Macrogen, USA) with internal primers 518F (forward, 5´-CCAGCAGCCGCGGTAATACG-3´) and 800R (reverse, 5´- TACCAGGGTATCTAATCC-3´). The sense and antisense strands were assembled by using the CAP contig assembly program tool of BIOEDIT 7.1.9 and further aligned in BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for homology identification.

Enzyme activity determinations

Qualitative assessment of chitinase activity

Coloidal chitin was prepared with commercial chitin (Merck-Aldrich, USA) by the Hsu and Lockwood modified method as reported by Castro *et al*. [13]. The resulting chitin solution was subsequently vacuum-dried and the precipitate was washed successively with distilled water with volumes adjusted to the initial volume to eliminate the residual acid. Then, it was sterilized in autoclave at 15 psi and 121 ºC for 15 min. The obtained colloidal chitin was used to supplement a culture media with the following concentrated solutions: 1) 30 g/L K_2HPO_4 and KH_2PO_4; 2) 15 g/L $MgSO_4 \cdot 7H_2O$; and 3) 5 g/L $CaCl_2$, 0.6 g/L $FeCl_3$ and 5 g/L NaCl (w/v). Then, actinomycetes were plated and incubated at 22 ºC for 5 to 8 days. Afterwards, the presence of hydrolysis halos produced by *Streptomyces* sp. was checked in the solid media.

Protease activity determination

Streptomyces sp. were plated in Minimal medium prepared with concentrated solution containing: 1g Peptone, 0.5 g NaCl, 0.5 g Meat extract, 0.24 g Gelatin, 1.5 g Agar and 100 mL distilled water. Plates were incubated at 22 ºC for 5 to 8 days [14], and then the presence of hydrolysis halos was evaluated.

Lipase activity determination

Streptomyces spp. strains were plated in Minimal medium prepared from concentrated solutions containing 1 mL Tween 20, 1 g Bacto peptone, 0.5 g NaCl, 0.01 g $CaCl_2 \cdot 2\ H_2O$, 1.5 g Agar, 100 mL distilled water, and plates were further incubated at 22 ºC for 5 to 8 days [14]. Afterwards, the presence of hydrolysis halos was determined in solid media.

Production of crude extracts by fermentation

The production of crude extract of selected actinomycetes was done using the ISP1 (International *Streptomyces* Project) culture medium, modified. *Streptomyces* spp. were reactivated at 28 ºC for 3 days in 3 mL of Tryptone soy broth (TSB). Subsequently, the content was used to inoculate the ISP1 liquid medium. Once inoculated, media were incubated under constant agitation (150 rpm) at 25 ºC for 7 days.

Extraction of secondary metabolites

The secondary metabolites from *Streptomyces* sp. bacterial strains Q-32, Q-45B, Q-60, Q-84, Q-85 were extracted during the stationary growth phase (idiophase) as described by Franco [14]. Subsequently, culture broth was centrifuged at 6000 rpm and 4 ºC for 20 min. The supernatant was collected and ethyl acetate was added at 1:1 ratio, and vigorously shaken for 2 h. After that period, the ethyl acetate phase was collected and let to evaporate at 40 ºC in a rotoevaporator (IsoLab, USA). The precipitate was then added with 10% methanol, and the resulting solution was stored at 5 ºC until used for antifungal activity determination against *Colletotrichum* spp.

Bioactive test of the bioactive compound and two commercial chemically synthesized fungicides against Colletothrichum *sp.*

PDA medium was prepared for evaluating and comparing the antagonistic activity by the diffusion well technique. For this, three 0.2-cm in diameter punchs were made on each plate, and they were filled with 40 µL of the crude bioactive compound from the five strains selected: Q-32, Q-45B, Q-60, Q-84 and Q-85.

Then, a disc with *Colletothrichum* sp. was place in the middle of the plate. The procedure was done in triplicates. Two commercial chemically synthetized fungicides, Amistar Top® and Opera®, were also tested, in order to comparatively evaluate the spectrum of action and the competitiveness degree of the *Streptomyces* spp. crude extracts. A completely randomized design was used, and the p value obtained in a variance analysis, and a Tukey's means comparison test was applied (< 0.05).

Results and discussion

Dual challenge of *Streptomyces* sp. and the phytopatogenic *Colletotrichum* sp. fungus

The five *Streptomyces* sp strains tested for its antifungal activity against *Colletotrichum* sp. by the dual challenge technique indicated that the five strains inhibited fungal growth. Specifically, strains Q-84 and Q-60 showed the highest inhibitory effects, reaching 92 and 88 % of inhibition, respectively. These results were statistically significant ($p < 0.05$) and similar according to the Tuckey's test (Figure 1). Conversely, the Q-32 strain showed the lowest inhibition (30 %).

The inhibitory capacity of *Streptomyces* sp. has been reported against several phytopathogens, including fungi. For instance, the *Streptomyces* hydrogenans DH16 strain was demonstrated to display a wide range antifungal activity against *Colletotrichum* spp., *Alternaria* spp., *C. herbarum* and *Exserohilum* sp. *in vitro*, with inhibition zones of 30-36 mm [15]. Moreover, there were isolated up to 70 actinomycetes with high inhibition levels (61.57 %) against *Colletotrichum* sp [16]. Tests evaluating *Streptomyces* spp. antagonism against pathogenic fungi *in vitro* by the dual challenge method have found antifungal activity of varied intensity against highly resistant fungal strains, mainly mediated by antibiotic synthesis

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(antibiosis), these compounds difusing on the culture medium and and ultimately inhibiting fungal growth [14]. In fact, such inhibition tests have shown that one of the compounds identified as antifungal agent is Actynomycin, which is a toxic secondary metabolite for most phytopathogenic fungi [17].

Particularly, antibiotics produced by *Streptomyces* sp. are formed during the transition of vegetative cells into propagules, resulting in the presence of such compounds within the spores, thereby inhibiting spore germination until the presence of more favorable conditions. After that growth phase, a fast development is attained, which is linked to the synthesis of antibiotics [18].

Comparative evaluation of the inhibitory activity of the crude bioactive compound to two commercial chemically synthesized fungicides against *Colletotrichum* sp.

The crude extracts obtained from five bacterial strains *Streptomyces* sp. were evaluated to two commercial chemically synthesized fungicides in the well diffusion test against *Colletotrichum* sp. It was evidenced that the crude extract from strain Q-84 displayed the highest inhibition percentage among the extracts (56 %), while strains Q-84 and Q-60 where relatively lower (51.33 and 44.0 %, respectively). However, all the three strains behaved statistically similar to the inhibition shown by Amistar Top® and Opera® (100 and 84 %, respectively), according to the Tukey's test, against *Colletotrichum* sp. (Figure 2).

Chemical fungicides are widely used to control several phytopathogenic agents. Nevertheless, the risk for environmental pollution still remains, with potential concerns on its impact on human health, the development of resistance against these compounds and phytotoxicity [19]. Many studies have focused on the discovery of new compounds of natural origin and environmentally friendly with activity against phytopathogens affecting economically relevant crops. There was reported that the strain *Streptomyces* sp. A1022 had a similar control against *Colletotrichum* sp., as compared to a treatment with Azoxystrobin (71.6 vs. 70 %, respectively) in pepper [20]. The crude extract of *Streptomyces* sp. MJM5763 was effective to control *Colletotrichum* sp. in Yam (*Dioscorea* sp.), decreasing the severity and foliar damage indexes down in 88 and 81 %, respectively [21]. In these two studies, the *Streptomyces* sp. strains became alternatives for the use of chemical fungicides for controlling *Colletotrichum* sp. In this regards, the wide variety of secondary metabolites of *Streptomyces* sp. could be applied for fungi control in different crops, as pest control alternatives [22].

Macroscopic and microscopic properties of the five bacterial strains

After incubation, the putative *Streptomyces* sp. bacterial strains were identified attending to their micro and microscopic properties. It was confirmed by microscopy that the five isolated strains (Figure 3) were Gram-positive bacteria, also showing morphological features consistent with the *Streptomyces* genera.

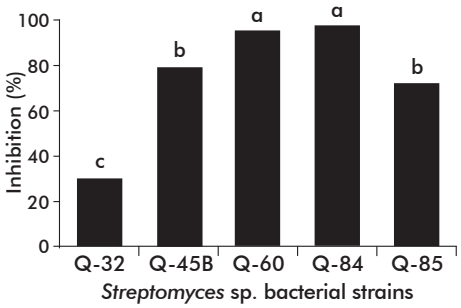

Figure 1. Inhibition of *Colletotrichum* sp. mycelial growth by bacterial strains of the genus *Streptomyces* sp. in the dual challenge test (Tukey's test). Different letters stand for statistically significant differences ($p < 0.05$).

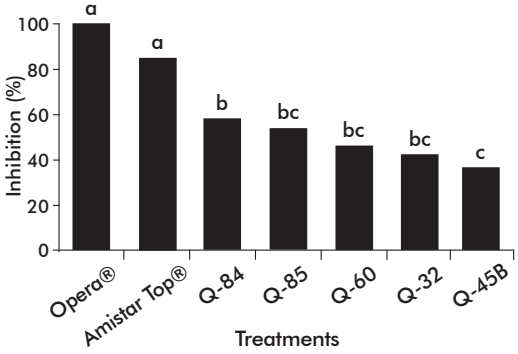

Figure 2. Inhibition of *Colletotrichum* sp. growth by crude extracts of bacterial strains of the genus *Streptomyces* sp. in the well diffusion test, compared to the commercial chemical fungicides Opera® and Amistar Top®. Different letters stand for statistically significant differences (Tukey's test; $p < 0.05$).

The Q-32 strain displayed a dry, dusty colony, dark reverse, the anverse initially white with white sporulation, and microscopically large and fragmented filaments, a sinuous mycelium with short conidia strands without spiral. The Q-45B strain had a creamy colony, with dark gray anverse, clear coffee-brown reverse, and microscopically filaments ramified of few conidia, most of them simple and non-stranded. In the case of the Q-60 strain, it was characterized by a dry colony, the anverse in gray with white edges, a dark gray reverse and a microscopic fragmented aerial mycelium, with fragmented vegetative mycelia and stranded or spiral conidia. The strain Q-84 showed a small colony, dry, dusty in appearance, with gray anverse and the reverse in dark orange as macroscopic features, and microscopically with a vegetative unfragmented mycelium and conidia strand of simple structure without spiral formation. Lastly, the Q-85 grew a dry, dusty colony, with a grayish-white anverse which turned to brown coffee over time, and microscopically with unfragmented vegetative mycelium and fragmented aerial mycelium with long stranded conidia.

The macro and microscopic observations made allowed us to describe the five strains as potentially belonging to the *Streptomyces* sp. genera, as showing intact substrate mycelia, a fragmented aerial

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mycelium, and the formation of conidia strands of coccus-like structure or bacilli-like with spiral disposition. On the other hand, the macroscopic observations showed dusty surface colonies, colored in white to whiter gray, hard in consistency and varied diffusible pigment. Likewise, most of the colonies shared a feature, consisting on a humid earth smell, indicating geosmin production [23].

Enzymatic activity tests of the five *Streptomyces* sp.

Following the distinct culture procedures for the three enzyme activities tested (lipase, protease and chitinase), the presence of enzymatic degradation halos was determined (Table 1).

As shown, all the five strains did not produce chitinases, with strains Q-60, Q-84 and Q85 producing lipases and proteases, and strains Q-45B and Q-32 only secreted lipases. Coincidently, strains Q-84, Q-60 and Q-85 showed the highest percentages of inhibition in the well diffusion test.

Previous studies have characterized the secretion of extracellular enzymes by *Streptomyces* sp. in large amounts, which are able to degrade organic compounds such as cellulose, lignin and protein substrates [24]. Those enzymes are able to degrade the fungal cell wall containing chitin [25, 26]. The five strains tested here, otherwise, showed no chitinase activity, while exhibiting acceptable inhibition percentages against *Colletotrichum* sp. In this regard, microorganisms do not depend on chitinase production to display antifungal control effects, since chitin is just one of the cell wall components of pathogenic fungi. For instance, in filamentous fungi, the cell wall is 20-30 % proteins [27]. This property is the base of the antifungal control strategies implemented against oomycetes of the genera *Phytophthora* spp. and *Pythium* spp., with 80 to 86 % of inhibition, respectively, against them [28]. Most of the effect of the metabolites produced by *Streptomyces* sp. is mediated by the interfering with the growth fungal hyphae, making the effect metabolite-specific [29, 30].

Genetic identification of the Q-84 strain

The Q-84 strain was genetically identified, by sequencing and homology analysis by BLAST, as belonging to the genus *Streptomyces* sp., showing a high homology (87.98 %) with the species *Streptomyces maritimus* strain MML1710, *Streptomyces* sp. strain RKBH-B124 and *Streptomyces rochei* strain Sal35, the three of them well known for their inhibitory capacity against phytopathogenic fungi [31, 32]. Considering the edaphic zone where this strain was isolated from, consisting of saline dessert soil (24.0 mhos electric conductivity) near the beach, and the production of lipases which is a distinctive property of actinomycetes from marine environments, it is suggested that the Q-84 strain could be *S. maritimus*. In this regard, soil sample characterization provided electric conductivity values of 24.9 mhos/cm, with pH 7.5, 0.7 % organic matter, 0.6 % $CaCO_3$ composition and sand-like soil. These environments are rich in polymeric substances and Actinomicetes spp. tend to secrete large amounts of lipases as an adaptive strategy [31, 32]. This is consistent with the results shown in table 2 regarding the enzymatic activity of the strains tested, while further characterization will be required since the homology percentage was below 95 %.

The identification of the BL02 result indicated that the organisms analyzed corresponds to a

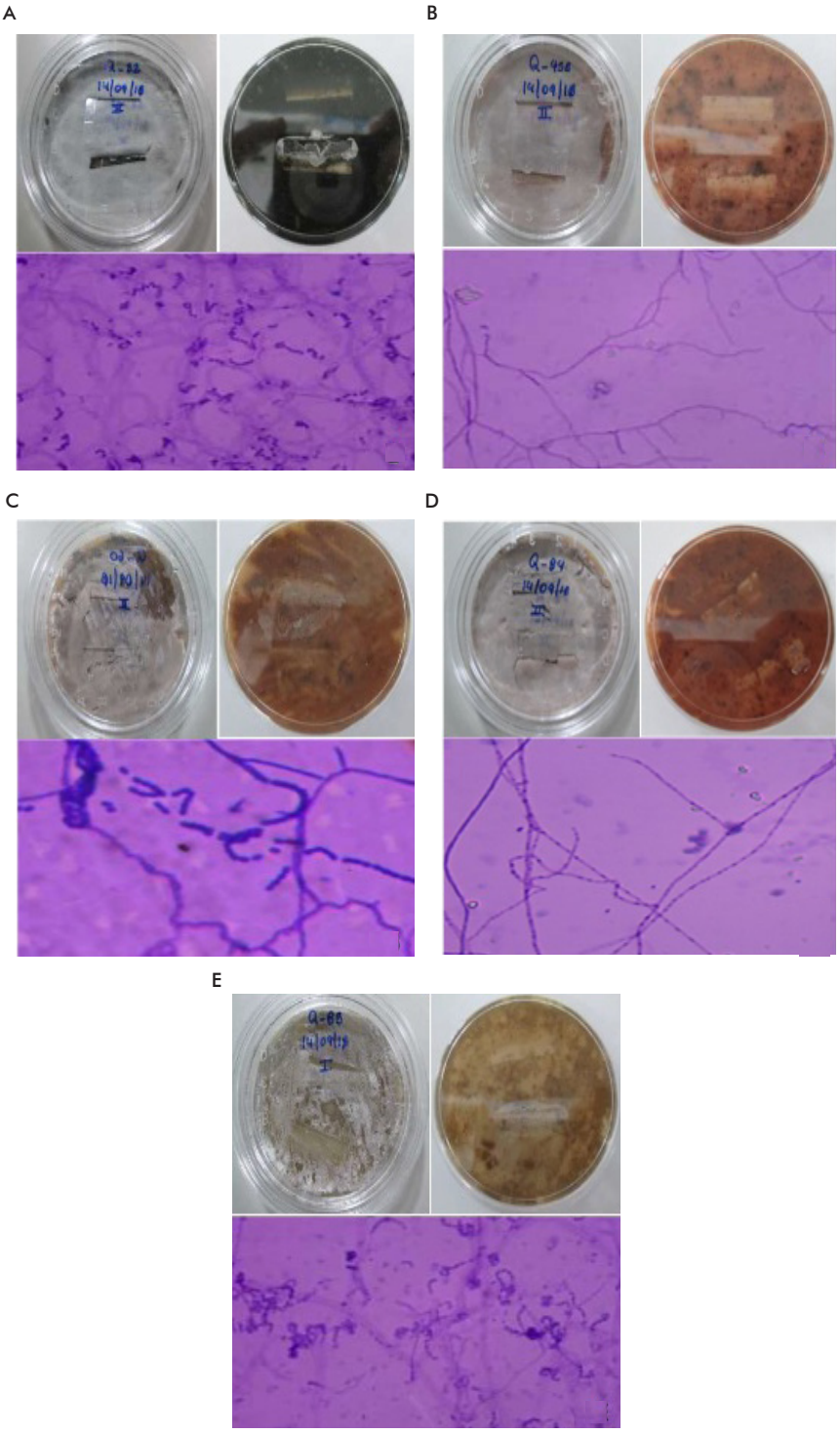

Figure 3. Macroscopic and microscopic properties of the five *Streptomyces* sp. bacterial strains identified with antifungal activity against *Colletotrichum* sp. A) Strain Q-32. B) Strain Q-45B. C) Strain Q-60. D) Strain Q-84. E) Strain Q-85.

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bacteria from the phylum Actinobacteria, order Streptomycetales, family Streptomycetaceae, genus *Streptomyces* sp.

Conclusions

The five *Streptomyces* sp. strains isolated from desert soil showed high antifungal activity against *Colletotrichum* sp. *in vitro*. Among them, the Q-84 strain showed the highest inhibition values both by the dual challenge test (91. 5 % inhibition) and the well diffusion test (56 %). This opens the possibility of using this strain for the integrated crop pest management of anthracnosis in relevant crops. The five bacterial strains were identified as belonging to the *Streptomyces* genus, based on their macroscopic and microscopic properties.

The strain Q-84, genetically identified with an 87.98 % accuracy at the 16S rRNA at PCR analysis, showed the highest inhibition values. Considering its enzymatic activity tested and the isolation zone, it may belong to the *Streptomyces maritimus*, to be refined in future studies.

Table 2. 16S rDNA partial sequencing data for the genetic identification of strain Q-84 of *Streptomyces* sp. bacteria

Description	Max score	Total score	Query cover (%)	E value	Identity (%)	Accesion number
Streptomyces maritimus strain MML1710 16S ribosomal RNA gene, partial sequence	2702	2702	99	0	99	MH084716.1
Streptomyces sp. Strain RKBH-B124 16S ribosomal RNA gene, partial sequence	2702	2702	99	0	99	KY362382.1
Streptomyces rochei strain Sal35 16S ribosomal RNA gene, partial sequence	2702	2702	99	0	99	KX440952.1

Conflicts of interest statement

The authors declare thar there are no conflicts of interest.

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