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DEPARTAMENTO DE AGRONOMÍA

**SELECTION OF ACTINOMYCETES STRAINS FOR THE  
CONTROL OF PHYTOPATHOGENIC SOIL-BORNE  
FUNGI, WITH SPECIAL REFERENCE TO ROOT ROT OF  
COMMON BEAN (*Phaseolus vulgaris* L.)**

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SELECCIÓN DE CEPAS DE ACTINOMICETOS PARA  
EL CONTROL DE HONGOS FITOPATÓGENOS DE  
SUELO, CON ESPECIAL REFERENCIA A LA  
PODREDUMBRE RADICAL DE LA JUDÍA COMÚN  
(*Phaseolus vulgaris* L.)

Programa de Doctorado: Ingeniería agraria, alimentaria, forestal y  
de desarrollo rural sostenible

Doctoranda: Miriam Díaz Díaz

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TITULO: *Selection of actinomycetes strains for the control of phytopathogenic soil-borne fungi, with special reference to root rot of common bean (Phaseolus vulgaris L.)*

AUTOR: *Miriam Díaz Díaz*

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**TÍTULO DE LA TESIS:** SELECCIÓN DE CEPAS DE ACTINOMICETOS PARA EL CONTROL DE HONGOS FITOPATÓGENOS DE SUELO, CON ESPECIAL REFERENCIA A LA PODREDUMBRE RADICAL DE LA JUDÍA COMÚN (*Phaseolus vulgaris* L.)

**DOCTORANDO/A:** MIRIAM DIAZ DIAZ

#### **INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS**

La doctoranda **Miriam Díaz Díaz** ha realizado satisfactoriamente y en los plazos previstos el trabajo presentado en esta Tesis Doctoral. La presente Tesis Doctoral se ha realizado dentro del programa internacional de la Asociación Universitaria Iberoamericana de Postgrado (AUIP) entre la Universidad Central 'Marta Abreu' de Las Villas (Cuba) y la Universidad de Córdoba (UCO, España). La mayoría de las actividades de investigación realizadas en esta Tesis Doctoral se han desarrollado en su universidad de origen, Universidad Central 'Marta Abreu' de Las Villas (Cuba), supervisadas por el Dr. Alexander Bernal, y apoyadas por sus directores de la UCO. Éstas han consistido en la caracterización de cepas de actinomicetos como potenciales agentes de biocontrol frente a la podredumbre radical de la judía común en Cuba. Todas estas actividades han resultado en dos publicaciones científicas en revistas indexadas por el Science Citation Index (SCI), y de alto impacto en la disciplina de fitopatología. Además, se está preparando una revisión sobre este tema que está en proceso de revisión para su publicación.

La doctoranda ha realizado una estancia de 6 meses en el Grupo Patología Agroforestal AGR-216 (Dpto. Agronomía, UCO), donde ha desarrollado un estudio de investigación complementario al tema de su Tesis Doctoral, evaluando las cepas de actinomicetos con las que trabajó en Cuba frente a la Verticilosis del olivo causada por *Verticillium dahliae*. Por las limitaciones del tiempo para presentar esta Tesis, este trabajo se muestra como borrador en el documento de Tesis y será también publicado próximamente en una revista científica de alto impacto. Además, durante el periodo de estancia en la UCO,

Miriam se ha adaptado perfectamente a nuestro laboratorio, manejando las técnicas empleadas, integrándose con sus compañeros y participando además en otros trabajos similares de investigación de otros doctorandos del grupo. A su vez, se ha integrado con el resto de doctorandos del Departamento de Agronomía participando en las actividades desarrollados por la Unidad de Excelencia María de Maeztu 2020-2023, como la realización de un seminario de actualidad exponiendo las actividades desarrolladas en su Tesis Doctoral.

Además de las publicaciones científicas, la doctoranda ha presentado dos comunicaciones orales sobre su trabajo, una en el XIV Simposio Internacional de Biotecnología Vegetal (Cuba, 2022), y otra en el XX Congreso de la Sociedad Española de Fitopatología (Valencia, España, 2022).

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 03 de marzo de 2023

Firma del/de los director/es

Fdo.: Antonio Trapero Casas

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## Summary

The common bean (*Phaseolus vulgaris* L.) is the most important crop for the Cuban population within the group of edible legumes with an annual production of 136,570 tonnes and yields ranging between 1.1 and 1.4 t ha<sup>-1</sup>. The environmental conditions, typical of subtropical countries, are favourable for the development and proliferation of a wide and heterogeneous soil microflora. Among them, the phytopathogenic soil-borne fungi *Macrophomina phaseolina* and *Rhizoctonia solani*, which are associated with bean root rot disease, causing important economic losses in this crop in Cuba. The low availability of active ingredients for the control of these plant pathogenic fungi, together with their intrinsic biology, typical of soil fungi, lead us to reconsider an integrated management strategy for the proper management of the disease. In this regard, the Centre for Bioactive Chemicals (CBQ) of the Central University "Marta Abreu" of Las Villas (UCLV) has a collection of 760 strains of bacteria classified as actinomycetes, microorganisms that have not been explored in Cuba so far as biological control agents (BCAs) with agricultural applications. Within the actinomycetes, *Streptomyces* is the most predominant genus in the terrestrial environment and the most studied for its properties as a biocontrol agent for plant pathogens and plant growth promoter. They are characterized by the production of biologically active primary and secondary metabolites and precursors of about 45 % of all bioactive compounds. Their enzymes allow them to chemically break down cellulose, lignin, chitin and protein-rich residues, as well as to physically bind soil particles together to form aggregates and prevent erosion.

Thus, the main objective of this PhD Thesis was to characterize 60 actinomycete strains *in vitro* and *in vivo* as BCAs against *M. phaseolina* and *R. solani*. Therefore, the first objective of this PhD Thesis was to carry out an exhaustive bibliographic review of the genus *Streptomyces* spp. as BCAs, with special emphasis on soil-borne pathogens. Here, aspects related to the phenotypic, biochemical and molecular characterisation of *Streptomyces*, as well as their mechanisms of action and their application in crop protection, were compiled.

The Objective 2 aimed to evaluate the effectiveness of 60 actinomycete strains as BCAs against *M. phaseolina* and *R. solani* *in vitro* by dual culture assays. The most effective strains were characterized according to their cellulolytic, chitinolytic and proteolytic extracellular enzyme activity, as well as their morphological, biochemical and molecular characters. The results showed that 66.6% and 41.0% of the actinomycete

strains significantly inhibited the mycelial growth of *M. phaseolina* and *R. solani*, respectively, compared with the control; and 30% of them showed a common effect against both pathogens. Significant differences were observed in their enzymatic and biochemical activity. Morphological and biochemical characters allowed us to identify all the strains as species belonging to the genus *Streptomyces*. Two *Streptomyces* strains, CBQ-EA-2 and CBQ-B-8, showed the highest effectiveness *in vitro*. Finally, the effect of seed treatments with both strains (CBQ-EA-2 and CBQ-B-8), using single or mixed applications, against the disease development was evaluated on seedlings of *P. vulgaris* cv. Quivicán grown in pots filled with soils inoculated with *M. phaseolina* and *R. solani*. Treatments combining the two *Streptomyces* strains (CBQ-EA-2 + CBQ-B-8) were able to significantly reduce the disease incidence and severity for both pathogen infections compared to the nontreated and inoculated control. Furthermore, they showed a similar effect to that observed for the BCA *Trichoderma harzianum* A-34 and the chemical Celest® Top 312 FS (Syngenta®; Basel, Switzerland) treatments, which were included for comparative purposes.

The Objective 3 was carried out to validate the results of the Objective 2 in three experimental fields with soils naturally infested by *M. phaseolina* and *R. solani*. In all experimental fields, significant differences in disease incidence and severity were observed between treatments compared to the nontreated controls. Overall, single treatments with *Streptomyces* sp. strain CBQ-EA-2 or *Streptomyces* sp. strain CBQ-B-8 showed significantly less effect in reducing disease incidence and severity than treatments combining the two strains (*Streptomyces* sp. CBQ-EA-2+ CBQ-B-8), regardless the experimental field. The three treatments with *Streptomyces* spp. were significantly more effective than those with Celest® Top 312 FS; in contrast, most of the *Streptomyces* treatments had a significantly lower effect than the BCA *T. harzianum* A-34. When we evaluated the yield and quality of the legumes, in general, the treatment combining the two strains (*Streptomyces* sp. CBQ-EA-2+ CBQ-B-8) showed similar results with the chemical Celest® Top 312 FS and with the BCA *T. harzianum* A-34.

Finally, the objective 4 was a complementary study carried out during a 6-month pre-doctoral stage at the University of Cordoba (Spain). The goal of this work was to evaluate the effect of six of the most effective *Streptomyces* strains from the Objective 1 as BCAs against Verticillium wilt of olive caused by *Verticillium dahliae*, one of the main olive diseases in Spain. For this purpose, trials were carried out under controlled conditions using two isolates of *Verticillium dahliae* (V-004 and V-323), evaluating the

effect of the *Streptomyces* strains on mycelial growth of the pathogen by dual culture as well as their effect on the viability of conidia and microsclerotia of the pathogen *in vitro*. In addition, their effect on the disease development was determined in olive plants inoculated with *V. dahliae* isolate V-323. The six *Streptomyces* sp. strains and the two BCAs *F. oxysporum* FO12 and *A. pullulans* AP08 used as effective control showed a significant effect on mycelial growth inhibition for both *V. dahliae* isolates V-004 and V-323 compared to the untreated control. The reference BCA FO12 was the most effective on mycelial growth inhibition followed by AP08 for the two *V. dahliae* isolates; while the *Streptomyces* sp. strains showed a moderate effect for both *V. dahliae* isolates. Among the *Streptomyces* strains evaluated, CBQ-EA-2 was the most effective inhibiting mycelial growth of *V. dahliae*.

The result generated through this PhD Thesis represents an important advance in the biocontrol of soil-borne pathogens of two crops, common bean and olive, of global economic importance through the use of *Streptomyces* spp. strains, which had not been previously explored in the two geographical areas where this PhD Thesis has been developed.



## Resumen

La judía común (*Phaseolus vulgaris* L.) es la especie vegetal más importante para la población cubana dentro del grupo de las leguminosas comestibles con una producción anual de 136.570 toneladas y rendimientos que oscilan entre 1,1 y 1,4 t ha<sup>-1</sup>. Las condiciones ambientales, típicas de países subtropicales, son favorables para el desarrollo y la proliferación de una amplia y heterogénea microflora del suelo, entre la que destacan los hongos fitopatógenos de suelo *Macrophomina phaseolina* y *Rhizoctonia solani*, asociados con la podredumbre de raíces de la judía, y que causan importantes pérdidas económicas en este cultivo en Cuba. La baja disponibilidad de materias activas para el control de estos hongos fitopatógenos, unida a su biología intrínseca, propia de hongos del suelo, nos conducen a replantearnos una estrategia de gestión integrada para el manejo adecuado de estas enfermedades. En tal sentido, el Centro de Bioactivos Químicos (CBQ), de la Universidad Central “Marta Abreu” de Las Villas (UCLV), cuenta con una colección de 760 cepas de bacterias clasificadas como actinomicetos, microorganismos no explorados en Cuba hasta el momento como agentes de control biológico (ACBs) con aplicaciones agrícolas. Dentro de los actinomicetos, *Streptomyces* es el género más predominante en el medio terrestre y el más estudiado por sus propiedades como agente de biocontrol de fitopatógenos y promotor del crecimiento de las plantas. Estos microorganismos se caracterizan por producir metabolitos primarios y secundarios, biológicamente activos y precursores de cerca del 45% de todos los compuestos bioactivos. Sus enzimas les permiten descomponer químicamente la celulosa, la lignina, la quitina y los residuos ricos en proteínas, así como unir físicamente las partículas del suelo para formar agregados y evitar la erosión.

Así, el objetivo principal de esta Tesis Doctoral fue caracterizar *in vitro* e *in vivo* 60 cepas de actinomicetos como ACBs frente a *M. phaseolina* y *R. solani*. Para ello, el primer objetivo de esta Tesis Doctoral fue realizar una exhaustiva revisión bibliográfica del género *Streptomyces* spp. como ACBs, con especial énfasis en patógenos de suelo, igualmente, se resumen aspectos relacionados con la caracterización fenotípica, bioquímica y molecular de *Streptomyces*, así como sus mecanismos de acción y su aplicación en la protección de cultivos.

El objetivo 2 fue evaluar la eficacia de 60 cepas de actinomicetos como ACBs frente a *M. phaseolina* y *R. solani* *in vitro* mediante ensayos de cultivo dual. Las cepas más eficaces se caracterizaron de acuerdo con su actividad enzimática extracelular celulolítica, quitinolítica y proteolítica, así como por sus caracteres morfológicos, bioquímicos y

moleculares. El 66,6 % y 41,0 % de las cepas de actinomicetos inhibieron el crecimiento micelial de *M. phaseolina* y *R. solani*, respectivamente, y 30 % de ellas mostraron un efecto común contra ambos patógenos. Se observaron diferencias significativas en su actividad enzimática y bioquímica. Los caracteres morfológicos y bioquímicos permitieron identificar todas nuestras cepas como pertenecientes al género *Streptomyces*. Las cepas *Streptomyces* CBQ-EA-2 y CBQ-B-8 mostraron la mayor eficacia *in vitro*. Por último, se evaluó el efecto de los tratamientos de semillas con ambas cepas sobre *M. phaseolina* y *R. solani* en plántulas de *P. vulgaris* cv. Quivicán. Los tratamientos que combinan las dos cepas de *Streptomyces* (CBQ-EA-2 + CBQ-B-8) fueron capaces de reducir significativamente la enfermedad causada por ambos patógenos en comparación con el control no tratado e inoculado. Además, mostraron un efecto similar al observado con *Trichoderma harzianum* A-34 y con los tratamientos Celest® Top 312 FS (Syngenta®; Basilea, Suiza), que se incluyeron con fines comparativos.

El objetivo 3 se llevó a cabo para validar los resultados anteriores en tres campos experimentales con suelos naturalmente infestados por *M. phaseolina* y *R. solani*. En todos los campos experimentales, se observaron diferencias significativas ( $P < 0,05$ ) en la incidencia y severidad de la enfermedad entre los tratamientos en comparación con los controles no tratados. En general, los tratamientos con *Streptomyces* sp. cepa CBQ-EA-2 o *Streptomyces* sp. cepa CBQ-B-8 solos mostraron un efecto significativamente menor en la reducción de la incidencia y la severidad que los tratamientos que combinaban las dos cepas (*Streptomyces* sp. CBQ-EA-2+ CBQ-B-8), independientemente del campo experimental. Los tres tratamientos con *Streptomyces* spp. resultaron significativamente más eficaces que los realizados con el producto químico Celest® Top 312 FS; por el contrario, la mayoría de los tratamientos con *Streptomyces* tuvieron un efecto significativamente menor que el ACB *T. harzianum* A-34. Cuando se evaluaron el rendimiento y calidad de las legumbres, en general, el tratamiento combinando las dos cepas (*Streptomyces* sp. CBQ-EA-2+ CBQ-B-8), mostró resultados similares con el producto químico Celest® Top 312 FS y con *T. harzianum* A-34.

Por último, el objetivo 4 fue un estudio complementario realizado durante una estancia predoctoral de 6 meses en la Universidad de Córdoba (España). En este trabajo se evaluó el efecto de seis de las cepas de *Streptomyces* más efectivas del Objetivo 1 como ACBs frente a la Verticilosis del olivo causada por *Verticillium dahliae*, una de las principales enfermedades del olivar en España. Para ello, se llevaron a cabo ensayos en condiciones controladas empleando dos aislados de *V. dahliae* (V-004 y V-323), evaluando

el efecto de las cepas sobre el crecimiento micelial del patógeno mediante cultivo dual, así como su efecto en la viabilidad de conidios y microesclerocios del patógeno *in vitro*. Además, se determinó su efecto frente al desarrollo de la enfermedad en plantas de olivo, empleando en este caso el aislado V-323 de *V. dahliae*. Las seis cepas de *Streptomyces* sp. y los dos ACBs *F. oxysporum* FO12 y *A. pullulans* AP08 utilizados como controles efectivos mostraron un efecto significativo sobre la inhibición del crecimiento micelial los dos aislados de *V. dahliae* V-004 y V-323 en comparación con el control no tratado. El ACB de referencia FO12 fue el más eficaz sobre la inhibición del crecimiento micelial seguido de AP08 para los dos aislados de *V. dahliae*; mientras que las cepas de *Streptomyces* sp. mostraron un efecto moderado, para ambos aislados de *V. dahliae*. De las cepas de *Streptomyces* sp evaluadas, fue CBQ-EA-2 la que mostró los mejores resultados en la inhibición del crecimiento micelial de *V. dahliae*.

El resultado generado a través de esta Tesis Doctoral representa un avance importante en el biocontrol de patógenos de suelo de dos cultivos de importancia económica mundial mediante el uso de cepas de *Streptomyces* spp., no explorado previamente en las dos zonas geográficas donde se ha desarrollado esta Tesis Doctoral.



## Abbreviations

AG-4\_HGI: anastomosis groups  
ANOVA: analysis of variance  
APDA: Potato dextrose agar acidified with lactic acid  
AUDPC: area under the disease progress curve  
BCAs: biological control agents  
BLAST: Basic local alignment search tool  
CSA: casein-starch agar  
CBQ: Centro de Bioactivos Químicos  
CCS: credit and services cooperative  
CFUs: colony forming units  
CIAP: Centro de Investigaciones Agropecuarias  
CI: consistency index  
DAGC: days after germination of cotyledons  
DAS: days after sowing  
D: defoliating pathotype  
DI: disease incidence  
DNA: desoxirribonucleico ácido  
dNTPs: deoxyribose nucleoside triphosphates  
DRBC: Rose Bengal dichloran agar with chloramphenicol  
DSstem: severity of the disease stem  
DSroot: severity of the disease root  
DS: disease severity  
FAO: Food and Agricultural Organization of the United Nations  
FC: field capacity  
HI: homoplasy index  
HSD: Tukey's honestly significant difference  
BI: Bayesian inference  
IBP: Instituto de Biotecnología de las Plantas  
INISAV: Instituto de Investigaciones de Sanidad Vegetal  
ISP2: International *Streptomyces* Project  
LSD: least significant difference  
MINAG: Ministerio de la Agricultura

MGI: Mycelial growth inhibition  
MGR: mycelial growth rate  
MgSO<sub>4</sub>: magnesium sulphate  
MR: methyl red  
MS: microsclerotia  
NaCl: sodium chloride  
NBRIP: National Botanical Research Institute Phosphate Medium  
NRAG: ramal standard  
rDNA: desoxirribonucleico ácido ribosomal  
RAUDPC: relative area under the disease progress curve  
RC: rescaled consistency index  
RGI: inhibition of conidial germination  
RGR: radial growth rate  
RH: relative humidity  
RI: retention index  
RNA: rribonucleico acido  
ND: non-defoliating pathotype  
NLP: number of legumes per plant  
NSL: number of seeds per legume  
NSP: number of seeds per plant  
PCR: polymerase chain reaction  
PDB: potato dextrose broth  
SDW: sterile distilled water  
TL: tree length  
TSA: tryptone soy agar  
TSB: tryptone soy broth  
UBS: seed-based business unit  
UBPC: basic unit of cooperative production  
UEB: Unidad Empresarial de Base  
VP: Voges Proskauer  
VWO: Verticillium wilt of olive

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# *Chapter 1*





## GENERAL INTRODUCTION

### The common bean crop and its diseases

The common bean (*Phaseolus vulgaris* L.) is one of the most important grain legumes in many areas of the world, providing a staple food with a diet rich in protein, dietary fibre, essential micronutrients and phytochemicals for more than 500 million people (Singh *et al.*, 2020). The global cultivated area of *P. vulgaris* reached 33.1 million hectares in the season 2019/2020, with a world production of 29 million tonnes and an average seed yield of 1557 kg ha<sup>-1</sup> (FAO 2021). Biotic and abiotic stresses that affects common bean crop in tropical regions, intensified by climate change lead to decreases in crop yields and legume quality (El-Mougy *et al.*, 2020). *Alternaria alternata*, *Colletotrichum truncatum*, *Fusarium oxysporum*, *Macrophomina phaseolina*, *Rhizoctonia solani*, and *Sclerotium rolfsii*, among others, are some of the plant pathogens affecting the crop (Were *et al.*, 2021). Among them, *M. phaseolina* and *R. solani* are considered the most prevalent pathogenic fungi associated with root rot diseases of common bean worldwide (Olaya and Abawi, 1996; Spedaletti *et al.*, 2017).

*Macrophomina phaseolina* (Ascomycota), causal agent of ashy stem blight, also affects roots and stems of host species via pycnidiospores and microsclerotia that persist in the soil, where the pathogen establishes the primary inoculum (Zhao *et al.*, 2020). Typical symptoms in common bean include dark, irregular lesions on cotyledons, wilting, systemic chlorosis, premature defoliation, epinasty and early maturity or death in adult plants (Olaya and Abawi, 1996). Late infections cause the appearance of grey areas on the stems, where microsclerotia and pycnidia of the fungus are produced. The occurrence of *M. phaseolina* in the seeds has major consequences since it causes the disqualification of legumes as propagation material (MINAG, 2008).

On the other hand, *R. solani* (Basidiomycota) is the causal agent of rhizoctonia blight, also commonly known as damping off (Abu-Tahon *et al.*, 2022). This soil-borne pathogen can affect more than 500 plant species, including cultivated and wild plants, and causes damping off in stands, necrotic lesions in roots, seeds and stems, as well as foliar lesions with a worldwide distribution (Spedaletti *et al.*, 2017; Muharrem *et al.*, 2020). This fungus affects young seedlings much more than adult plant tissues. On the stem and hypocotyl of affected plants, reddish-brown cankers of various sizes appear, usually delimited by a dark border, which later become rough, dry up and destroy plant tissues

(Muharrem *et al.*, 2020). It also attacks the roots causing foot rot of the plants González (1988).

The management of soil-borne pathogens including both *R. solani* and *M. phaseolina* is usually difficult, not only due to their intrinsic biology, but also because the lack of effective active ingredients. Thus, the use and extension of eco-friendly control methods such as biological control is required, not only to prevent plant diseases, but also contributing markedly to soil preservation and conservation (Muharrem *et al.*, 2020).

Likewise, a wide diversity of microorganisms including bacteria such as *Agrobacterium*, *Bacillus* (Sendi *et al.*, 2020), *Burkholderia*, *Pseudomonas* and *Rhizobium* (Ferreira *et al.*, 2020) and fungi such as *Trichoderma* (Ketta and Hewedy, 2021) have already been evaluated under controlled conditions for their effectiveness as biological control agents (BCAs) against soil-borne pathogens such as those associated with root rot diseases of common bean (Eke *et al.*, 2019; Bedine *et al.*, 2020). Other microorganisms, although to a lesser extent, have been evaluated under field conditions (Volpiano *et al.*, 2018; Eke *et al.*, 2019).

### **Starting hypothesis and justification of the PhD Thesis**

In addition to the microorganisms mentioned above and known for their proven potential as BCAs, much of the research in recent decades has focused on actinomycetes and their potential as BCAs. Actinomycetes are a heterogeneous group of gram-positive bacteria, inhabiting different environments, both terrestrial and aquatic, belonging to the phylum Actinomycetota. They are the most abundant microorganisms in the soil, generally reaching densities of  $10^6$ - $10^9$  cells per gram of soil (Barka *et al.*, 2015; Gomes *et al.*, 2018). They are distinguished by producing extracellular enzymes with proteolytic activity, cellulolytic, lignolytic and chitinolytic, improve soil physical properties, contribute to water retention, prevent erosion, degrade and reduce certain types of pollutants, such as pesticide residues (Kannabiran, 2017; Otani *et al.*, 2022). Within Actinomycetota, the genus *Streptomyces* stands out for its characteristics. Its applications range from the production of antibiotics, anti-tumour, immunosuppressants and anti-parasitic drugs to biotechnological applications (Takahashi and Nakashima, 2018; Donald *et al.*, 2022). Interestingly, *Streptomyces* strains have been also used in agriculture as insecticides, fungicides and herbicides, as well as plant growth-promoting endophytes to improve crop yields and as biocontrol agents for plant diseases, especially against soil-

borne plant pathogenic fungi (Franco *et al.*, 2010; Hayat *et al.*, 2010; Doolotkeldieva *et al.*, 2015; Gomes *et al.*, 2018; Gebily *et al.*, 2021; Rajeeswari *et al.*, 2021; Carlucci *et al.*, 2022). Recently, several *in vitro* techniques were used to evaluate the antagonistic activity of *S. griseus* (DG5), *S. rochei* (DG4) and *S. sampsonii* (DG1) against *S. sclerotiorum*, causal agent of white mould disease affecting green beans (*Phaseolus vulgaris* L.), The dual confrontation, cell-free filtrate and volatile compound activity, led to a test under greenhouse conditions where *Streptomyces* spp, either alone or in combination significantly reduced the incidence of disease relative to the untreated control (Gebily *et al.*, 2021). Similarly, *S. albidoflavus* CARA17, by root immersion in *Foeniculum vulgare* seedlings infected by *A. rolfsii*, *F. oxysporum*, *Plectosphaerella ramiseptata*, *S. sclerotiorum* and *V. dahliae*, showed efficacy in disease reduction (Carlucci *et al.*, 2022).

The use of the biological potential of actinomycetes, through the production, use and marketing of biological fungicides such as MYCOSTOP MIX® and MYCOSTOP® (*Streptomyces griseoviridis* K61; Sanogo and Lujan, 2022), Actinovate® (*Streptomyces lydicus* WYEC 108\*; Hernández-Cruz *et al.*, 2020), have been useful for the control of *Rhizoctonia*, *Phytophthora*, *Fusarium*, *Pythium* on legume species and other crops. However, the use of actinomycetes as ACBs has not yet studied for the control of root rot diseases of common bean in Cuba, where no biological products based on actinomycetes has been developed so far. In this way, considering the properties of actinomycetes described above, the Centro de Bioactivos Químicos has a collection of actinomycetes strains isolated in the central region of Cuba, that deserve to be explored as a potential biological alternative to be included in the integrated disease management programme against soil-borne phytopathogens in common bean crop. In parallel, evaluating the effect of actinomycetes against one of the major olive pathogens in Spain, *Verticillium dahliae*, is necessary to search for alternatives to *Verticillium* wilt of olive.







## OBJECTIVES

Against this background, the general objective of this PhD Thesis was to characterize *in vitro* and *in vivo* 60 actinomycetes strains as biological control agents against soil pathogens, with special emphasis on the control of common bean root rot associated with the soil-borne pathogens *Macrophomina phaseolina* and *Rhizoctonia solani*.

Thus, the specific objectives were:

1. To carry out a comprehensive literature review on the use of actinomycetes as biocontrol agents against soil-borne pathogens. This objective is addressed in **Chapter 2**.
2. To characterize 60 actinomycetes strains *in vitro* against *M. phaseolina* and *R. solani* by dual culture assays as well as their cellulolytic, chitinolytic and proteolytic extracellular enzymatic activity and by their morphological, biochemical and molecular characteristics. In addition, the effect of seed treatments of *P. vulgaris* cv. Quivicán with *Streptomyces* spp. strains in soil inoculated with *M. phaseolina* and *R. solani* was evaluated under semi-controlled conditions. This objective is addressed in **Chapter 3**.
3. To validate the results *in planta* of Objective 2 in three experimental fields with soils naturally infested with *M. phaseolina* and *R. solani*. This objective is addressed in **Chapter 4**.
4. To evaluate the effect of the six most effective *Streptomyces* spp. strains of the Objective 2 against *Verticillium dahliae*. To this end, *in vitro* experiments were conducted to determine their effect on the mycelial growth of the pathogen by dual confrontation, and to assess the viability of conidia and microsclerotia of the pathogen. Their biocontrol effectiveness on disease development was also evaluated in cotton and olive plants inoculated with *V. dahliae*. This objective is addressed in **Chapter 5**.

## OUTLINE OF THE THESIS

In the **Chapter 2** of this PhD Thesis, a comprehensive literature review on actinomycetes as potential biocontrol agents against soil-borne plant pathogens has been carried out. This first step was necessary to document us about the general characteristics of actinomycetes with special emphasize in the potential of *Streptomyces* spp. as biological control agents against soil-borne plant pathogenic fungi.

In the **Chapter 3**, 60 actinomycetes strains were evaluated by means of dual cultures against *M. phaseolina* and *R. solani*. This study was necessary to test whether the actinomycetes strains were able to inhibit the mycelial growth of both phytopathogens *in vitro*. Then, the extracellular enzyme activity (chitinolytic, cellulolytic and proteolytic) was qualitatively assessed, and 11 of them were characterised morphologically and biochemically, and the two most effective strains were identified by molecular techniques as *Streptomyces* sp. These aspects were necessary to have a more complete characterisation of the strains and to have elements to decide which ones we would select for further studies under semi-controlled conditions. Subsequently, the effect of two most effective *Streptomyces* sp. strains against the disease progress was evaluated under semi-controlled conditions on seedlings of *P. vulgaris* cv. Quivicán inoculated with *M. phaseolina* or *R. solani*. In addition, the main objective of the **Chapter 4** was to validate the above results under field conditions. To this end, the same biological treatments using *Streptomyces* sp. strains evaluated *in planta* in the Chapter 3 were assessed in experimental fields of *P. vulgaris* cv. Quivicán established in soils naturally infested by *M. phaseolina* and *R. solani*. Here, disease incidence and severity as well as crop yield and legume quality was evaluated.

Finally, the **Chapter 5** aims to evaluate the effect of the most effective *Streptomyces* sp. strains from Chapters 3 and 4 as biocontrol agents against *Verticillium dahliae* by means of dual culture assays, *in vitro* assays in naturally infested soils, and bioassays on olive and cotton plant inoculated with *V. dahliae*.

Chapters 2, 3 and 4 have been published in peer-reviewed journals (see **Appendix: Scientific production**), while Chapter 5 has not yet been published, as it is part of a complementary study carried out during a 6-month pre-doctoral stay at the University of Cordoba (Spain) conducted from September, 2022 to May, 2023, but a similar format has been applied for it. This last chapter will be also published in a peer-review journal next year.

## *Chapter 2*





**Actinomycetes as potential biocontrol agents against soil-borne fungal plant pathogens: A review**

**ABSTRACT**

Actinomycetes are unicellular, saprophytic, gram-positive bacteria with a high guanine + cytosine (G+C) content in their DNA and with physiological characteristics that resemble those of many fungal species. Actinomycetes are ubiquitous in both sweet and salty terrestrial and aquatic ecosystems, where they are associated with sediments, especially organic matter undergoing decomposition. They exhibit proteolytic, cellulolytic, lignolytic and chitinolytic enzymatic activity since their enzymes allow them to chemically break down protein-rich materials, cellulose, lignin and chitin. The largest genus belonging to the class Actinomycetes is *Streptomyces*; to date, 846 species with validly published names have been assigned to this genus. Actinomycetes, particularly *Streptomyces* spp., are used in agriculture as insecticides, fungicides, and herbicides as well as plant growth-promoting endophytes to improve crop performance. In addition, they are a significant component of the microbial population of the rhizosphere, contribute to plant biofertilization, and play an important role as biocontrol agents against plant diseases, especially diseases caused by soil-borne plant pathogenic fungi. This review extends the current understanding of how *Streptomyces* spp. contribute to the biocontrol of soil-borne pathogens. Here, we provide updates on recent studies *in vitro*, *in vivo* and in the field that evaluate the role of a broad diversity of *Streptomyces* species in combating soil-borne pathogens in a wide diversity of crops. Detailed information about the phenotypic, biochemical and molecular characterization of *Streptomyces* species, as well as their mechanisms of action and their application in crop protection, is also provided.

**KEYWORDS:** biological control, crop protection, soil-borne diseases, *Streptomyces* spp., sustainable agriculture

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## **2.1. INTRODUCTION**

The soil is considered an important ecological niche in which a broadly diverse group of beneficial microorganisms establishes a complex soil network and dynamic and nonrenewable system. The activity of these microorganisms is essential for preserving soil properties as well as for enhancing the productivity of agricultural systems (Viti *et al.*, 2008). Nevertheless, increased demand for global production due to the need to supply food to the growing worldwide population has led over time to the development of intensive agricultural production, causing environmental, abiotic and biotic stresses (Goswami *et al.*, 2016).

In the framework of traditional agricultural systems, the management of agricultural soils, including application of nutrients, extensive soil cultivation and the use of pesticides against soil-borne diseases, among others, has a significant impact on soil microbial communities (Vitanovic, 2012). The resulting changes in soil microbial communities may cause long-term disruption of the functioning of plant and soil ecosystems (Viti *et al.*, 2008).

It is estimated that biotic stresses caused by plant pathogenic microorganisms such as viruses, bacteria, fungi and other parasites reduce annual agricultural yields by at least 30%. Among plant pathogenic microorganisms, fungi in the broad sense, including true fungi and oomycetes, are the most frequent cause of two-thirds of all plant diseases (Goswami *et al.*, 2016). The Honduran Foundation for Agriculture Research (FHIA) estimates that 5-25% of crops in developed countries and 20-50% of crops in developing countries are lost to fungal diseases (FHIA, 2007).

Within the context of this review, plant pathogenic fungi can be classified according to the parts of plants that they infect and according to the method through which they are disseminated. In this sense, we find two major groups: i) airborne plant pathogens that affect the aerial parts of plants and are easily and quickly dispersed, mainly through the air and in raindrops; and ii) soil-borne plant pathogens that normally affect the roots and basal stems of plants but may also be vascular in nature, affecting the xylem of the plant; soil-borne pathogens remain in the soil, where they survive for a long time and are dispersed slowly by means of irrigation, soil movement, cultural practices, and other routes (Agrios, 2005).

## **2.2. Plant diseases caused by soil-borne fungal pathogens**

### *2.2.1. Root and crown rot diseases*

This type of disease affects the roots and/or crowns of plants, causing necrosis or rot symptoms in both herbaceous and woody crops (Fig. 2.1 A-E). A broad diversity of fungal species representing almost all fungal taxa have been associated with the disease. These fungi are necrotrophs whose parasitic relationship with the host varies greatly. They act as soil inhabitants with a dominant saprophytic phase as well as soil invaders or root inhabitants with little or no capacity for saprophytic competition (García-Jiménez *et al.*, 2010; Bellgard *et al.*, 2019). In general, their presence is favoured by excess moisture, and they are able to survive in soil for long periods of time in the absence of host plants because they form resistant structures within (García-Jiménez *et al.*, 2010; Khan and Abid, 2017).

In herbaceous crops, the most serious diseases caused by this group of fungi are *i*) root and crown rot of legumes caused by species of *Fusarium*, *Phytophthora*, *Macrophomina*, *Rhizoctonia* and *Pythium* (Bodah, 2017; Viteri *et al.*, 2017); *ii*) a serious root disease of cereals known as “take-all disease of wheat and barley” that is caused by *Gaeumannomyces graminis* var. *tritici* and by *Fusarium* spp. (Tyburski, 2014); *iii*) root rot of pepper caused by *Phytophthora capsici* (Majid *et al.*, 2016); and *iv*) root rot of various horticultural and ornamental plants caused by species of *Rhizoctonia*, *Sclerotinia* and *Sclerotium* (Friberg *et al.*, 2005). Among the pathogens that cause root and crown rot disease in woody crops, there are two main categories that are distinguished according to whether the pathogens affect the rootlets or the woody roots of the plants. Of the pathogens in the first category, *Phytophthora* spp., which mainly affect fruit and olive trees, are considered one of the pathogens with the highest incidence (Sánchez-Hernández *et al.*, 2001); they also affect several forest species, including oak and chestnut trees (Sánchez-Hernández *et al.*, 2004; Bellgard and Smith, 2019). Among the rots affecting woody roots, those caused by *Armillaria mellea* and *Rosellinia necatrix* affect large numbers of fruit trees and forest species (Bodah, 2017).

### *2.2.2. Damping-off diseases*

“Damping-off” refers to the death of a seedling before or shortly after emergence due to decomposition of the root and/or lower stem; it is common to distinguish between preemergence damping-off and postemergence damping-off (Doohan, 2005; Bodah, 2017) (Fig. 2.1F-H). This condition affects 5 to 80% of seedlings, resulting in direct costs

due to damage to seeds or seedlings and reducing the growth and the number of healthy plants, as it affects both nurseries and fields (Lamichhane *et al.*, 2017).

The plant pathogens associated with damping-off include those belonging to certain genera of oomycetes, i.e., *Phytophthora* and *Pythium*, and fungi, i.e., ‘*Cylindrocarpon*’ anamorphs, *Fusarium*, *Rhizoctonia* and *Sclerotium*. These fungi inhabit the soil and have a high capacity for survival as saprophytes or through the formation of resistance structures; they usually have a wide range of hosts (Lamichhane *et al.*, 2017; Majeed *et al.*, 2018).

Environmental and management conditions commonly lead to increased severity of the infections caused by these pathogens. Grey mould (*Botrytis cinerea*) and some abiotic conditions that favour the occurrence of damping-off are generally related to excessive soil moisture, excessive overhead misting, lower soil temperatures before emergence, higher soil temperatures after emergence, and overcrowded flats or unelevated seedbeds. Many of these infections involve *Pythium*, *Phytophthora*, *Fusarium*, *Sclerotinia* and *Rhizoctonia*, which together account for almost two-thirds of the fungal disease problems of forest species in the genera *Ceratonia*, *Pinus*, *Quercus*, and others and those of ornamental and horticultural species. In the case of field crops, the most important failures caused by these pathogens occur during the birth of legumes (chickpeas, beans, lentils, and beans), beets, cotton and wheat (Lamichhane *et al.*, 2017).

### 2.2.3. Vascular wilt diseases

According to the American Phytopathological Society (APS), wilt refers to the drooping of leaves from stems due to a lack of water, i.e., an inadequate water supply or excessive transpiration caused by a vascular disease that interrupts normal water uptake (Fig. 2.1I-K). Fungal vascular wilt is a major plant syndrome that is of enormous economic importance. Some fungal diseases, such as Dutch elm disease, have been devastating in their effects worldwide. The earliest reports of vascular diseases appeared in the 1880s; thus, these diseases have been the focus of intensive study, both applied and basic, for more than 140 years (Velásquez-Valle and Schwartz, 1997; Dorjey *et al.*, 2017; Sandani and Weerahewa, 2018).

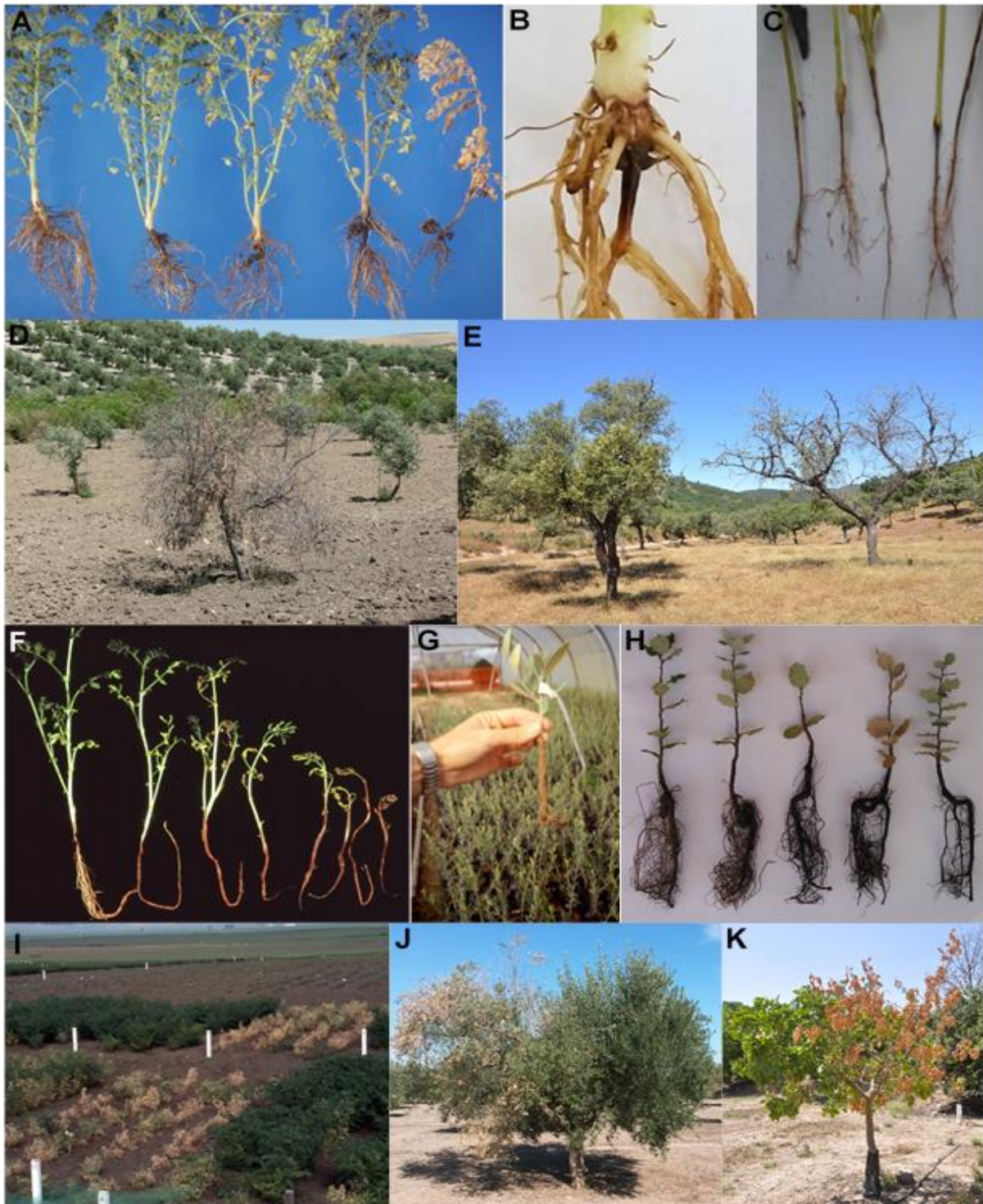
Among the most important vascular wilt diseases are i) *Fusarium* wilt disease caused by numerous formae speciales of a single species, *Fusarium oxysporum*; although this species is currently considered a complex species, some researchers prefer to divide

it into several species of *Fusarium* (Lombard *et al.*, 2015); ii) *Verticillium* wilt disease, which is mainly caused by two species, *Verticillium dahliae* and *V. albo-atrum*; and iii) Dutch elm disease caused by *Ophiostoma ulmi* (syn. *Graphium ulmi*) (Elagamey *et al.*, 2017; Kuswinanti and Junaid, 2019).

Several mechanisms, including mechanical plugging, the activity of toxins that alter the tracheal fluids, alterations in internal growth patterns due to imbalances in the levels of plant growth hormones, enzymatic weakening of pit membranes and cell walls, and leakage of materials from the xylem parenchyma into xylem vessels and resulting collapse of the vessels, may explain vascular wilt diseases caused by both bacteria and fungi (Zhang *et al.*, 2016; Dorjey *et al.*, 2017; Sandani and Weerahewa, 2018).

*Fusarium* wilts are among the most economically important diseases that cause significant losses during the cultivation of legumes, cucumbers, bananas and tomatoes (Raza *et al.*, 2017; Sinha *et al.*, 2018). In several studies, *P. capsici*, *Ralstonia solanacearum* and *F. oxysporum* were identified as causal agents of wilt in tomato and chili (Dorjey *et al.*, 2017; Sandani and Weerahewa, 2018). Wilt caused by *F. oxysporum* f. sp. cubense is a common disease in banana (Azam *et al.*, 2017). *F. oxysporum*, *F. solani*, *V. dahliae*, *Rhizoctonia solani* and *Pythium* spp. affect olive plants (Hibar *et al.*, 2017); *V. dahliae*, which causes *Verticillium* wilt, is the most important vascular pathogen in olive (López-Escudero and Mercado-Blanco, 2011).

In general, plant pathogenic fungi fall within the wide range of microorganisms that cause great harvest losses in world agriculture. Humans require sufficient safe and nutritious food in their diets, and this has contributed to the deterioration of agricultural soils due to the use of large amounts of inorganic and organic pesticides. This has occurred for more than six decades and has led to two major problems: (i) an increase in chemical residues that are potentially toxic to humans and (ii) proliferation of resistant plant pathogens (Viti *et al.*, 2008; Sarwar, 2015; Altieri *et al.*, 2017; Tuğrul, 2019).



**Figure 2.1.** Symptoms of root and crown (A-E), damping off (F-H) and vascular wilt (I-K) diseases. Root and crown symptoms in A) chickpea (*Cicer arietinum*) caused by *Macrophomina phaseolina*, B, C) common bean (*Phaseolus vulgaris*) associated with *M. phaseolina* and *Rhizoctonia solani*, D) olive (*Olea europaea*) caused by *Phytophthora megasperma*, and E) holly oak (*Quercus ilex*) caused by *P. cinnamomi*. Damping-off in F) chickpea caused by *Pythium ultimum*, G) olive caused by *Py. irregularre*, and H) holly oak caused by *P. cinnamomi*. Vascular wilt symptoms in I) chickpea caused by *Fusarium oxysporum* f.sp. *ciceris*, and in J) olive and K) pistachio (*Pistacia vera*) caused by *Verticillium dahliae* (Photos A, D-K courtesy of A. Trapero).

In the area of crop protection, the most sustainable agricultural management calls for a reduction in the use of agrochemicals. The microorganisms that often live in association with plants provide a potential alternative method of plant pathogen control. Thus, the use of microorganisms in the biological control of soil-borne plant pathogens is a research challenge (Altieri *et al.*, 2017; Díaz-Díaz *et al.*, 2022).

### 2.3. Biological control agents against soil-borne pathogens

The International Organization for Biological Control (IOBC) defines biological control as "the use of living organisms or their products to prevent or reduce loss or damage caused by harmful organisms". Biological control can significantly reduce the application of agrochemicals when the problem is addressed in the framework of integrated pest management (IPM) (Pottorff and Panter, 2009). This type of control has many advantages, including (i) few or no harmful side effects of the natural enemies on other organisms; (ii) pest resistance to biological control is very rare; (iii) biological control is usually long-term but permanent; (iv) pesticide treatment is substantially reduced; (v) the cost–benefit ratio is favourable; and (vi) secondary pests are avoided (Cook, 1988).

The mechanisms of action of biological control agents (BCAs) include mycoparasitism, production of enzymes, antibiotics and volatile organic compounds (VOCs), competition for nutrients and space, induction of plant host resistance, and the ability to prevent colonization of specific host tissues by plant pathogens through endophytic colonization by BCA (Brimner and Boland, 2003). Fungal species belonging to the genus *Trichoderma* and bacterial species belonging to the genera *Bacillus* and *Pseudomonas* are considered the most important BCAs of soil-borne plant pathogens. The antagonistic properties of *Trichoderma* depend on the activation of multiple direct and indirect mechanisms that may act in a coordinated manner (Benítez *et al.*, 2004; Gajera *et al.*, 2013). The genera *Bacillus* and *Pseudomonas* are used as biofertilizers and as BCAs since they produce secondary metabolites that are involved in the suppression of plant pathogens as well as in the induction of plant growth (Elkins *et al.*, 2004; O'Callaghan, 2016; Radhakrishnan *et al.*, 2017).

Nonpathogenic strains of *F. oxysporum* and organic amendments from agroindustrial waste have also been described as potential BCAs against soil-borne plant pathogens in arable and woody crops (Varo-Suárez *et al.*, 2018; Mulero-Aparicio *et al.*,

2019; 2020). However, the study of actinomycetes has aroused interest in recent years, and the use of such organisms could provide a biological alternative for the control of soil-borne plant pathogens (Bubici, 2018; Gopalakrishnan and Srinivas, 2019; Djebaili *et al.*, 2021; Rajeeswari *et al.*, 2021; Díaz-Díaz *et al.*, 2022).

### 2.3.1. Actinomycetes as biocontrol agents

Actinomycetes constitute a heterogeneous group of gram-positive bacteria that possess a high content of guanine + cytosine (G+C) in their DNA and have physiological characteristics that resemble those of many fungal species. They are extremely diverse and are currently included in the phylum Actinomycetota. This phylum is one of the largest of the 37 major phyla classified in the domain Bacteria and comprises 6 classes, 33 orders, 75 families and 526 genera. The largest genus is *Streptomyces*, which belongs to the class Actinomycetes, the order Streptomycetales and the family Streptomycetaceae; in this genus, 846 species with validly published names have been registered thus far (Parte *et al.*, 2020; Feeney *et al.*, 2022).

Actinomycetes are ubiquitous in terrestrial and aquatic ecosystems, both sweet and salty, where they are associated with sediments, especially organic matter that is undergoing decomposition. They are the most abundant microorganisms in the soil, usually reaching densities of  $10^6$ - $10^9$  cells per gram of soil (Barka *et al.*, 2015; Gomes *et al.*, 2018). They exhibit proteolytic, cellulolytic, lignolytic and chitinolytic enzymatic activity since they contain enzymes that allow them to chemically break down protein-rich residues, cellulose, lignin and chitin. They also increase the formation of macroaggregates that improve soil physical properties such as structure, consistency, porosity and water-binding capacity, thereby preventing erosion and degrading and reducing the numbers of certain types of contaminants such as pesticide residues (Kannabiran, 2017; Otani *et al.*, 2022).

Actinomycetes, particularly *Streptomyces* spp., are well known for producing as secondary metabolites numerous bioactive compounds that are widely used in human medicine (e.g., antibiotics, antitumour, immunosuppressant, and antiparasitic drugs) and in biotechnological applications (Takahashi and Nakashima, 2018; Donald *et al.*, 2022). Actinomycetes are also used in agriculture as insecticides, fungicides, and herbicides and as plant growth-promoting endophytes that improve crop performance. In addition, they are a significant component of the microbial population of the rhizosphere and of plant biofertilization, where they play an important role as biocontrol agents for plant diseases,

especially diseases caused by soil-borne plant pathogenic fungi (Franco *et al.*, 2010; Hayat *et al.*, 2010; Doolotkeldieva *et al.*, 2015; Gomes *et al.*, 2018; Rajeeswari *et al.*, 2021).

### 2.3.2. Isolation and characterization of Actinomycetes

#### 2.3.2.1. Isolation and collection: culture media

Published protocols for the isolation of actinomycete strains from soil or from marine sediment samples indicate that 5-10 g of sample must be collected at a substrate depth of 2-8 cm and stored at room temperature until processing. One gram of the collected substrate was added to 9 mL of sterile distilled water ( $10^{-1}$  dilution), and the sample was subjected to two treatments: *i*) it was placed in a water bath at 70 °C for 15 min (Shivabai and Gutte, 2019; Tiwari *et al.*, 2021); and *ii*) it was chemically treated by placing it in a 1.5% phenol solution for 30 min at 30 °C (Bredholt *et al.*, 2008; Shivabai and Gutte, 2019). After completion of the chemical treatment, serial dilutions of the sample were made to achieve a final dilution of  $1 \times 10^{-3}$  g ml<sup>-1</sup>. From each dilution, 100 µl was plated in triplicate on Petri dishes containing starch-casein agar (ACA), a selective medium for actinobacteria (Faisal and Al-Obaidi, 2021), and yeast extract (YE), a standard medium for *Streptomyces* isolation and characterization (Faisal and Al-Obaidi, 2021). Gause (SG) synthetic agar medium is also used as an oligotrophic medium in the isolation of bioactive actinomycetes (Yang *et al.*, 2019). To inhibit the growth of other microorganisms, antibiotics such as cycloheximide (10 µg ml<sup>-1</sup>), nystatin (20 µg ml<sup>-1</sup>) and nalidixic acid (20 µg ml<sup>-1</sup>) were added to the media listed above. Colonies with characteristics typical of actinomycetes appear after incubation of the plates at 28 °C for 7-30 days. Individual colonies can then be selected and stored at 4 °C in solid ACA or EL medium supplemented with 10% glycerol (Shivabai and Gutte, 2019).

As mentioned above, it is common practice to add antimicrobial substances to the culture medium to prevent contamination of collected samples during actinomycetes isolation. Imada *et al.* (2010) added antibiotics such as nalidixic acid (20 mg ml<sup>-1</sup>) and cycloheximide (50 mg ml<sup>-1</sup>) to the medium used to culture marine sediment samples isolated from the neritic zone of the sea around Japan and supplemented the medium with various concentrations of NaCl. Similarly, Alharbi *et al.* (2012) described the use of starch-casein agar (SCA) supplemented with streptomycin sulfate at 40 µl ml<sup>-1</sup> and

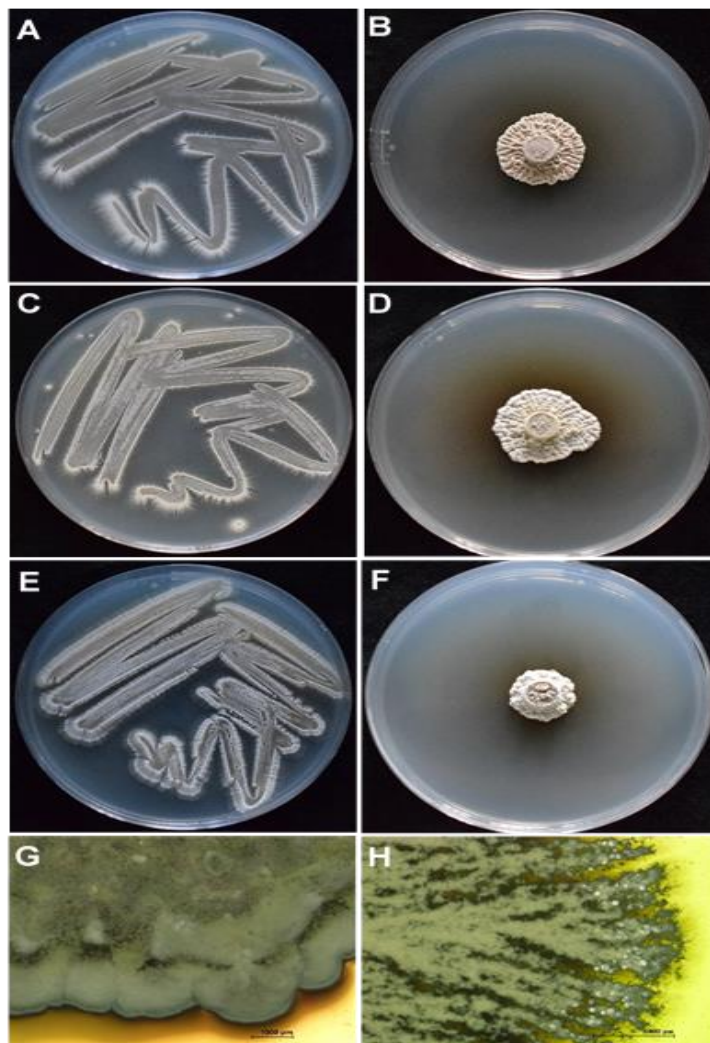
griseofulvin at 50  $\mu\text{l ml}^{-1}$ . Sharifi and Bipinraj (2019) conducted similar trials using SCA containing tetracycline and fluconazole, both at 30  $\mu\text{g ml}^{-1}$ .

### 2.3.2.2. Morphological characterization

Numerous types of culture media in which growth, pigment formation, aerial mycelium and substrate colour can be observed are used to study the morphological characteristics of actinomycetes. Among the types of solid medium used in the morphological characterization of actinomycetes are those described by the International *Streptomyces* Project (ISP); they include yeast extract agar (ISP-2), oat agar (ISP-3), inorganic salt/starch agar (ISP-4), asparagine glycerol agar (ISP-5), yeast iron peptone agar (ISP-6), detyrosine agar (ISP-7), casein agar (ACA), actinomycetes isolation agar (AIA), nutrient agar (NA), and xylan agar (XA) (Bérdy, 2005; Singh *et al.*, 2017; Priya *et al.*, 2018; Ali *et al.*, 2018; Charousová *et al.*, 2019; Qi *et al.*, 2019; Chaiharn *et al.*, 2020).

The macroscopic morphology of the actinomycete colonies that form on these culture media is highly diverse; the colonies may have a dry texture with whitish tones, and some mature colonies produce two types of mycelia, a vegetative mycelium that appears brown, blackish, white–grey, pink, or yellow and an aerial mycelium with colours that range from whitish–grey to cream or yellow and give off a peculiar smell similar to that of wet soil (Fig. 2.2). The most important characteristic of the genus *Streptomyces* is the formation of dusty colonies with spiral spore chains and a well-developed substrate mycelium with filamentous branches; most of its aerial mycelia appear granular or powdery after incubation of the cultures for 7 days (Cheema *et al.*, 2016; Ali *et al.*, 2018; Priya *et al.*, 2018; Charousová *et al.*, 2019; Chaiharn *et al.*, 2020; Díaz-Díaz *et al.*, 2022).

Liquid media such as starch broth (SB), malt yeast extract broth (YEMEB), inorganic salted starch broth (ISSB), nutrient broth (NB), and tryptic soybean broth (CST) are also used; when grown in liquid media, actinomycetes form lump-like structures (Van Wezel *et al.*, 2000).



**Figure 2.2.** Two-weeks-old colonies of *Streptomyces* strains CBQ-EA-2 (A, B), CBQ-B-8 (C, D), and CBQ-EA-12 (E, F) growing on ACA (A, C, E) and on PDA (B, D, F) at 28°C in the dark; (G, H) detail of the mycelium developed by *Streptomyces* strain CBQ-EA-2 on PDA growing at the same conditions.

### 2.3.2.3. Biochemical characterization

As aerobic gram-positive bacteria, various strains of actinomycetes can grow at pH 5.0-8.0 (pH optimum 7.0) and at temperatures ranging from 21 to 44 °C (optimum 28-43 °C). Tolerance to NaCl depends on the origin of the strain and ranges from 0% to 10% NaCl. Actinomycetes can be used as a sole source of carbon L-rhamnose, L-arabinose, D-glucose, D-ribose, D-raffinose, D-cellulose, melibiose, maltose, sucrose, soluble starch, inositol, salicin and xylan. The strains may reduce nitrate and produce tyrosinase and are gelatine-liquefying and degrade Tween 20 and 80. They produce urease, melanin, and H<sub>2</sub>S, hydrolyse starch, and break down cellulose. In addition, actinomycetes may use L-phenylalanine, ammonium sulfate, L-hydroxyproline, L-cysteine, histidine, glycine, valine and ammonium oxalate as their sole source of nitrogen (Shirling and Gottlieb, 1966; Bérdy, 2005; Cheema *et al.*, 2016; Sharma *et al.*, 2016;

Bawazir *et al.*, 2017; Qi *et al.*, 2019). Other biochemical tests that can be used to confirm the identification of actinomycetes include the catalase test, the indol test, the methyl red test, the Voges-Proskauer (VP) test, the motility test, the triple sugar-iron agar (TSI) test and the citrate utilization test (Srivastav and Pofali, 2018; Díaz-Díaz *et al.*, 2022).

Melanin formation is the most reliable test for the characterization of members of the genus *Streptomyces*, and this test was included in the international *Streptomyces* project (ISP). Cheema *et al.* (2016) showed positive results for melanin production in strains M5, M72, M511, M712, W101, M54, M710 and M91, thus characterizing them as belonging to the genus *Streptomyces*. They also showed that glucose is the best source of carbon for these strains (all of the isolated strains were able to use it), followed by fructose (94%), sucrose (73%), mannitol (70%), arabinose (57%) and galactose (27%).

In addition to macroscopic and microscopic identification of actinomycetes, multiple biochemical tests can be used for better characterization. For example, Srivastav and Pofali Srivastav and Pofali (2018) subjected strains of actinomycetes isolated from the rice rhizosphere in the agricultural area of Tamilnadu, India to the catalase test, the indol test, the methyl red test, the Voges-Proskauer (VP) test, gelatine hydrolysis, starch hydrolysis, urea hydrolysis, a test of the ability to produce acid from different hydrogen sulfide (H<sub>2</sub>S) sugars, the mobility test, the triple sugar iron (TSI) agar test and the citrate usage test. The results of these tests confirmed the major properties of the actinomycetes (Srivastav and Pofali, 2018).

On the other hand, Qi *et al.* (2019) showed that the *Streptomyces* sp. strain SCA3-4 shows an increased growth rate when cultured on certain types of culture medium, including yeast extract agar (ISP-2 or YE), oat agar (ISP-3), inorganic salt-starch agar (ISP-4), tyrosine agar (ISP-7), Gause No. 1 pink–white agar and potato dextrose agar (PDA) over a pH range of 5.0-8.0 and at temperatures between 21 and 44 °C. Its tolerance to NaCl was less than 5% (w/v), and it was able to reduce nitrate, produce tyrosinase, liquefy gelatine and degrade Tween 20 and 80.

#### 2.3.2.4. Molecular identification

Taxonomic classification of *Streptomyces* species based on morphological, biochemical, and physiological characterization is often problematic. Thus, molecular approaches, including phylogenetic analysis, must be used to identify species of *Streptomyces*. The rRNA gene sequences that encode ribosomal subunits (16S, 23S and

5S rRNA) are the target of phylogenetic analysis because of the highly conserved structure of rRNA in all cells throughout evolution (Law *et al.*, 2018). Several authors have indicated that amplification of 1.5 kb of the 16S rRNA gene using the universal primer pair 27f (5'AGAGTTGATCMTGCTCAG3') and 1492r (5'TACGGYTACCTTGACTT3') is sufficient to identify *Streptomyces* species (Qi *et al.*, 2019; Chaiharn *et al.*, 2020). However, according to Srivastav and Pofali (2018), the 16S rRNA gene has been extensively studied, and analysis of 16S gene sequences has proven sensitivity for the taxonomic and phylogenetic identification of most bacteria; unfortunately, its sensitivity for discerning closely related species within the genus *Streptomyces* is poor. These authors recommend use of the 23S rRNA gene to identify actinobacterial strains since it amplifies approximately 3000 bp at better resolution than can be obtained using the 16S rRNA gene (Han *et al.*, 2012). The use of protein-coding genes such as *gyrB* and *trpB* and the use of both ribosomal and protein-coding genes to provide better resolution for the taxonomic classification of *Streptomyces* has also been studied. Indeed, Rong and Huang (2012) demonstrated that the use of multilocus sequence analysis (MLSA) data for the five housekeeping genes *atpD*, *gyrB*, *recA*, *rpoB* and *trpB* is a valuable alternative way of clarifying the phylogenetic relationships of *Streptomyces* species. These authors also demonstrated that there is a high correlation between evolutionary distances calculated using representative MLSA data and the extent of DNA–DNA hybridization (DDH) between *Streptomyces* strains. This shows that the multilocus sequence scheme is appropriate for inferring phylogeny within a *Streptomyces* community and for constructing a theory-based taxonomy that can be used in further analysis of the ecology and bioprospecting of these microorganisms (Rong and Huang, 2012). The difficulties that arise in distinguishing *Streptomyces* species are probably due to the fact that speciation of these microorganisms has been driven by the evolution of their production of secondary metabolites; this evolution is quite complicated, as it may have been shaped by widespread gene exchange and recombination (Rong and Huang, 2012).

The existing literature on the use of actinomycetes as BCAs in crop protection indicates that most of the actinomycete strains that have been collected worldwide and found to be associated with antagonistic effects against plant pathogens have been identified as *Streptomyces* spp. Thus, *Streptomyces* is the predominant actinobacterial genus to which biocontrol activity has been attributed in most studies, followed by

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*Saccharopolyspora*, *Nocardia*, *Frankia*, *Mycobacterium*, *Microbispora*, *Micromonospora*, *Actinomadura*, *Actinoplanes* and *Amycolatopsis* (Gopalakrishnan *et al.*, 2019). For this reason, the present review focuses on *Streptomyces*. The main *Streptomyces* species and strains characterized worldwide as BCAs against soil-borne pathogens are summarized in Table 2.1. The most relevant studies in which the activity of *Streptomyces* spp. strains as potential BCAs against soil-borne pathogens has been evaluated are described below. These studies are organized according to the modes of action that they discuss and according to the experimental conditions used.

### 2.3.3. Mechanisms of action

#### 2.3.3.1. Direct mechanisms

##### 2.3.3.1.1. Production of secondary metabolites

Metabolic activity in actinomycetes is often accompanied by the production of secondary metabolites (SM) that are highly chemically diverse and play a major role in bacterial maintenance, signalling and habitat colonization (González *et al.*, 2005; Cordovez *et al.*, 2015). The secondary metabolites found in *Streptomyces* can be broadly divided into four classes: *i*) compounds that possess regulatory activity, including growth factors, morphogenic compounds and siderophores, and plant-promoting rhizobia; *ii*) antagonistic agents, including antiprotozoals, antibacterials, antifungals, and antivirals; *iii*) agrobiological agents, including insecticides, pesticides and herbicides; and *iv*) pharmacological agents, including agents with neurological activity, immunomodulators, compounds with antitumor activity and enzyme inhibitors. Almost 17% of biologically active secondary metabolites (almost 7,600 of 43,000 known metabolites) have been found in *Streptomyces* (Harir *et al.*, 2018).

*Streptomyces* spp. produce an exceptionally large number of secondary metabolites, including VOCs. Several types of compounds, including alcohols, aldehydes, carboxylic acids, esters, ketones, sulfur compounds and various terpenes, have been detected. Most of the VOCs have been shown to be specifically found in certain *Streptomyces* strains, and 45 of the VOCs are produced by all the strains tested. Geosmin (trans-1,10-dimethyl-trans-9-decalol, RI 1423) is a common VOC (Cordovez *et al.*, 2015; Gebily *et al.*, 2021). Cordovez *et al.* (2015) tested the antifungal activity of VOCs against *R. solani* and showed that the radial growth of hyphae was reduced by 57% and 41% after one and two days, respectively, of exposure. Those investigators also tested whether

*Streptomyces* VOCs promote plant growth by exposing 7-day-old *Arabidopsis thaliana* seedlings to the VOCs produced by each strain. In that test, they observed no negative effects on the growth of the seedlings after two weeks of exposure; in contrast, VOCs from 10 of the 12 strains significantly increased shoot biomass, and VOCs from 8 of the 12 strains significantly increased root biomass compared with the control (Cordovez *et al.*, 2015).

#### 2.3.3.1.2. Antibiotic and antifungal activity

Antimicrobial resistance has emerged as a major challenge to human, animal and plant health. Due to the evolution of multidrug-resistant strains, the study of natural resources, especially actinomycetes, has attracted the interest of researchers. Actinomycetes produce approximately two-thirds of all known antibiotics. Most of these antibiotics are produced by the genus *Streptomyces* as secondary extracellular metabolites that are normally secreted into the culture medium and serve as precursors for the biosynthesis of anticancer agents (e.g., doxorubicin), immunosuppressants (e.g., rapamycin), human antifungals (e.g., amphotericin B), antibacterials (e.g., daptomycin), and antiparasitic agents (e.g., ivermectin) (Kekuda *et al.*, 2010; Newman and Cragg, 2012; Braña *et al.*, 2019).

The use of actinomycetes that produce compounds with antibacterial properties in agriculture has been increasing for over a decade. In one such study, El Karkouri *et al.* (2010) isolated *S. cinereoruber* from Moroccan soil samples and evaluated its antibacterial activity by measuring the inhibition halo around the actinomycete strain when it was cultured with *Erwinia chrysanthemi* strain 3937VIII, the causal agent of soft-rot disease in tomatoes.

In addition, *in vitro* studies conducted by Dezfully *et al.* (2018) in an attempt to measure the antibiotic activity of the *S. chartreusis* strain ACTM-8 isolated from soil samples showed that this strain possesses antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis* and *Enterobacter aerogenes* but does not inhibit the growth of *Escherichia coli* or prevent the growth of the plant pathogenic filamentous fungi *F. sporotrichioides*, *F. equiseti.*, *F. poae* and *F. graminearum*. An ethyl acetate extract of the ACTM-8 strain showed the highest antibiotic activity against *F. graminearum*, followed by *F. poea*, *F. sporotrichioides*, *F. equeseti*, *F. nivale*, *E. aerogenes*, *B. subtilis* and *Staphylococcus aureus*.

Multiple investigations have demonstrated that actinomycetes possess antifungal activity against phytopathogenic fungi that cause significant agricultural losses worldwide. For example, Qi *et al.* (2019) evaluated ethyl acetate extracts of *Streptomyces* sp. strain SCA3-4 by the agar well diffusion method and found broad-spectrum antifungal activity against 13 plant pathogenic fungi. The investigators then determined the minimum inhibitory concentrations (MICs) of the extracts against *Colletotrichum fragariae* strain ATCC58718, *Pyricularia oryzae* strain ATCC52352 and *R. solani* strain ATCC76144; the lowest and highest values were obtained against *C. fragariae* and *P. oryzae*, respectively. Moreover, treatment with the raw extracts inhibited spore germination, destroyed the structure of the cell membrane and decreased the number of organelles in the cytoplasm of *F. oxysporum* f. sp. cubense race 4 Tropical (Foc TR4, ATCC76255) (Qi *et al.*, 2019).

In another study, He *et al.* (2020) found an *in vitro* inhibitory effect of fermentation broth used to grow the A217 actinomycetes strain against *Phytophthora capsici*, *B. cinerea*, *Sclerotinia sclerotiorum*, *F. oxysporum*, *Pseudomonas syringae* and *Xanthomonas campestris*; in these experiments, they observed significant antibiotic effects against all of the listed fungi and bacteria.

Recently, Díaz-Díaz *et al.* (2022) reported the results of *in vitro* evaluation by dual culture of the activities of 60 actinobacterial strains against *R. solani* and *M. phaseolina*; 40 and 25 of the evaluated strains inhibited mycelial growth of *M. phaseolina* and *R. solani*, respectively, and strains CQB-EA2 and CQB-CD-24 were the most effective against the two plant pathogens.

### 2.3.3.2. Indirect mechanisms

#### 2.3.3.2.1. Siderophore production

Siderophores are defined as relatively low-molecular-weight compounds that are ferric ion-specific chelating agents produced by bacteria and fungi growing under conditions of low iron stress (Neilands, 1995). The vast majority of nitrogen-fixing microorganisms produce siderophores that allow them to obtain iron. Iron is an element that is necessary for N fixation, since the enzyme nitrogenase, which is composed of several protein units, requires 36 iron atoms to function properly (Sylvia, 2005). In general, based on the importance of iron in the vital functions of microorganisms, the efficiency of the capture mechanisms they generate is understood. Aerobic bacteria and

facultative aerobes possess high-affinity iron transport systems and excrete siderophores that facilitate efficient uptake of this element (Jurkevitch *et al.*, 1992).

*Streptomyces* strains have the ability to produce iron compounds, siderophores, that deprive pathogens of iron (Tokala *et al.*, 2002). The ability to produce siderophores gives the biocontrol agent a competitive advantage in diverse environments such as rhizospheres in which soluble iron is scarce (Mullen, 2004). In a representative study of this mechanism, Fatmawati *et al.* (2019) evaluated the abilities of 26 strains of actinomycetes to produce siderophores after agar inoculation into Chrome Azurol S medium (CAS). The formation of an orange halo around the actinomycete colonies indicated the production of siderophores. In 2020, Chaiharn *et al.* (2020) evaluated the production of siderophores by 27 strains of *Streptomyces* spp. isolated from the rice rhizosphere and found that some of the evaluated strains were positive, producing orange halos of different sizes around the colonies when grown in CAS culture medium.

#### 2.3.3.2.2. Chitinase production

The production of extracellular enzymes by antagonistic actinobacteria involved in degradation of the cell walls of plant pathogenic agents is one of the best documented biological control mechanisms, especially with respect to mechanisms against plant pathogenic fungi (Goswami *et al.*, 2016; Singh *et al.*, 2018). Chitin, a polymer composed of N-acetylglucosamine units, is one of the most abundant biomaterials in nature. It is widely distributed, particularly as a structural polysaccharide in the external skeletons of insects and crustaceans and in the cell walls of fungi (Shinya and Fukamizo 2017). Chitin is degraded by the enzyme chitinase. Thus, evaluation of chitinase production by antagonistic microorganisms is of great significance, as these microorganisms play a trophic role related to cell wall synthesis, nutrition, and parasitism in the control of plant pathogenic fungi (Hamid *et al.*, 2013; Rathore and Gupta 2015).

Rashad *et al.* (2017) isolated *S. griseorubens* E44G from cultivated soil samples and demonstrated its chitinolytic activity using the plate seeding method on colloidal chitin agar. After molecular characterization and phylogenetic analysis of its chitinase, analogy with other chitinases isolated from other antagonistic microorganisms was demonstrated. Fatmawati *et al.* (2019) also used the plate method to qualitatively assess the chitinolytic activity of actinomycete strains isolated from the rhizosphere of soybean

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cultures. Each strain was evaluated in triplicate, and the results revealed that all strains except ASR 55 were chitinase-positive.

Song *et al.* (2020) used a fermentation broth instead of solid medium to support auxin production in an attempt to measure the chitinolytic activity of *Streptomyces* strains and found that the *Streptomyces* strain JT- possessed the highest levels of chitinase.

Recently, Díaz-Díaz *et al.* (2022) used culture on colloidal chitin agar to evaluate the chitinolytic activities of 31 actinobacterial strains; they reported that 20 of the strains showed chitinolytic halos and that *Streptomyces* CBQ-EA-2 displayed the highest chitinolytic activity.

### 2.3.3.2.3. Induced resistance (IR)

As living organisms, plants have the ability to respond to various environmental, light-related, gravitational, physical and chemical factors, and this as a whole allows their survival. A wide variety of chemical stimuli produced by microorganisms associated with plants, including saprophytes, plant growth promoters, and virulent and nonpathogenic strains, induce plant defenses through biochemical changes that increase resistance against subsequent infection through different phytopathogens, both in the rhizosphere and in the phyllosphere, and even protect against attack by phytophagous insects (Roopa and Gadag, 2019; Llorens and Agustí-Brisach, 2022). The interaction between endophytic actinomycetes and plants results in a number of specific biochemical, physiological and molecular events (Li *et al.*, 2020).

Inducing defense mechanisms in plants by prior application of a biological inducer is a potential ecofriendly plant protection strategy. These metabolites serve as defense mechanisms for plants that allow them to resist biotic and abiotic stresses, and plants may even mount specific defense responses against diseases caused by plant pathogenic microorganisms (Kuldau and Bacon, 2008).

An example of a study that demonstrates the effectiveness of actinomycetes as inducers of host resistance is the study conducted by Jogaiah *et al.* (2016). Those investigators showed that seven strains of actinomycetes that had been applied by coating and soaking seeds of *Pennisetum glaucum* reduced the incidence of downy mildew. After inoculation of the plants with the pathogen, they concluded that plants treated with *S. griseus* SJ\_UOM-07-09 and *S. roseum* SJ\_UOM-18-09 showed the highest degree of protection. In addition, analysis of the roots of plants treated with either of these strains

showed abundant growth of actinomycetes at 9 and 12 days post-inoculation, and they concluded that both strains were able to colonize roots internally, leading to an increase in the induced resistance.

Several additional studies of the induction of resistance by *Streptomyces* that involve experiments conducted in planta are described in the following sections.

#### 2.3.4. Effect of *Streptomyces* spp. against soil-borne pathogens in planta under semicontrolled conditions

In planta bioassays performed under semicontrolled conditions (i.e., in a greenhouse, shadehouse, or similar environment) provide a quick and easy way to assess the efficacy of microorganisms as BCAs. Furthermore, experimentation in greenhouses provides tools for the adaptation of techniques and procedures that can support evaluations performed under field conditions. Below, we describe several representative studies that have evaluated the effect of actinomycetes on soil-borne pathogens in several crops under semicontrolled conditions. Additional information is provided in Table 2.1.

In 2009, El-Tarabily *et al.* (2009) evaluated the potential of three actinomycetes, *Actinoplanes campanulatus*, *Micromonospora chalcea* and *S. spiralis*, against damping-off of cucumber roots caused by *Pythium aphanidermatum*. The three actinomycetes were able to colonize cucumber roots endophytically, and they promoted plant growth and reduced disease severity in both seedlings and mature cucumber plants. Treatment with a combination of the three isolates resulted in significantly better suppression of the disease than treatment with any of the individual actinomycetes alone.

Goudjal *et al.* (2014) showed that *S. mutabilis* strain CA-2 and *S. cyaneofuscatus* strain AA-2 reduced the severity of damping-off caused by *R. solani* in tomato plants, promoted plant growth, and increased the fresh weight of the seedlings as well as the length of the treated seedlings compared to the untreated control.

Balaraju *et al.* (2016) demonstrated that foliar spray or soil-drenching treatment with paromomycin, an antibiotic derived from *Streptomyces* sp. strain AG-P 1441, controlled *Phytophthora* blight and soft rot disease caused by *Phytophthora capsici* and *Pectobacterium carotovorum*, respectively, in chili pepper. In addition, the treatment slightly promoted growth and increased chlorophyll content, and induction of resistance by paromomycin treatment was proven by the expression of the pathogenesis-related (PR)

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genes PR-1,  $\beta$ -1,3-glucanase, chitinase, PR-4, peroxidase, and PR-10. Hassan *et al.* (2017) evaluated the efficacy of 77 strains of actinomycetes against *Alternaria brassicicola* in cabbage seedlings. Seven days after inoculation, control plants (untreated seedlings) showed severe disease symptoms, whereas 11 of the 77 evaluated strains were able to suppress the development of disease, and three of the strains (MBCY58-1, MBCN56-1 and MBCN152-1) reduced the disease severity (DS) by 33.0%, 56.0% and 60.0%, respectively. Scanning electron microscopy revealed that the hyphae of MBCN152-1 multiplied on the surface of the seedlings and penetrated their epidermal cells. Based on the results, these authors concluded that the strain MBCN152-1, identified as an *S. humidus*-related species, is a potential BCA that can be used to control *A. brassicicola* on cabbage plug seedlings.

Sarwar *et al.* (2019) compared the effects of three pathogenic strains of *S. scabies*, AJ-7, AJ-10 and AC469, and a nonpathogenic strain of *S. violaceusniger*, AC12AB, in potato tubers. These authors concluded that the nonpathogenic strain *S. violaceusniger* AC12AB acted as a growth promoter by increasing sprout and root length and tuber weight, while the *S. scabies* strains were pathogenic to potato tubers. The nonpathogenic strain was also able to reduce disease severity by up to 90%.

Chaiharn *et al.* (2020) evaluated the potential effects of several *Streptomyces* spp. strains as BCAs against *P. oryzae* *in vitro* using dual culture assays. The four *Streptomyces* strains that were found to be most effective (W1, PC 12, D 4.1 and D 4.3) were tested on *Oryza sativa* that had been inoculated with *P. oryzae*. The *Streptomyces* sp. strain PC12 was the most effective at reducing the disease severity, reducing it by 64% with respect to the nontreated and inoculated controls at 60 days after inoculation.

He *et al.* (2020) compared the effects of *Streptomyces* sp. strain A217 and the chemical metalaxyl against pepper blight caused by *Phytophthora capsici*. The results showed that treatment with fermentation broth in which *Streptomyces* spp. strain A217 had been grown was significantly more effective in disease control than did treatment with the chemical.

Díaz-Díaz *et al.* (2022) reported that treatment of seeds with a mixture of two *Streptomyces* sp. strains (CBQ-EA-2 + CBQ-B-8) significantly reduced root rot disease in common bean caused by *M. phaseolina* and *R. solani*. The effectiveness of the treatment was similar to that of the commercial BCA *T. harzianum* A-34 and that of the chemical Celest® Top 312 FS.

Carlucci *et al.* (2022) demonstrated the effectiveness of root immersion treatment with *S. albidoflavus* CARA17 against infection of seedlings of *Foeniculum vulgare* by *F. oxysporum*, *Plectosphaerella ramiseptata*, *S. sclerotiorum*, *Sclerotium rolfsii* and *V. dahliae*. Finally, Elshafie and Camele (2022) showed that treatment of tomato plants with actinomycetes isolates (*Streptomyces* spp. and *Streptomyces atratus*) promoted plant growth. In particular, seedlings treated with *S. atratus* showed a moderate disease incidence compared with the positive control. These authors also showed that *Streptomyces* sp. and *S. atratus* produced amylase and pectinase at high and moderate levels, respectively.

### 2.3.5. Effect of *Streptomyces* spp. on soil-borne pathogens in the field

Many of the studies conducted with actinomycetes have been performed in the laboratory, others were conducted under semicontrolled conditions, and few have reached the field. It should not be neglected that a vitally important part of the management and application of a product of biological origin is, without doubt, its evaluation under field conditions. The introduction of BCAs in the field requires assessment of their efficacy in controlling the target organism, determination of their impact on plant health and biodiversity, evaluation of their impact on human and/or animal health with respect to possible induction of allergies and vectoring of diseases, and determination of their ecological impact on the environment, including their effects on nontarget organisms (Loomans, 2021). Below, we describe several representative studies in which the effects of actinomycetes against soil-borne pathogens that affect several crops have been evaluated in the field. Additional information is provided in Table 2.1.

In 2013, the protective effects of several *Streptomyces* spp. strains (StB-11, StB-12 and StB-6) against *V. dahliae* infection in the field were evaluated. These strains were found to reduce disease severity in tomato crops by 48.2%, 35% and 32.6%, respectively, but they did not show any effect against *V. dahliae* in aubergine crops (Bubici *et al.*, 2013).

Khucharoenphaisan *et al.* (2016) evaluated the protective effect of *S. malaysiensis* strain LB35 against *Phytophthora* spp. in cassava for two months under field conditions. Four months after treatment, the plants that had been treated with *S. malaysiensis* LB35 were the tallest, and the treatment was effective in preventing the disease compared with the nontreated control (Khucharoenphaisan *et al.*, 2016).

Similar results were obtained in Egypt in a study in which *S. griseorubens* E44G was applied to tomato plants. Its application significantly increased all growth and yield parameters, and the treated plants had a lower incidence of *Fusarium* wilt disease than did the untreated infected plants, suggesting that *S. griseorubens* E44G is a potential biological control agent (Rashad *et al.*, 2017).

Álvarez-Pérez *et al.* (2017) evaluated the activity of 58 endophytic and 94 rhizospheric actinobacteria isolated from *Vitis vinifera* against *Diplodia seriata* and *Dactylonectria macrodidyma* in an *in vitro* bioassay. The strains that yielded the best results were also evaluated against *Phaeoconiella chlamydospora* and *Phaeoacremonium minimum*. The best rhizospheric and endophytic strains were then applied to nursery grafts. A field trial conducted over a period of three years resulted in the identification of an endophytic strain, *Streptomyces* sp. VV/E1, and two rhizospheric strains, *Streptomyces* sp. VV/R1 and *Streptomyces* sp. VV/R4, that produced significant reductions in the rates of infection of *Dactylonectria* sp. *Ilyonectria* sp., *P. chlamydospora* y *P. minimum*. The reduction in infection rate was related to a significant reduction in the mortality of the grafted plants in the nursery.

Sarwar *et al.* (2019) demonstrated that *S. violaceusniger* strain AC12AB reduced the incidence of potato common scab disease and the incidence of rotting in potato tubers by 83.0% and that it increased yield by 26.8% and significantly increased the attributes used to measure potato plant growth (including sprout/root length, tuber number, and tuber weight).

Gebily *et al.* (2021) evaluated the effectiveness of *S. griseus* strain DG5, *S. rochei* strain DG4 and *S. sampsonii* strain DG1 against *S. sclerotiorum* in common bean. All three strains showed significant disease reduction compared to the untreated control. In addition, the effectiveness of the treatments was higher when the *Streptomyces* strains were mixed; combinations of *S. rochei* DG4 + *S. griseus* DG5 and *S. sampsonii* DG1 + *S. griseus* DG5 were the most effective.

Finally, treatment of seeds with a mixture of the two *Streptomyces* spp. strains CBQ-EA-2 and CBQ-B-8 described above was also evaluated in three experimental fields of common bean crops that experienced natural infestation by *M. phaseolina* and *R. solani*. The effectiveness of the treatment was also compared with those of the BCA *T. harzianum* A-34 and the chemical Celest® Top 312 FS. The results obtained in the field were used to validate the previous results obtained under semicontrolled conditions since

the effectiveness of the treatment with *Streptomyces* strains in reducing disease incidence and severity was similar to those observed for the commercial BCA *T. harzianum* A-34 and the chemical Celest® Top 312 FS. In addition, plants treated with *Streptomyces* strains exhibited increased yield parameters (Díaz-Díaz *et al.*, 2023).

Despite the fact that a number of previous studies designed to evaluate the effectiveness of actinomycetes as BCAs in crop protection have been conducted, to date only a few commercial products based on actinomycetes have been shown to have important effects on biocontrol. Among them, we can highlight Actinovate® (Natural Industries, Inc.), which contains spores of *S. lydicus* strain WYEC 108 and is recommended for use against *Pythium*, *Rhizoctonia*, *Phytophthora*, *Verticillium*, *Fusarium*, *Botrytis* and *Monilinia* (Himmelstein *et al.*, 2014), and Mycostop® (Verdara Oy, Finland), which contains *S. griseoviridis* strain K61 and has shown effectiveness against several soil-borne pathogens, including *Fusarium* sp. (White *et al.*, 1990; Lahdenperä *et al.*, 1991). Thus, further research and technology transfer are needed to involve private companies in the development of potentially effective commercial products based on *Streptomyces* strains as potential biocontrol tools against soil-borne pathogens.

**Table 2.1.** *Streptomyces* species used in the biocontrol of soil borne fungal plant pathogens

<b><i>Streptomyces</i> spp. (strain)</b>	<b>Pathogen</b>	<b>Experimental conditions</b>	<b>Mechanisms of action</b>	<b>References</b>
<i>Streptomyces achromogenes</i> (3B44)	<i>Rhizoctonia solani</i>	- <i>In vitro</i> by antifungal activity of Volatile Organic Compounds (VOC) produced by the <i>Streptomyces</i> strain - <i>In vivo</i> in <i>Arabidopsis thaliana</i>	Antibiosis <i>in vitro</i> and plant growth-promoting effects	Cordovez <i>et al.</i> , 2015
<i>S. albidoflavus</i> (VT111I)	<i>Fusarium oxysporum</i> f.sp. <i>lactucae</i> <i>Phytophthora</i> sp. <i>Pythium ultimum</i> <i>Rhizoctonia solani</i> <i>Sclerotinia sclerotiorum</i> <i>Thielaviopsis basicola</i>	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Kunova <i>et al.</i> , 2016
<i>S. albidoflavus</i> (CARA17)	<i>Sclerotium rolfsii</i> <i>F. oxysporum</i> <i>Plectosphaerella ramiseptata</i> <i>S. sclerotiorum</i> <i>Verticillium dahliae</i>	- <i>In vitro</i> by dual confrontation - <i>In vivo</i> in <i>Phoeniculum vulgare</i>	Antibiosis <i>in vitro</i>	Carlucci <i>et al.</i> , 2022
<i>S. albidoflavus</i> (St-220)	<i>F. oxysporum</i>	- <i>In vitro</i> by dual confrontation - <i>In vivo</i> in <i>Salvia miltiorrhiza</i>	Antibiosis <i>in vitro</i> e <i>in vivo</i> and plant growth-promoting effects	Du <i>et al.</i> , 2022
<i>S. anulatus</i> (CX14W)	<i>F. oxysporum</i> f. sp. <i>lactucae</i> <i>S. sclerotiorum</i>	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Kunova <i>et al.</i> , 2016
<i>S. anulatus</i> (CMJ58I)	<i>F. oxysporum</i> f. sp. <i>lactucae</i> <i>Phytophthora</i> sp. <i>P. ultimum</i> <i>R. solani</i> <i>T. basicola</i> <i>S. sclerotiorum</i>	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Kunova <i>et al.</i> , 2016

<i>S. atratus</i> (3A41)	<i>R. solani</i>	- <i>In vitro</i> by antifungal activity of VOC produced by the <i>Streptomyces</i> strain - <i>In vivo</i> in <i>A. thaliana</i>	Antibiosis <i>in vitro</i> and plant growth-promoting effects	Cordovez <i>et al.</i> , 2015
<i>S. atratus</i> (RS3)	<i>S. sclerotiorum</i>	- <i>In vivo</i> in tomato	Antibiosis <i>in vivo</i> and plant growth-promoting effects	Elshafie and Camele, 2022
<i>S. avermitilis</i> (NAIMCC-B-01412)	<i>F. oxysporum</i> <i>Macrophomina</i> sp. <i>Sclerotium</i> sp.	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Panneerselvam <i>et al.</i> , 2021
<i>S. canus</i> (NAIMCC-B-01342)	<i>F. oxysporum</i> <i>Macrophomina</i> sp. <i>Sclerotium</i> sp.	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Dezfully <i>et al.</i> , 2018
<i>S. chartreusis</i> (ACTM-8)	<i>F. acuminatum</i> <i>F. anthophilum</i> <i>F. avenaceum</i> <i>F. crookwellense</i> <i>F. equiseti</i> <i>F. graminearum</i> <i>F. nivale</i> <i>F. poae</i> <i>F. sambucinum</i> <i>F. semitectum</i> <i>F. sporotrichioides</i> <i>F. venenatum</i>	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Dezfully <i>et al.</i> , 2018
<i>S. cinnamonensis</i> (NAIMCC-B-01413)	<i>F. oxysporum</i> <i>Macrophomina</i> sp. <i>Sclerotium</i> sp.	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Panneerselvam <i>et al.</i> , 2021
<i>S. ciscaucasicus</i> (W47, W214)	<i>R. solani</i>	- <i>In vitro</i> by antifungal activity of VOC produced by the <i>Streptomyces</i> strain - <i>In vivo</i> in <i>A. thaliana</i>	Antibiosis <i>in vitro</i> and plant growth-promoting effects	Cordovez <i>et al.</i> , 2015
<i>S. collinus</i> (S-13)	<i>Fusarium</i> sp.	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Al-Askar <i>et al.</i> , 2012

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	<i>Macrophomina</i> sp.			
	<i>Rhizoctonia</i> sp.			
<i>S. corchorusii</i> (AUH-1)	<i>B. dothidea</i> <i>F. oxysporum</i> f.sp. <i>cucumerinum</i> <i>F. oxysporum</i> f.sp. <i>niveum</i> <i>F. oxysporum</i> f.sp. <i>vasinfectum</i> <i>Ph. parasitica</i> var. <i>nicotianae</i> <i>Ph. capsici</i> <i>R. solani</i> <i>V. dahliae</i>	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Yang <i>et al.</i> , 2019
<i>S. cyaneofuscatus</i> (ZY153, AA-2)	<i>V. dahliae</i>	- <i>In vitro</i> by dual confrontation - <i>In vivo</i> in <i>Gossypium hirsutum</i> L. -In the field in <i>G. hirsutum</i> L.	Antibiosis <i>in vitro</i> and plant growth-promoting effects	Xue <i>et al.</i> , 2013
<i>S. cyaneofuscatus</i> (AA-2)	<i>R. solani</i>	- <i>In vitro</i> by dual confrontation - <i>In vivo</i> in <i>Lycopersicon esculentum</i>	Antibiosis <i>in vivo</i> and plant growth-promoting effects	Goudjal <i>et al.</i> , 2014
<i>S. cyaneus</i> (ZEA171)	<i>F. oxysporum</i> f.sp. <i>lattucae</i> <i>Phytophthora</i> sp. <i>P. ultimum</i> <i>R. solani</i> <i>S. sclerotiorum</i> <i>T. basicola</i>	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Kunova <i>et al.</i> , 2016
<i>S. eurocidicus</i> (NRRL-B1676)	<i>Diaporthe aspalathi</i> <i>Macrophomina phaseolina</i>	- <i>In vitro</i> by dual confrontation - <i>In vivo</i> in <i>Glycine max</i>	Antibiosis <i>in vitro</i> and plant growth-promoting effects	Bercovich <i>et al.</i> , 2022
<i>S. flavotricini</i> (Z-13)	<i>V. dahliae</i>	- <i>In vitro</i> by dual confrontation - <i>In vivo</i> in <i>G. hirsutum</i> -In the field in <i>G. hirsutum</i>	antibiosis <i>in vitro</i> and plant growth-promoting effects	Xue <i>et al.</i> , 2013

<i>S. flavotricini</i> (25)	<i>Sc. rolfsii</i>	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i> with cell-free cultures	Li <i>et al.</i> , 2017
<i>S. flavoviridis</i> (SF 1)	<i>Botrytis cinerea</i>	-In the field in <i>Vicia fabae</i> cv. Giza 3	Antibiosis <i>in vivo</i> and plant growth-promoting effects	El-Shatoury <i>et al.</i> , 2020
<i>S. globosus</i> (UAE1)	<i>T. punctulata</i>	- <i>In vivo</i> by culture filtrate	Antibiosis <i>in vitro</i>	Saeed <i>et al.</i> , 2017
<i>S. griseorubens</i> (E44G)	<i>F. oxysporum</i>	- <i>In vitro</i> by dual confrontation and by effect of purified chitinase enzyme - <i>In vivo</i> in <i>L. esculentum</i>	Antibiosis <i>in vitro</i> and plant growth-promoting effects	Rashad <i>et al.</i> , 2017
<i>S. griseoviridis</i> (K61)	<i>Alternaria brassicicola</i> <i>B. cinerea</i> <i>Fusarium</i> spp. <i>F. oxysporum</i> f.sp. <i>dianthi</i>	- <i>In vivo</i> in <i>Brassica oleracea</i> cv. <i>capitata</i> , <i>Dianthus caryophyllus</i> , <i>Lactuca sativa</i> , <i>Triticum durum</i> , <i>T. compactum</i>	Antibiosis <i>in vivo</i>	White <i>et al.</i> , 1990
<i>S. griseus</i> (DG5)	<i>S. sclerotiorum</i>	- <i>In vitro</i> by dual confrontation - <i>In vitro</i> by culture filtrate - <i>In vitro</i> by antifungal activity of VOC produced by the <i>Streptomyces</i> strain - <i>In vivo</i> in <i>Phaseolus vulgaris</i>	Antibiosis <i>in vitro</i>	Gebily <i>et al.</i> , 2021
<i>S. griseus</i> (SJ_UOM-07-09)	<i>Sclerospora graminicola</i>	- <i>In vivo</i> in <i>Pennisetum glaucum</i> - <i>In vivo</i> by induced resistance in <i>P. glaucum</i>	Antibiosis <i>in vivo</i>	Jogaiah <i>et al.</i> , 2016
<i>S. globisporus</i> subsp. <i>globisporus</i> (C28)	<i>Sc. rolfsii</i>	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i> with cell-free cultures	Li <i>et al.</i> , 2017
<i>S. hygroscopicus</i> var. <i>geldanus</i> (NRRL3602)	<i>R. solani</i>	- <i>In vivo</i> in <i>Pisum sativum</i>	Antibiosis <i>in vivo</i>	Rothrock and Gottlieb, 1984
<i>S. hygroscopicus</i> (3B40)	<i>R. solani</i>	- <i>In vitro</i> by antifungal activity of VOC produced by the <i>Streptomyces</i> strain - <i>In vivo</i> in <i>A. thaliana</i>	Antibiosis <i>in vivo</i>	Cordovez <i>et al.</i> , 2015
<i>S. hygroscopicus</i> (SRA14)	<i>Colletotrichum gloeosporioides</i>	- <i>In vitro</i> by cell-free culture filtrates	Antibiosis <i>in vitro</i>	Prapagdee <i>et al.</i> , 2008

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	<i>Sc. rolfsii</i>			
<i>S. hygrosopicus</i> (PACCH24)	<i>Sc. rolfsii</i>	- <i>In vitro</i> by dual confrontation - <i>In vivo</i> in <i>Capsicum</i> sp.	Antibiosis <i>in vitro</i> and <i>in vivo</i>	Pattanapitpaisal and Kamlandharn, 2012
<i>S. humiferus</i> (W99)	<i>R. solani</i>	- <i>In vitro</i> by antifungal activity of VOC produced by the <i>Streptomyces</i> strain - <i>In vivo</i> in <i>A. thaliana</i>	Antibiosis <i>in vitro</i> and plant growth-promoting effects	Cordovez <i>et al.</i> , 2015
<i>S. kanamyceticus</i> (B-49)	<i>V. dahliae</i>	- <i>In vitro</i> by dual confrontation - <i>In vivo</i> in <i>G. hirsutum</i> -In the field in <i>G. hirsutum</i>	Antibiosis <i>in vitro</i> and plant growth-promoting effects	Xue <i>et al.</i> , 2013
<i>S. laceyi</i> (W62)	<i>R. solani</i>	- <i>In vitro</i> by antifungal activity of Volatile organic compounds (VOC) produced by the <i>Streptomyces</i> strain - <i>In vivo</i> in <i>A. thaliana</i>	Antibiosis <i>in vitro</i> and plant growth-promoting effects	Cordovez <i>et al.</i> , 2015
<i>S. lividans</i> (1326)	<i>R. solani</i>	- <i>In vitro</i> by antifungal activity of Volatile organic compounds (VOC) produced by the <i>Streptomyces</i> strain - <i>In vivo</i> in <i>A. thaliana</i>	Antibiosis <i>in vitro</i> and plant growth-promoting effects	Cordovez <i>et al.</i> , 2015
<i>S. malaysiensis</i> (LB35)	<i>Phytophthora</i> spp.	- <i>In vitro</i> by dual confrontation -In the field in <i>Manihot esculenta</i>	Antibiosis <i>in vitro</i> and plant growth-promoting effects	Khucharoenphaisan <i>et al.</i> , 2016
<i>S. mexicanus</i> (W126, W75.5)	<i>R. solani</i>	- <i>In vitro</i> by antifungal activity of Volatile organic compounds (VOC) produced by the <i>Streptomyces</i> strain - <i>In vivo</i> in <i>A. thaliana</i>	Antibiosis <i>in vitro</i> and plant growth-promoting effects	Cordovez <i>et al.</i> , 2015
<i>S. mirabilis</i> (3A18)	<i>R. solani</i>	- <i>In vitro</i> by antifungal activity of Volatile organic compounds (VOC) produced by the <i>Streptomyces</i> strain - <i>In vivo</i> in <i>A. thaliana</i>	Antibiosis <i>in vitro</i> and plant growth-promoting effects	Cordovez <i>et al.</i> , 2015
<i>S. mutabilis</i> (CA-2)	<i>R. solani</i>	- <i>In vitro</i> by dual confrontation - <i>In vivo</i> in <i>Lycopersicum esculentum</i>	Antibiosis <i>in vivo</i> and plant growth-promoting effects	Goudjal <i>et al.</i> , 2014

<i>S. pactum</i> (Act12)	<i>Sc. rolfsii</i>	- <i>In vitro</i> by dual confrontation	antibiosis <i>in vitro</i> with cell-free cultures	Li <i>et al.</i> , 2017
<i>S. padanus</i> (PMS-702)	<i>R. solani</i>	- <i>In vitro</i> by dual confrontation - Determination of minimum inhibitory concentrations (MIC) - <i>In vivo</i> in <i>B. oleracea</i> cv. <i>capitata</i>	Antibiosis <i>in vitro</i>	Shih <i>et al.</i> , 2003
<i>S. plicatus</i> (101)	<i>V. dahliae</i>	- <i>In vitro</i> by dual confrontation - <i>In vitro</i> by crude extract	Antibiosis <i>in vitro</i>	Shahidi Bonjar and Aghighi, 2005
<i>S. rochei</i> (ACTA1551)	<i>F. oxysporum</i>	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Kanini <i>et al.</i> , 2013
<i>S. rochei</i> (X-4)	<i>V. dahliae</i>	- <i>In vitro</i> by dual confrontation - <i>In vivo</i> in <i>G. hirsutum</i> -In the field in <i>G. hirsutum</i>	Antibiosis <i>in vitro</i> and plant growth-promoting effects	Xue <i>et al.</i> , 2013
<i>S. rochei</i> (CMJ57I)	<i>F. oxysporum</i> f.sp. <i>lactucae</i> <i>S. sclerotiorum</i>	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Kunova <i>et al.</i> , 2016
<i>S. rochei</i> (WZS1-1)	<i>F. graminearum</i>	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Han <i>et al.</i> , 2018
<i>S. rochei</i> (DG4)	<i>S. sclerotiorum</i>	- <i>In vitro</i> by dual confrontation - <i>In vitro</i> by culture filtrate - <i>In vitro</i> by antifungal activity of VOC produced by the <i>Streptomyces</i> strain - <i>In vivo</i> in <i>Ph. vulgaris</i>	Antibiosis <i>in vitro</i>	Gebily <i>et al.</i> , 2021
<i>S. roseum</i> (SJ_UOM-18-09)	<i>S. graminicola</i>	- <i>In vivo</i> in <i>P. glaucum</i> - <i>In vivo</i> by induced resistance in <i>P. glaucum</i>	Antibiosis <i>in vivo</i>	Jogaiah <i>et al.</i> , 2016
<i>S. sampsonii</i> (DG1)	<i>S. sclerotiorum</i>	- <i>In vitro</i> by dual confrontation - <i>In vitro</i> by culture filtrate - <i>In vitro</i> by antifungal activity of VOC produced by the <i>Streptomyces</i> strain - <i>In vivo</i> in <i>Ph. vulgaris</i>	Antibiosis <i>in vitro</i>	Gebily <i>et al.</i> , 2021

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<i>S. senoensis</i> (420)	<i>Sc. rolfsii</i>	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i> with cell-free cultures	Li <i>et al.</i> , 2017
<i>S. sindenensis</i> (263)	<i>Magnaporthe oryzae</i>	- <i>In vitro</i> by dual confrontation -Determination of minimum inhibitory concentrations (MIC) - <i>In vivo</i> in <i>Oryza sativa</i>	Antibiosis <i>in vitro</i> and <i>in vivo</i>	Zarandi <i>et al.</i> , 2009
<i>S. spororaveus</i> (RDS28)	<i>A. alternata</i> <i>B. cinerea</i> <i>F. solani</i> <i>F. verticillioides</i> <i>R. solani</i>	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Khair, 2011
<i>Streptomyces</i> sp. (RC 1071)	<i>C. gloeosporioides</i> <i>F. graminearum</i> <i>F. oxysporum</i> <i>F. solani</i> <i>Fusarium</i> sp.	- <i>In vitro</i> by dual confrontation - <i>In vitro</i> by crude extract	Antibiosis <i>in vitro</i>	Gomes <i>et al.</i> , 2001
<i>Streptomyces</i> sp. (g10)	<i>F. oxysporum</i>	- <i>In vitro</i> by dual confrontation - <i>In vivo</i> in <i>Musa</i> sp.	Antibiosis <i>in vitro</i>	Getha <i>et al.</i> , 2005
<i>Streptomyces</i> sp. (AC 26-1B)	<i>C. gloeosporioides</i> <i>Curvularia eragrostides</i>	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Soares <i>et al.</i> , 2006
<i>Streptomyces</i> sp. (EN27, EN28)	<i>Fusarium</i> sp. <i>Gaeumannomyces</i> sp. <i>Pythium</i> sp. <i>Rhizoctonia</i> sp.	- <i>In vitro</i> by endophytic colonization of the plant and visualization by microscopy - <i>In vivo</i> in <i>A. thaliana</i>	<i>In vitro</i> colonization, antibiosis <i>in vivo</i> and plant growth-promoting effects	Otani <i>et al.</i> , 2022
<i>Streptomyces</i> sp. (J2 and B11)	<i>Sc. rolfsii</i>	- <i>In vitro</i> by dual confrontation - <i>In vitro</i> by culture filtrate - <i>In vivo</i> in <i>Beta vulgaris</i>	Antibiosis <i>in vitro</i> and <i>in vivo</i> and plant growth-promoting effects	Errakhi <i>et al.</i> , 2009
<i>Streptomyces</i> sp. (RO3)	<i>Penicillium digitatum</i> <i>Geotrichum candidum</i>	- <i>In vitro</i> by activity of metabolites - <i>In vivo</i> by activity of metabolites	Antibiosis <i>in vitro</i> and <i>in vivo</i>	Maldonado <i>et al.</i> , 2010
<i>Streptomyces</i> sp. (JDA1-JDA9)	<i>A. alternata</i> <i>C. truncatum</i>	- <i>In vitro</i> by dual confrontation - <i>In vivo</i> in <i>Glycine max</i>	Antibiosis <i>in vitro</i> Plant growth promoting (PGPR)	Dalal <i>et al.</i> , 2014

	<i>F. oxysporum</i> <i>M. phaseolina</i> <i>R. solani</i> <i>Sc. rolfsii</i>			
<i>Streptomyces</i> sp. (KS62, KS55, KS112, KS58, KS310)	<i>F. oxysporum</i> f.sp. <i>ciceris</i>	- <i>In vitro</i> by dual confrontation - <i>In vivo</i> in <i>Cicer arietinum</i>	Antibiosis <i>in vitro</i> and <i>in vivo</i> Plant growth promoting (PGPR)	Amini <i>et al.</i> , 2016
<i>Streptomyces</i> sp. (ACITM-1)	<i>C. truncatum</i> <i>F. oxysporum</i> <i>M. phaseolina</i> <i>R. solani</i>	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Singh <i>et al.</i> , 2016
<i>Streptomyces</i> sp. (SAI-13, SAI-29, VAI-7, VAI-40)	<i>F. oxysporum</i> f.sp. <i>ciceris</i> <i>M. phaseolina</i> <i>Sc. rolfsii</i>	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Sreevidya <i>et al.</i> , 2016
<i>Streptomyces</i> sp. (AG-P 1441)	<i>P. carotovorum</i> <i>Ph. capsici</i>	- <i>In vivo</i> by induced resistance by Paromomycin in <i>Capsicum annum</i>	Antibiosis <i>in vivo</i>	Balaraju <i>et al.</i> , 2016
<i>Streptomyces</i> sp. (VV/E1, VV/R1, VV/R4)	<i>Dactylonectria</i> sp. <i>Ilyonectria</i> sp. <i>P. chlamydospora</i> <i>P. minimum</i>	-In the field in <i>Vitis vinifera</i>	Antibiosis <i>in vivo</i>	Álvarez-Pérez <i>et al.</i> , 2017
<i>Streptomyces</i> sp. (RC 87B)	<i>F. graminearum</i>	-In the field in <i>T. aestivum</i>	Undetermined	Palazzini <i>et al.</i> , 2017
<i>Streptomyces</i> sp. (RML-A23a, RML-B41, RML-B41b, RML-B42)	<i>F. oxysporum</i> f.sp. <i>radicis-passiflorae</i>	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Priya <i>et al.</i> , 2018
<i>Streptomyces</i> sp. (N2)	<i>R. solani</i>	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Wu <i>et al.</i> , 2019
<i>Streptomyces</i> sp. (CACIS-1.5CA)	<i>Alternaria</i> sp. <i>Aspergillus</i> sp. <i>Botrytis</i> sp. <i>C. gloeosporioides</i> <i>Colletotrichum musae</i> <i>Colletotrichum</i> sp. <i>Rhizoctonia</i> sp.	- <i>In vitro</i> by dual confrontation - <i>In vitro</i> by inhibition of spore germination by bioactive extract	Antibiosis <i>in vitro</i>	Evangelista-Martínez <i>et al.</i> , 2020

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	<i>Rhizopus</i> sp.			
<i>Streptomyces</i> sp. (ASR53)	<i>R. solani</i>	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Fatmawati <i>et al.</i> , 2020
<i>Streptomyces</i> sp. (UC1A-3)	<i>Alternaria</i> sp. <i>F. oxysporum</i> <i>Macrophomina</i> sp. <i>M. oryzae</i>	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Manigundan <i>et al.</i> , 2020
<i>Streptomyces</i> sp. (W1, PC 12, D 4.1 and D 4.3)	<i>Pyricularia</i> sp.	- <i>In vitro</i> by dual confrontation - <i>In vivo</i> in <i>O. sativa</i>	Antibiosis <i>in vitro</i> and plant growth-promoting effects	Chaiharn <i>et al.</i> , 2020
<i>Streptomyces</i> sp. (A217)	<i>R. solani</i> <i>P. grisea</i> <i>C. lagenarium</i> <i>P. capsici</i> <i>B. cirerea</i> <i>E. turcicum</i> <i>S. sclerotiorum</i> <i>F. oxysporum</i>	- <i>In vitro</i> by dual confrontation - <i>In vivo</i> in <i>Actinidia deliciosa</i>	Antibiosis <i>in vitro</i>	He <i>et al.</i> , 2020
<i>Streptomyces</i> sp. (H2, H3)	<i>P. aphanidermatum</i>	- <i>In vitro</i> by dual confrontation - <i>In vivo</i> in <i>L. esculentum</i>	Antibiosis <i>in vitro</i> and plant growth-promoting effects	Hassanisaadi <i>et al.</i> , 2021
<i>Streptomyces</i> sp. (CBQ-EA-2, -B-8)	<i>M. phaseolina</i> <i>R. solani</i>	- <i>In vitro</i> by dual confrontation - <i>In vivo</i> in <i>Ph. vulgaris</i>	Antibiosis <i>in vitro</i>	Díaz-Díaz <i>et al.</i> , 2022
<i>Streptomyces</i> sp. (AC1)	<i>S. sclerotiorum</i>	- <i>In vivo</i> in <i>L. esculentum</i>	Antibiosis <i>in vivo</i> and plant growth-promoting effects	Elshafie and Camele, 2022
<i>Streptomyces</i> sp. (Y20)	<i>Aspergillus</i> sp. <i>B. cinerea</i> <i>C. musae</i> <i>C. gloeosporioides</i> <i>Colletotrichum</i> sp. <i>F. oxysporum</i> <i>Fusarium</i> sp. <i>Lasiodiplodia</i> sp. <i>Sclerotium</i> sp.	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Rejón-Martínez <i>et al.</i> , 2022
<i>Streptomyces</i> sp. (FXP04)	<i>P. infestans</i>	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Fu <i>et al.</i> , 2022

		- <i>In vivo</i> in <i>Solanum tuberosum</i>		
<i>Streptomyces</i> sp. (CBQ-EA-2, CBQ-B-8)	<i>M. phaseolina</i> <i>R. solani</i>	-In the field in <i>Ph. vulgaris</i>	Antibiosis <i>in vivo</i>	Díaz-Díaz <i>et al.</i> , 2023
<i>Streptomyces</i> sp. (S30)	<i>R. solani</i>	- <i>In vitro</i> by metabolites - <i>In vivo</i> in <i>L. esculentum</i> and <i>Cucumis sativus</i>	Antibiosis <i>in vitro</i> The effect of metabolites on tomato and cucumber seedlings growth was not significant	Cao <i>et al.</i> , 2004
<i>Streptomyces</i> sp. (J-2, B-5, B-11, B-33, B-40, B-42, B-62, D-29, D-35, D-75)	<i>Sc. rolfsii</i> <i>B. cinerea</i> <i>F. oxysporum</i> <i>V. dahliae</i>	- <i>In vitro</i> by dual confrontation - <i>In vivo</i> in <i>Beta vulgaris</i> - <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i> e <i>in vivo</i> and plant growth-promoting effects	Errakhi <i>et al.</i> , 2007
<i>Streptomyces</i> sp. (KAI-32, KAI-90; RB-6, RB-24, RB-115)	<i>M. phaseolina</i> <i>R. bataticola</i>	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Gopalakrishnan <i>et al.</i> , 2011
<i>Streptomyces</i> sp. (CAI-24, CAI-121, CAI-127, KAI-32, KAI-90)	<i>F. oxysporum</i> f.sp. <i>ciceri</i>	- <i>In vitro</i> by dual confrontation - <i>In vivo</i> in <i>C. arietinum</i> -In the field in <i>C. arietinum</i>	Antibiosis <i>in vivo</i>	
<i>Streptomyces</i> sp. (StB-3, StB-6, StB-11, StB-12)	<i>V. dahliae</i>	- <i>In vitro</i> by dual confrontation - <i>In vivo</i> in <i>S. melongena</i> -In the field in <i>S. melongena</i>	Antibiosis <i>in vitro</i> and <i>in vivo</i>	Bubici <i>et al.</i> , 2013
<i>Streptomyces</i> sp. (APA2, AASH48, AAH5 3,APC70)	<i>Alternaria</i> sp. <i>Colletotrichum</i> sp. <i>Fusarium</i> sp. <i>Rhizoctonia</i> sp.	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Dávila-Medina <i>et al.</i> , 2013
<i>Streptomyces</i> sp. (CB-75)	<i>A. tenuissima</i> <i>B. cinerea</i> <i>C. acutatum</i> <i>C. fragariae</i> <i>C. gloeosporioides</i> <i>C. musae</i> <i>Curvulatia fallax</i>	- <i>In vitro</i> by dual confrontation -Determination of MIC	Antibiosis <i>in vitro</i>	Chen <i>et al.</i> , 2018

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	<i>F. oxysporum</i>			
<i>Streptomyces</i> sp. (M2A2)	<i>R. solani</i>	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Caviedes <i>et al.</i> , 2021
<i>S. spiralis</i> (17)	<i>P. aphanidermatum</i>	- <i>In vivo</i> in <i>C. sativus</i>	Antibiosis <i>in vivo</i> and plant growth-promoting effects	El-Tarabily <i>et al.</i> , 2009
<i>S. sundarbansensis</i> (WZS2-1)	<i>F. graminearum</i>	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Han <i>et al.</i> , 2018
<i>S. tendae</i> (RDS16)	<i>F. oxysporum</i> <i>M. phaseolina</i> <i>Sc. rolfsii</i>	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Al-Askar <i>et al.</i> , 2013
<i>S. toxytricini</i> (vh22, vh55)	<i>R. solani</i>	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Patil <i>et al.</i> , 2010
<i>S. vinaceusdrappus</i> (S5MW2)	<i>R. solani</i>	- <i>In vitro</i> by dual confrontation - <i>In vivo</i> in <i>L. esculentum</i>	Antibiosis <i>in vitro</i> e <i>in vivo</i> and plant growth-promoting effects	Yandigeri <i>et al.</i> , 2015
<i>S. vinaceusdrappus</i> (LSCH-10C)	<i>Pyricularia oryzae</i>	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Law <i>et al.</i> , 2017
<i>S. violaceusniger</i> (G10)	<i>F. oxysporum</i>	- <i>In vitro</i> by dual confrontation	Antibiosis <i>in vitro</i>	Getha and Vikineswary, 2002
<i>S. violaceusniger</i> (AC12AB)	<i>S. scabies</i>	- <i>In vitro</i> by disk diffusion assay - <i>In vivo</i> in <i>potato</i> -In the field in <i>S. tuberosum</i>	Antibiosis <i>in vitro</i>	Sarwar, 2015
<i>S. variabilis</i> (SF 2)	<i>B. cinerea</i>	-In the field in <i>Vicia fabae</i>	Antibiosis <i>in vivo</i> and plant growth-promoting effects	El-Shatoury <i>et al.</i> , 2020
<i>S. viridodiastaticus</i> (73)	<i>S. minor</i>	- <i>In vitro</i> by dual confrontation - <i>In vivo</i> in <i>Lactuca sativa</i>	Antibiosis <i>in vitro</i> e <i>in vivo</i>	El-Tarabily <i>et al.</i> , 2000

## 2.4. Conclusions and future perspectives

Actinomycetes are excellent colonizers of the plant rhizosphere and possess great potential as BCAs for soil-borne plant pathogens and as plant growth promoters. They are environmentally friendly and constitute a significant component of the rhizosphere microbial population, and they are capable of colonizing plant root surfaces and of colonizing plants endophytically, giving them a vital quality through which they can act against a wide variety of soil-borne pathogens. Actively proteolytic, their enzymes allow them to chemically break down residues rich in cellulose, lignin, chitin and protein, and in addition to the enormous number of bioactive metabolites they produce, they synthesize extracellular enzymes that attack the cell walls of plant pathogenic fungi and have the ability to produce spores that are resistant to dry heat, desiccation, UV and  $\gamma$ -radiation. In particular, species belonging to the genus *Streptomyces* help preserve the stability and diversity of farming communities, reduce synthetic input and help farmers adapt to a rapidly changing world in which agricultural intensification, land use and climate change increase the risk of infection by devastating epiphytes. Based on the studies conducted to date on the potential effects of *Streptomyces* as BCAs against a broad diversity of plant diseases, there is no doubt that these microorganisms are a potential ecofriendly alternative in crop protection. However, most of the studies included in this review have been conducted under controlled laboratory conditions, and only a small percentage of the results reported here have been confirmed in the field. Thus, the future of actinomycetes as commercial BCAs requires improved and increased evaluation of potential *Streptomyces* strains in the field to demonstrate their effectiveness as biocontrol tools

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## *Chapter 3*





**Characterization of actinobacterial strains as potential biocontrol agents against *Macrophomina phaseolina* and *Rhizoctonia solani*, the main soil-borne pathogens of *Phaseolus vulgaris* in Cuba**

**ABSTRACT**

*Macrophomina phaseolina* and *Rhizoctonia solani* are considered two major soil-borne pathogens of *Phaseolus vulgaris* in Cuba. Their management is difficult, not only due to their intrinsic biology as soil-borne pathogens, but also because the lack of active ingredients available against these pathogens. Actinobacteria, a heterogeneous bacterial group traditionally known as actinomycetes have been reported as promising biological control agents (BCAs) in crop protection. Thus, the main objective of this study was to evaluate the effectiveness of 60 actinobacterial strains as BCAs against *M. phaseolina* and *R. solani* *in vitro* by dual culture assays. The most effective strains were characterized according to their cellulolytic, chitinolytic and proteolytic extracellular enzymatic activity, as well as by their morphological and biochemical characters *in vitro*. Forty and 25 out of the 60 actinobacteria strains inhibited the mycelial growth of *M. phaseolina* and *R. solani*, respectively, and 18 of them showed a common effect against both pathogens. Significant differences were observed on their enzymatic and biochemical activity. The morphological and biochemical characters allow us to identify all our strains as species belonging to the genus *Streptomyces*. *Streptomyces* strains CBQ-EA-2 and CBQ-B-8 showed the highest effectiveness *in vitro*. Finally, the effect of seed treatments by both strains was also evaluated against *M. phaseolina* and *R. solani* infections in *P. vulgaris* cv. Quivicán seedlings. Treatments combining the two *Streptomyces* strains (CBQ-EA-2 + CBQ-B-8) were able to reduce significantly the disease severity for both pathogen infections in comparison with the non-treated and inoculated control. Moreover, they showed similar effect than that observed for *Trichoderma harzianum* A-34 and with Celest® Top 312 FS (Syngenta®; Basilea, Switzerland) treatments, which were included for comparative purposes.

**KEYWORDS:** ashy stem blight; biological control; common bean; rhizoctonia blight; *Streptomyces* spp.

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### 3.1. INTRODUCTION

The common bean (*Phaseolus vulgaris* L.) is one of the most important grain legumes in many areas of the world, providing a diet rich in protein, dietary fiber, essential micronutrients and phytochemicals for more than 500 million people (Singh *et al.*, 2020). The global cultivated surface of *P. vulgaris* reached 33.1 million hectares in the season 2019/2020, with an annual production of 28.9 million metric tons (FAOSTAT, 2021). Common bean is the most important plant species for Cuba population within the group of edible legumes with an annual production of 169,900 tons. Together with rice (*Oryza sativa* L.), they form the basis of the daily diet in this geographic area (Ulloa *et al.*, 2011).

In countries with a subtropical climate, the environmental conditions are favorable for the development and proliferation of a vast and heterogeneous soil microflora, including complexes of fungal species associated with root rot diseases such as *Alternaria alternata* (Fr.) Keissl., *Colletotrichum truncatum* (Schwein.) Andrus & W.D. Moore, *Fusarium oxysporum* Schldl., *Macrophomina phaseolina* (Tassi) Goid., *Rhizoctonia solani* J.G. Kühn, and *Sclerotium rolfsii* Sacc. (Were *et al.*, 2021). In addition, González (1988), pointed out that the incidence and severity of root rot diseases caused by these fungi depend on the climatic factors prevailing at each sowing time, as well as the characteristics of the microclimates existing in each region of the country where common beans are grown. Among them, *M. phaseolina* and *R. solani* are considered the most prevalent fungal pathogens associated with root rot diseases of common bean worldwide (Olaya and Abawi, 1996; Spedaletti *et al.*, 2017).

*Macrophomina phaseolina* (Ascomycota), causal agent of ashy stem blight, also affects roots and stems of host species via pycnidiospores and microsclerotia that persist in the soil, where the pathogen establishes the primary inoculum (Zhao *et al.*, 2020). Typical symptoms in common bean include dark, irregular lesions on cotyledons, wilting, systemic chlorosis, premature defoliation, epinasty and early maturity or death in adult plants (Olaya and Abawi, 1996). Late infections cause the appearance of grey areas on the stems, where microsclerotia and pycnidia of the fungus are produced. The occurrence of *M. phaseolina* in the seeds has major consequences since it causes the disqualification of legumes as propagation material (MINAG, 2008). For instance, six tons of common bean and three tons of broad bean to be used as planting material in the province of Villa Clara were disqualified between 2006–2007 because they were affected by *M. phaseolina* (González, 2007).

On the other hand, *R. solani* (Basidiomycota) is the causal agent of rhizoctonia blight, also commonly known as damping off (Abu-Tahon *et al.*, 2022). This soil-borne pathogen can affect more than 500 plant species, including cultivated and wild plants, and causes damping off in stands, necrotic lesions in roots, seeds and stems, as well as foliar lesions with a worldwide distribution (Spedaletti *et al.*, 2017; Muharrem *et al.*, 2020). This fungus affects Young seedlings much more than adult plant tissues. On the stem and hypocotyl of affected plants, reddish-brown cankers of various sizes appear, usually delimited by a dark border, which later become rough, dry up and destroy plant tissues (Muharrem *et al.*, 2020). It also attacks the roots causing foot rot of the plants González (1988). The management of soil-borne pathogens including both *R. solani* and *M. phaseolina* is usually difficult, not only due to their intrinsic biology, but also because the lack of effective active ingredients. Thus, the use and extension of eco-friendly control methods such as biological control is required, not only to prevent plant diseases, but also contributing markedly to soil preservation and conservation (Muharrem *et al.*, 2020).

Microorganisms belonging to genera *Bacillus* (bacteria) and *Trichoderma* (fungi) are the most commonly used biological control agents (BCAs) against soil-borne plant pathogenic fungi (Sabaté *et al.*, 2019; Torres *et al.*, 2016). Within this context, species belonging to *Trichoderma* fungal genus have been studied since 1930, and their use has been successfully applied directly to the soil or by seed treatments (Mayo-Prieto *et al.*, 2021). On the other hand, since the last century, bacteria belonging to the genus *Bacillus* have been used as BCAs due to their ability to colonize the rhizosphere of plants and inhibit the growth and development of plant pathogens. In addition, they are used as plant growth promoters (Torres *et al.*, 2016). At the same time, the ability of these bacteria to form endospores gives them resistance to climatic changes, which is an important characteristic for inoculum production (Sabaté *et al.*, 2019). In addition to these well-known BCAs, research in the last decades highlights the benefits of the actinobacteria (*Streptomyces* spp. mainly) and their potential as BCAs (e.g., *Streptomyces griseoviridis*, *S. lydicus*) against soil-borne pathogens, such as species of *Rhizoctonia*, *Phytophthora*, *Fusarium*, and *Pythium* in legumes and other crops (Vurukonda *et al.*, 2021). Actinobacteria, which have been traditionally known as actinomycetes, are a heterogeneous group of aerobic, filamentous and Gram-positive bacteria. Traditionally, the main genera isolated from soil samples are *Micromonospora*, *Nocardia*, and *Streptomyces*. The genus *Streptomyces* is represented in nature by the largest number of

species among the family Actinomycetaceae (Olanrewaju and Babalola, 2019). This genus, as a colonizer of the rhizosphere, is able to: (i) act as BCA of plant pathogenic fungi, (ii) produce siderophores, (iii) produce plant growth promoting substances, (iv) promote nodulation, (v) produce biodegradative enzymes such as chitinases, cellulases, glucanases, peroxidases, and (vi) assist *Rhizobium* bacteria in iron assimilation, or in nitrogen fixation in legumes, which indirectly contributes to the promotion of plant growth (Vurukonda *et al.*, 2021).

As we mentioned above, ashy stem blight and rhizoctonia blight are considered the main diseases of *P. vulgaris* in Cuba since they are associated in a complex disease of this crop that causes root rot and plant death. The control management strategies already available against this complex disease are not enough for its optimum control in the frame of the sustainable agriculture. Thus, it is necessary to explore new alternatives towards biological control of these diseases. Therefore, actinobacteria could play an important role as BCAs against the main causal agents of the disease, *M. phaseolina* and *R. solani*. However, the effect of actinobacteria as BCAs against plant pathogenic fungi is still uncertain. Consequently, no biological based compounds on actinobacteria have been developed so far. Likewise, the ‘Centro de Bioactivos Químicos’ Universidad Central “Marta Abreu” de Las Villas (Cuba) has a wide collection of actinobacterial strains isolated in the central region of the country, which may be explored as a new biological alternative to be included in the integrated disease management program against soil-borne plant pathogens in the common bean crop. Therefore, the main goal of this study was to evaluate 60 actinobacterial strains for their effectiveness as BCAs against *M. phaseolina* and *R. solani* by *in vitro* dual-cultures assays and finally to select several actinobacterial strains with high efficiency of reduction the viability of both pathogens *in vitro*, and the disease progress in planta. We expect to select several actinobacterial strains with high efficacy on reducing the viability of *M. phaseolina* and *R. solani* *in vitro*, and the disease progress *in planta*.

## **3.2. MATERIALS AND METHODS**

### *3.2.1. Actinobacterial strains and growth conditions*

A total of 60 actinobacterial strains isolated from different substrates or geographical areas of west-central Cuba were included in this study. They were recovered from rhizosphere (21), stem (15) or root (9) samples from a wide diversity of hosts, among

other sources (Table 1), and stored in the laboratory at 4 °C for no more than 72 h until processing. For isolation of actinobacteria from rhizosphere samples, 1 g of each sample was suspended in 9 mL of sterile distilled water (SDW) by vortexing and incubated in water bath at 55°C for 6 min. Subsequently, serial dilutions (up to 10<sup>-5</sup>) were performed. The same procedure was carried out with stem or root samples, but they were previously macerated in a mortar with sterile sand. In all cases, 100 µL aliquots of each dilution were spread in 9.0 cm diameter Petri dishes containing casein-starch agar (CSA) supplemented with filtered cycloheximide (100 µg/mL) and nalidixic acid (30 µg/mL) (El Karkouri *et al.*, 2019). The inoculated Petri dishes were incubated at 28°C for 28 days in darkness. Based on macroscopic characters i.e., texture, appearance, surface with or without aerial mycelium, colonies of actinobacteria were selected, transferred to CSA, and incubated as described before. Subsequently, spore suspensions were obtained from the pure cultures of each selected strain, and they were kept in 2 mL translucent screw-capped microtubes (Zhejiang Runlab Technology Co., Taizhou, China) at -20 °C in 20% glycerol for further studies (Bernal *et al.*, 2015). The collection belongs to the Microbiology Laboratory of the CBQ of the Universidad Central “Marta Abreu” de Las Villas (Cuba).

**Table 3.1.** Origen of actinobacterial strains used in this study.

Strain*	Isolation Substrate	Origin (Location, State)	Year of collection
CBQ-RS-3	Sediment	River Seibabo, Villa Clara	2007
CBQ-A-2	Rhizosphere	Arco Iris, V. Clara	2007
CBQ-EA-2 <sup>x,y,z</sup>	Endophytic, stem of <i>Mosiera bullata</i>	Arco Iris, V. Clara	2008
CBQ-B-8 <sup>x,y,z</sup>	Rhizosphere, Carbonated brown	Botanical Garden UCLV, V. Clara	2008
CBQ-J-4 <sup>x,y</sup>	Rhizosphere Ferrallitic red	River Seco, Jibacoa, Manicaragua, V. Clara	2008
CBQ-A-9	Rhizosphere	Arco Iris, V. Clara	2008
CBQ-A-17	Rhizosphere	Arco Iris, V. Clara	2008
CBQ-C-5	Rhizosphere	Cienfuegos	2008
CBQ-C-7	Rhizosphere	Cienfuegos	2008
CBQ-B-1	Rhizosphere	Botanical Garden UCLV, V. Clara	2008
CBQ-E-5	‘Fangos de Elguea’	Corralillo, V. Clara	2009
CBQ-B-41	Rhizosphere	Botanical Garden UCLV, V. Clara	2009
CBQ-B-44	Rhizosphere	Botanical Garden UCLV, V. Clara	2009
CBQ-Be-29	Rhizosphere	Escambray, Bernal	2010
CBQ-EC-3 <sup>y</sup>	Endophytic	Coge Finca, Camajuaní, V. Clara	2010
CBQ-EC-5	Endophytic, stem of <i>Petiveria alliacea</i> .	V. Clara	2010
CBQ-Be-36	Rhizosphere	Escambray, Bernal	2010
CBQ-EBe-3	Endophytic, root of <i>Hibiscus elatus</i>	Bernal, Herradura, Manicaragua, V. Clara	2010
CBQ-Cy-5	Rhizosphere	Key I, V. Clara	2010
CBQ-CYM-2	Rhizosphere	Salinas, V. Clara	2010
CBQ-EA-29	Endophytic, stem	Arco Iris, V. Clara	2011

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CBQ-EBa-1	Endophytic, root	Banao, S. Spíritus	2011
CBQ-EB-5	Endophytic	Botanical Garden UCLV, V. Clara	2011
CBQ-EBa-22	Endophytic, rtem	Banao, S. Spíritus	2011
CBQ-EBe-15	Endophytic, root	Planta Escambray, Bernal	2011
CBQ-EBe-16	Endophytic, root	Planta Escambray, Bernal	2011
CBQ-EBe-20	Endophytic, root	Planta Escambray, Bernal	2011
CBQ-EA-3 <sup>y</sup>	Endophytic	Arco Iris. V. Clara	2011
CBQ-EB-27 <sup>y</sup>	Endophytic, Stem	Jardín Botánico UCLV, V. Clara	2011
CBQ-EA-12 <sup>x,y</sup>	Endophytic, leaf of <i>Mosiera bullata</i> ,	Arco Iris. V. Clara	2011
CBQ-EC-18 <sup>y</sup>	Endophytic, stem of <i>Petiveria alliacea</i> .	Coge Finca, Camajuaní, V. Clara	2011
CBQ-ECa-24 <sup>y</sup>	Endophytic, root	Caguanes, S. Spíritus	2011
CBQ-EBa-5 <sup>x,y</sup>	Endophytic, root	Banao. S. Spíritus	2011
CBQ-EBa-21 <sup>x,y</sup>	Endophytic, root of <i>Piper aducum</i>	Banao. S. Spíritus	2011
CBQ-Ni-24 <sup>y</sup>	Endophytic, stem	Nicho, V. Clara	2011
CBQ-Ni-32 <sup>y</sup>	Endophytic, stem	Nicho, V. Clara	2011
CBQ-ESFe-12 <sup>y</sup>	Endophytic, stem of <i>Fleurya cuneata</i> ,	Loma Sta Fé, V. Clara	2012
CBQ-ESFe-5 <sup>y</sup>	Endophytic, stem of <i>Fleurya cuneata</i> ,	Loma Sta Fé, V. Clara	2012
CBQ-ESFe-10 <sup>y</sup>	Endophytic, stem of <i>Fleurya cuneata</i> ,	Loma Sta Fé, V. Clara	2012
CBQ-ESFe-11 <sup>y</sup>	Endophytic, stem of <i>Fleurya cuneata</i> ,	Loma Sta Fé, V. Clara	2012
CBQ-WP-14 <sup>y</sup>	Sediment, <i>Clarias batrachus</i> ,	V. Clara	2012
CBQ-ESFe-4 <sup>x,y</sup>	Endophytic, leaf of <i>Piper aduncum</i>	Loma Santa Fé. V. Clara	2012
CBQ-Wni-21 <sup>y</sup>	Sediment	River Nicho, V. Clara	2012
CBQ-Amb-3	Endophytic, Stem of <i>Cecropia adenopu</i> ,	V. Clara	2013
CBQ-CB-14 <sup>x,y</sup>	Sediment	Caves de Bellamar, Matanzas	2013
CBQ-OSS-4 <sup>y</sup>	Endophytic	Topes de Collantes, S. Spíritus	2013
CBQ-Plat-3 <sup>y</sup>	Endophytic, Stem of <i>Comocladia platyphylla</i> .	V. Clara	2013
CBQ-Plat-4 <sup>y</sup>	Endophytic, Root of <i>Comocladia platyphylla</i> ,	V. Clara	2013
CBQ-CB-3 <sup>y</sup>	Sediment	Caves de Bellamar, Matanzas	2013
CBQ-Plat-2 <sup>x,y</sup>	Endophytic, stem of <i>Comocladia platyphylla</i> .	V. Clara	2013
CBQ-OSS-3 <sup>x,y</sup>	Endophytic, leaf of <i>Ossanum</i>	Topes de Collantes, S. Spíritus	2013
CBQ-CB-4 <sup>y</sup>	Sediment	Caves de Bellamar, Matanzas	2013
CBQ-CD-12	Rhizosphere	Key Las Dunas, V. Clara	2014
CBQ-CD-19	Rhizosphere	Key Las Dunas, V. Clara	2014
CBQ-CD-21	Rhizosphere	Key Las Dunas, V. Clara	2014
CBQ-CD-23	Rhizosphere	Key Las Dunas, V. Clara	2014
CBQ-CD-25	Rhizosphere	Key Las Dunas, V. Clara	2014
CBQ-CD-24 <sup>x,y</sup>	Rhizosphere	Key Las Dunas. V. Clara	2014
CBQ-Mg-6 <sup>y</sup>	Endophytic, tem of <i>Rhizophora mangle</i>	Mégano, La Habana	2014
CBQ-SFe-5 <sup>y</sup>	Rhizosphere	Sta Fé, V. Clara	2015

<sup>a</sup> Strains selected for biochemical characterization. <sup>b</sup> Strains selected for qualitative determination of their chitinolytic, cellulolytic and proteolytic activity. <sup>c</sup> Strains selected for in planta bioassays. \* All actinobacterial strains used in this study were collected in Cuba by Dr. C. R. Medina-Marrero (CBQ: 'Centro de Bioactivos Químicos').

### 3.2.2. *In vitro* effect of actinobacterial strains against *Macrophomina phaseolina* and *Rhizoctonia solani*: dual culture assays

All the 60 actinobacterial strains (Table 3.1) were evaluated for their effectiveness inhibiting mycelial growth of *M. phaseolina* isolate CCIBP-Mp1 and *R. solani* isolate

CCIBP-Rh1 by means *in vitro* dual culture assays. The two pathogenic fungi were obtained from the collection of plant pathogenic fungi of the Instituto de Biotecnología de las Plantas (IBP) of the Universidad Central Marta Abreu de Las Villas (Cuba), where are maintained growing on PDA at 5 °C in darkness. These isolates were selected due to their high aggressiveness previously tested in the common bean crop (Díaz, 2011). Prior to conduct the dual culture assay, the 60 actinobacterial strains were grown on CSA (pH = 7) at 30 °C for seven days in darkness. The inoculum of *M. phaseolina* and *R. solani* was prepared by seeding suspensions of mycelial fragments of each isolate on Potato Dextrose Agar (PDA; BioCen, Bejucal, Mayabeque, Cuba) at 28°C for three days in darkness. *In vitro* dual culture assays were conducted in 9.0 cm in diameter Petri dishes with PDA (Sellem *et al.*, 2017). To this end, a 7.0 mm in diameter mycelial plug of the pathogen was placed at one end of the plate, and another 7.0 mm in diameter mycelial plug of the actinobacterial strain was plated at 50.0 mm apart at the opposite end. Additionally, 7.0 mm in diameter mycelial plugs of *M. phaseolina* or *R. solani* isolates were seeded in the center of PDA plates without actinobacteria as a positive growth control. All Petri dishes were incubated at 28 °C in total darkness, and the radial mycelial growth of the two plant pathogens was assessed every 24 h, until seven days of incubation (Misk and Franco, 2011; Cuesta *et al.*, 2012). There were three replicated Petri dishes per actinobacterial strain (n = 60) and plant pathogen (n = 2) or control (n = 2) combination in a completely randomized design [(60 actinobacterial strains×2 fungal pathogens×3 Petri dishes) + (2 control×3 Petri dishes) = 366 Petri dishes in total]. The experiment was performed three times under similar conditions. For each fungal pathogen, the percentage of the inhibition of mycelial growth was calculated using the following formula:

$$\text{Mycelial growth inhibition (MGI) (\%)} = [(RGR-rgr)/RGR] \times 100$$

where ‘rgr’ is the radial growth of *M. phaseolina* or *R. solani* in dual culture with each actinobacterial strain, and ‘RGR’ is the radial growth rate of the control treatment (fungal pathogen isolates growing on PDA without actinobacterial strains).

### 3.2.3. Qualitative evaluation of enzyme activities of actinobacterial strains

Of the 60 strains analyzed *in vitro* (3.2.2), the 31 most effective were selected to determine their chitinolytic, cellulolytic, proteolytic activity (Table 6). For chitinolytic activity, all the strains were grown on Colloidal Chitin Agar culture medium (pH = 7) at 28°C for seven days in darkness (Kawase *et al.*, 2004). For cellulolytic activity, the strains

were grown on ISP<sub>2</sub> (International *Streptomyces* Project) (Meena *et al.*, 2013) with cellulose (1%, w/v) (pH = 7.2) also at 28°C for seven days in darkness; then a congo red solution (1%) was added as developer for 15 min; and finally, the congo red solution was removed and NaCl solution (1 M) was added for 15 min (El-Sersy *et al.*, 2010). For proteolytic activity, the strains were grown on ISP<sub>2</sub> with 1% skimmed milk at 30 °C for seven days in darkness (Ara *et al.*, 2012). For each parameter evaluated, there were three replicated Petri dishes per strain in a completely randomized design (93 Petri dishes in total), and the experiment was performed three times under similar conditions. In all cases, after seven days of incubation, the halo surrounding the colonies of the actinobacterial strains was measured (mm) from the center of the inoculated mycelial disc.

#### 3.2.4. *Phenotypic characterization*

Taking into account the macroscopic appearance of the 60 actinobacterial strains evaluated for their effectiveness on MGI of the two pathogens in this study, a total of 11 strains (Table 3.1) were selected as representative of the main groups with slightly differences on the colony morphology to complete their macro- and microscopic morphological characterization. These strains were grown on CSA as described above, and then, macroscopic colony characters such as presence and color of aerial mycelium, as well as substrate color, shape, elevation, edges and consistency of colonies were recorded (Shirling and Gottlieb, 1966; Bergey, 2005). Subsequently, microscopic observations were conducted under optical microscope (LABOMED®, Fremont, CA, USA). Bacterial cell observations were carried out on fresh and stained preparations (simple and Gram staining) to define the shape, clustering and response to Gram stain (Bergey, 2005). Additional microscopic features such as aerial and vegetative mycelium, mycelial fragmentation, or clumping of spores were recorded by microcultures with lactophenol blue as a contrast stain (Franco-Correa, 2009), and they were compared with those described in Bergey's Manual of Bacteriological Determination (Oskay *et al.*, 2004). There were three replicated Petri dishes per strain in a completely randomized design (33 Petri dishes in total), and the experiment was performed three times under similar conditions.

#### 3.2.5. *Biochemical characterization and assimilation of carbon sources*

The biochemical characterization using traditional techniques of the same 11 actinobacterial analyzed in the Section 3.2.4 (Table 3.1) was evaluated by applying the following tests: catalase, acid production by using different carbohydrate sources (e.g., glucose, mannitol, dextrose, fructose, maltose, raffinose, sucrose and xylose), casein hydrolysis, citrate utilization, indole test, and gelatin hydrolysis (Selvakumar *et al.*, 2012). The ability to produce hydrolytic enzymes for the utilization of polysaccharides such as starch was also determined. The hydrolysis of urea to reveal the activity of the enzyme urease Saxena *et al.* (2015), methyl red (MR) and Voges Proskauer (VP) tests were carried out according to the ISP (Shirling and Gottlieb, 1966). There were three replicated Petri dishes per strain in a completely randomized design (33 Petri dishes in total), and the experiment was performed three times under similar conditions.

#### 3.2.6. Molecular characterization

The actinobacterial strains CBQ-EA-2 and CBQ-B-8 were grown in tryptone-soyabroth (BioCen) at 30°C for three days, and centrifuged at 16,000 rpm. DNA was extracted from the resulting pellet using the PureLink™ Genomic DNA Mini Kit reagent (Invitrogen, Waltham, MA, USA), following the manufacturer's instructions. The universal primers 27f and 1492r (Coombs and Franco, 2003) for eubacteria were used to amplify the 16S rRNA gene via Polymerase Chain Reaction (PCR). Each reaction mixture contained each primer at 20 µM, dNTPs at 10 µM, 5 µL of 10X MgSO<sub>4</sub> and buffer, dimethyl sulfoxide (5%), 1 µg of genomic DNA and 1 unit of taq DNA polymerase, for a final volume of 50 µL. PCR steps included an initial denaturation at 94°C for 3 min, followed by 30 cycles at 94°C for 30 s, 47°C for 33 s and 72°C for 90 s and a final extension step at 72°C for 7 min. PCR products were run through 1% agarose gel electrophoresis stained with RedSafe™ dye (iNtRON Biotechnology), followed by purification using the PureLink™ kit (Invitrogen, Waltham, MA, USA) and determination of amplicon quality by spectrophotometry (NanoDrop 2000, ThermoScientific; Waltham, MA, USA). Sequencing was carried out on the ABI310 Prism automated sequencer (Applied Biosystems; Waltham, MA, USA), and the resulting sequences were compared with those in the GenBank database using the BLAST (Basic Local Alignment Search Tool) algorithm to identify closely related sequences (Altschul *et al.*, 1990; Cole *et al.*, 2005). The consensus sequences were uploaded to GenBank data base (Table 3.1).

### 3.3. Effect of actinobacterial strains against *Macrophomina phaseolina* and *Rhizoctonia solani* infections *in planta*

#### 3.3.1. Plant material

Seedlings of the common bean (*P. vulgaris* L.) of cv. Quivicán (white testa) were used in this study. The seeds used are registered in the official list of commercial cultivars (MINAG, 2016) from the 'UEB Semillas Villa Clara'. Prior to conduct the experiments, the viability of seeds was tested estimating the percentage of germination (%) using a humid chamber at 100% of relative humidity (RH). The seeds were previously disinfected in a serial wash by dipping them first in a 70% ethanol solution for 5 min, then in a 1.5% sodium hypochlorite solution for 15 min, and finally, three times in distilled water for 20 min.

#### 3.3.2. Biological control agents and inoculum preparation

The actinobacterial strains CBQ-EA-2 and CBQ-B-8 were selected to conduct the experiments *in planta* because they were considered as representative of the strains showing high (CBQ-EA-2; MGI = 70.4 and 77.4% for *M. phaseolina* and *R. solani*, respectively) and moderate (CBQ-B-8; MGI = 63.1 and 69.0% for *M. phaseolina* and *R. solani*, respectively) effectiveness to both pathogens in the dual culture assays. In addition, their morphological, biochemical, and extracellular enzymatic characteristics together with their molecular characterization were also taken into account to ensure that they belong to *Streptomyces* genus together. To prepare the inoculum of the two strains for seed treatments (see below), 20  $\mu\text{L}$  of the original spore suspension preserved at  $-20^{\circ}\text{C}$  in 20% glycerol were firstly added in a 5 mL sterile plastic tubes with tryptone soy broth (BioCen) and incubated at  $28^{\circ}\text{C}$  for 48 h (Hamdali *et al.*, 2008). Then, they were transferred to 250 mL Erlenmeyer flasks with 100 mL of tryptone soy broth and shaken in a Gerhardt orbital shaker at  $28^{\circ}\text{C}$  at a speed of 120 G for 3 days. Finally, the inoculum of each actinobacterial strain was adjusted at  $1 \times 10^8$  spores  $\text{mL}^{-1}$  using a hemocytometer.

Additionally, *T. harzianum* strain A-34 belonging to the Plant Health Research Institute (INISAV, La Habana, Cuba) was also included in this experiment as a BCA for comparative purposes. The selected strain is the active ingredient of a bioproduct for the control of phytopathogenic soil fungi, foliar diseases and nematodes commonly used in Cuba (Stefanova *et al.*, 2014). To prepare the inoculum of *T. harzianum* A-34 for seed treatments (see below), sterile 250 mL Erlenmeyer flasks with 100 mL of Potato Dextrose

Broth (PDB; BioCen) were inoculated by adding five 10-mm in diameter mycelial plugs of *T. harzianum* A-34 obtained from the active margin of colonies previously grown on PDA at 28 °C in darkness for 72 h. Then, the inoculated Erlenmeyer flask were shaken as described above, and the inoculum was adjusted at  $1 \times 10^8$  spores  $\text{mL}^{-1}$ .

### 3.3.3. Soil inoculation with *Macrophomina phaseolina* and *Rhizoctonia solani*

The effectiveness of the selected BCAs was evaluated in planta against *M. phaseolina* isolate CCIBP-Mp 2, and *R. solani* isolate CCIBP-Rh1. To prepare the inoculum of both isolates, 1-L Erlenmeyer flasks were filled with 200 g of an artificial substrate (risk husk, part rice grain and distilled water; 3:1:0.5, weight:weight:volume) and sterilized at 120 °C for 1 h. Subsequently, the flasks were seeded with five 1.0-cm in diameter of mycelial plugs of *M. phaseolina* isolate CCIBP-Mp 2 or *R. solani* CCIBP-Rh1 anastomosis groups (AG-4\_HGI), taken from the edge of the active growing colonies previously grown on PDA as described before. The inoculated flasks were incubated at 28°C in darkness for 10 days, and they were manually shaken each 2 days to favor the homogeneous colonization of the substrate (Suryawanshi *et al.*, 2020). In this study, a medium washed fluffy brown soil (Hernández *et al.*, 2015) non-sterilized and sterilized (120°C for 20 min in cycles of three consecutive days, and subsequent sterility testing) was used in this study. In all cases, and for each pathogen, the inoculation was carried out at 2% by homogenizing the colonized substrate with the soil (Hernández Pérez *et al.*, 2018). Subsequently, plastic pots were filled with 1.5 Kg of this mix. After 48 h of mix preparation (soil + colonized substrate), four common bean seeds previously treated were sown per plastic pot, and soil moisture was kept at 80% of the field capacity (FC).

### 3.3.4. Seed treatments, growth conditions and experimental design

Seed treatments were conducted by dipping the seeds for 30 min in the following suspensions: (i) actinobacterial strain CBQ-EA-2 at  $1 \times 10^8$  spores  $\text{mL}^{-1}$ ; (ii) actinobacterial strain CBQ-B-8 at  $1 \times 10^8$  spores  $\text{mL}^{-1}$ ; (iii) a mix of the actinobacterial strains CBQ-EA-2 and CBQ-B-8 at  $1 \times 10^8$  spores  $\text{mL}^{-1}$  global concentration; (iv) *T. harzianum* strain A-34 at  $1 \times 10^8$  spores  $\text{mL}^{-1}$ ; and (v) Celest® Top 312 FS (Syngenta®; Basilea, Switzerland) prepared in a water suspension of 192 mL of active ingredient per kg of seeds. The latter chemical compound was included for comparison purposes. Additionally, seeds dipped for 30 min in SDW were also included as non-treated control seeds, and lots of non-treated seeds were sowed in plastic pots with inoculated soil

(treatment (vi): positive control) as well as in plastic pots with non-inoculated soil (treatment (vii): negative control).

After more than 50% of the seeds emerged, seedlings were treated every three days by wetting the substrate with 1 mL of the respective biological treatment (actinobacterial or *T. harzianum*) adjusted to  $1 \times 10^8$  spores  $\text{mL}^{-1}$  until the end of the experiment [28 days after sowing (das)]. Both positive and negative controls and the chemical treatment were wetted every three days with 1 mL of SDW. For each pathogen, a split-plot design was used with soil ( $n = 2$ ; sterilized and nonsterilized) as the main plot factor and treatments ( $n = 7$ ) as sub-plot factor; with ten pots (replicates) per treatment, and 4 seeds per replicate ( $n = 40$ ). They were maintained in a CBQ greenhouse at  $28^\circ\text{C}$ , 70% RH and  $1100 \mu\text{mol m}^{-2} \text{s}^{-1}$  light intensity.

### 3.3.5. Disease severity assessment

For treated seed lings inoculated with *M. phaseolina*, disease severity (DS) was assessed at 35 days after inoculation using the following DS rating scale: 1 = no visible disease symptoms; 3 = wilt restricted to cotyledons, lower stem tissues with small necrotic lesions; 5 = 10% of hypocotyl and lower stem tissues showing lesions, fungal fruiting structures starting the development in the affected tissues, 7 = 25% of hypocotyl and lower stem tissues showing lesions, with development of fungal fruiting structures in the affected tissues; and 9 =  $\geq 50\%$  of hypocotyl and lower stem tissues with lesions, with abundant development of fungal fruiting structures (Van Schoonhoven and Pastor Corrales, 1987). Subsequently, a DS index was estimated using the following formula:

$$\text{DS (\%)} = \left[ \sum_{i=1}^9 n_i (st_i) / (N \times K) \right] \times 100$$

In which  $n_i$  = number of seedlings in the DS development stage  $i$ ,  $st_i$  = value of the DS stage (1–9),  $N$  = total number of plants assessed, and  $K$  = largest scale level (9) (Townsend and Heuberger, 1943).

Regarding treated seedlings inoculated with *R. solani*, DS was evaluated separately on stem and roots tissues at 28 days after inoculation by using the following DS rating scales: (i) DSstem: 1 = absence of lesions on hypocotyl, 2 = superficial lesions (yellow-brown discoloration) on hypocotyl, 3 = deep tissue lesions, and 4 = seedlings dead or wilted (Bradley *et al.*, 2001); (ii) DSroot: 0 = healthy seedlings, 1 = yellowish-brown discoloration near hypocotyl, 2 = yellowish-brown discoloration plus lesions or

brown spots near hypocotyl, 3 = entirely brown surface or lesions covering more than 75% of root surface, and 4 = pre-emergence damping off, seedlings dead or wilted (Keijer *et al.*, 1997). Subsequently, a DS index was estimated for each tissue using the following formulas:

$$DS_{stem} (\%) = \left[ \sum_{i=1}^4 n_i (st_i) / (N \times K_{stem}) \right] \times 100$$
$$DS_{root} (\%) = \left[ \sum_{i=1}^5 n_i (st_i) / (N \times K_{root}) \right] \times 100$$

in which  $n_i$  = number of stems or roots in the DS development stage  $i$ ,  $st_i$  = value of the DS stage (1–4 and 0–4 for stems and roots, respectively),  $N$  = total number of plants assessed, and  $K$  = largest scale level (4 in all cases) (Townsend and Heuberger, 1943). Furthermore, for each combination of soil and treatment, the incidence of disease (DI; % of affected plants) and mortality (% of dead plants) were estimated at 28 days after inoculation.

Ungerminated seeds or plants with lesions on the hypocotyl, roots and/or stem were subjected to wet chamber and microscope preparations to confirm the identity of the inoculated pathogens.

### 3.3.6. Data analyses

Data from the repetitions of each experiment were combined after checking for homogeneity of the experimental error variances by the F test ( $P \geq 0.05$ ). Subsequently, data were tested for normality and homogeneity of variances prior to conduct analyses of variance (ANOVA). For the dual culture assay, factorial ANOVA was conducted with MGI as dependent variable, and actinobacterial strains, fungal pathogens and their interaction as independent variables. Significant differences were observed for the two independent variables as well as for their interaction ( $P < 0.0001$  in any cases). Thus, independent ANOVA were conducted to determine differences between actinobacterial strains against each fungal pathogen. For each fungal pathogen, mean values were compared using Tukey's honestly significant difference (HSD) tests at  $p = 0.05$  (Steel and Torrie 1985). For the enzymatic activity, data of the halo (mm) for each of the three parameters evaluated were analyzed separately by the non-parametric Kruskal-Wallys test due to the assumptions of normality and homogeneity of variances were not fulfilled even though logarithmically, arcsine or square root transformation of the data were conducted. Data from the actinobacterial strains that not develop halo (0.0 mm) were

excluded from the statistical analysis in any cases. Mean values were compared using Dunn's comparisons test at  $P = 0.05$ . In the in planta experiment, data of total DS (seedlings inoculated with *M. phaseolina*), and DSstem and DSroot (seedlings inoculated with *R. solani*) were tested for normality and homogeneity of variances prior to conduct analyses of variance (ANOVA). Data from negative control were omitted since no symptoms were observed in all cases. For each dependent variable, a split-plot ANOVA was conducted with soil ( $n = 2$ ) as main-plot factor and treatments ( $n = 6$ ) as the subplot factor. Due to significant differences were observed in all cases for the two independent variables as well as for their interaction ( $P < 0.005$ ), independent ANOVA were conducted to determine differences between treatments for each disease. The treatment means of total DS, or DSstem and DSroot were compared according to Fisher's protected LSD test at  $P = 0.05$  (Steel and Torrie 1985). For both inoculated plants with *M. phaseolina* and *R. solani*, data on the final DI (% of affected plants) and mortality (% of dead plants) were analyzed by multiple comparisons for proportions tests at  $P = 0.05$  (Zar, 2010). Additionally, for plants inoculated with *R. solani*, the Pearson correlation coefficients ( $r$ ) between the DSstem and DSroot were calculated using the average values of the two variables for each of the treatment evaluated in sterilized or non-sterilized soil ( $n = 6$  in each type of soil). All data analyses were conducted using Statistix 10 (Analytical Software 2013).

### 3.4. RESULTS

#### 3.4. 1. In vitro effect of actinobacterial strains against *Macrophomina phaseolina* and *Rhizoctonia solani*: dual culture assays

For both fungal pathogens *M. phaseolina* and *R. solani*, significant differences between actinobacterial strains were observed on their effectiveness in the Mycelial Growth Inhibition (MGI; %) ( $P < 0.001$  in both cases). Regarding their effect against *M. phaseolina* isolate CCIBP-Mp1, 40 out of the 60 strains tested showed antagonistic activity against the pathogen. For this group of 40 strains, the MGI ranged from  $70.4 \pm 1.23$  to  $3.24 \pm 1.01\%$  for CBQ-EA-2 and CBQ-ESFe-11, respectively. The most effective strains against *M. phaseolina* were CBQ-EA-2, -Plat-2 and -CD-24 with mean MGI values of  $70.4 \pm 1.23$ ,  $66.6 \pm 0.78$  and  $64.6 \pm 1.48\%$ , respectively. On the other hand, 25 out of the 60 actinobacterial strains tested showed antagonistic effect against *R. solani* isolate CCIBP-Rh1. For this group of 25 strains, the MGI ranged from  $78.3 \pm 0.37$  to  $5.6 \pm 0.47\%$  for CBQ-EA-12 and CBQ-C-5, respectively. The most effective strains against *R. solani*

were CBQ-EA-12, -EA-2 and -CD-24 with mean MGI values of  $78.3 \pm 0.37$ ,  $77.4 \pm 1.20$  and  $75.4 \pm 1.22\%$ , respectively. In addition, 19 out of the 60 actinobacteria strains evaluated showed a MGI efficacy higher than 50% for both phytopathogenic fungi, with the strains CBQ-EA-2 (MGI =  $70.4 \pm 1.23$  and  $77.4 \pm 1.20\%$  for *M. phaseolina* and *R. solani*, respectively) and CBQ-CD-24 (MGI =  $64.6 \pm 1.48$  and  $75.4 \pm 1.22\%$  for *M. phaseolina* and *R. solani*, respectively) showing the highest common effectiveness for the two pathogens. At the same time, 5% of the total actinobacteria tested did not show any effect on MGI for any of the two pathogens evaluated (Table 3.2; Figure 3.1).

**Table 3.2.** Antagonistic effect of the 60 actinobacterial strains on mycelial growth of *Macrophomina phaseolina* and *Rhizoctonia solani* in dual cultures.

Actynobacterial Strain	(MGI; %) <sup>a,b</sup>	
	<i>Macrophomina phaseolina</i>	<i>Rhizoctonia solani</i>
CBQ-CB-14	$60.9 \pm 1.63$	$69.8 \pm 1.40$
CBQ-EA-2	$70.4 \pm 1.23$	$77.4 \pm 1.20$
CBQ-EA-12	$46.2 \pm 1.32$	$78.3 \pm 0.37$
CBQ-OSS-3	$62.8 \pm 1.55$	$61.2 \pm 0.53$
CBQ-ESFe-4	$52.0 \pm 2.00$	$63.7 \pm 1.19$
CBQ-CD-24	$64.6 \pm 1.48$	$75.4 \pm 1.22$
CBQ-EBa-5	$60.5 \pm 1.65$	$45.9 \pm 1.32$
CBQ-EBa-21	$54.0 \pm 1.11$	$55.7 \pm 2.01$
CBQ-Plat-2	$66.6 \pm 0.78$	$37.3 \pm 1.91$
CBQ-WP-14	$56.7 \pm 1.81$	$37.1 \pm 0.40$
CBQ-B-8	$63.1 \pm 1.54$	$69.0 \pm 1.63$
CBQ-J-4	$28.1 \pm 1.71$	$35.4 \pm 2.70$
CBQ-CB-3	$40.5 \pm 0.91$	$40.1 \pm 1.35$
CBQ-EA-3	$44.4 \pm 0.65$	$0.0 \pm 0.00$
CBQ-EB-27	$0.0 \pm 0.00$	$0.0 \pm 0.00$
CBQ-EC-3	$32.2 \pm 0.60$	$0.0 \pm 0.00$
CBQ-EC-18	$36.0 \pm 1.30$	$54.6 \pm 0.91$
CBQ-ECa-24	$29.9 \pm 1.51$	$0.0 \pm 0.00$
CBQ-ESFe-5	$31.6 \pm 1.31$	$0.0 \pm 0.00$
CBQ-ESFe-10	$35.8 \pm 1.25$	$0.0 \pm 0.00$
CBQ-ESFe-11	$3.24 \pm 1.01$	$0.0 \pm 0.00$
CBQ-ESFe-12	$38.9 \pm 1.32$	$43.5 \pm 1.92$
CBQ-Mg-6	$8.7 \pm 0.42$	$0.0 \pm 0.00$
CBQ-Ni-24	$21.2 \pm 0.58$	$0.0 \pm 0.00$
CBQ-Ni-32	$5.9 \pm 0.34$	$0.0 \pm 0.00$
CBQ-OSS-4	$8.9 \pm 0.30$	$14.3 \pm 2.38$
CBQ-Plat-3	$20.4 \pm 1.52$	$0.0 \pm 0.00$
CBQ-Plat-4	$24.2 \pm 1.43$	$0.0 \pm 0.00$
CBQ-SFe-5	$4.0 \pm 0.11$	$0.0 \pm 0.00$
CBQ-Wni-21	$28.3 \pm 1.55$	$0.0 \pm 0.00$
CBQ-RS-3	$4.7 \pm 0.19$	$0.0 \pm 0.00$
CBQ-A-2	$0.0 \pm 0.00$	$0.0 \pm 0.00$
CBQ-A-9	$0.0 \pm 0.00$	$52.7 \pm 2.98$

CBQ-A-17	9.7 ± 0.33	32.6 ± 2.30
CBQ-Amb-3	0.0 ± 0.00	0.0 ± 0.00
CBQ-B-1	0.0 ± 0.00	35.0 ± 1.73
CBQ-B-41	0.0 ± 0.00	21.5 ± 0.63
CBQ-B-44	0.0 ± 0.00	41.5 ± 0.43
CBQ-Be-29	0.0 ± 0.00	0.0 ± 0.00
CBQ-Be-36	0.0 ± 0.00	0.0 ± 0.00
CBQ-C-5	0.0 ± 0.00	5.6 ± 0.47
CBQ-C-7	0.0 ± 0.00	17.8 ± 1.04
CBQ-CB-6	17.8 ± 1.78	0.0 ± 0.00
CBQ-CD-12	0.0 ± 0.00	0.0 ± 0.00
CBQ-CD-19	3.4 ± 0.32	0.0 ± 0.00
CBQ-CD-21	38.2 ± 1.26	0.0 ± 0.00
CBQ-CD-23	26.6 ± 1.56	0.0 ± 0.00
CBQ-CD-25	3.5 ± 0.36	0.0 ± 0.00
CBQ-Cy-5	0.0 ± 0.00	0.0 ± 0.00
CBQ-CYM-2	0.0 ± 0.00	0.0 ± 0.00
CBQ-E-5	0.0 ± 0.00	0.0 ± 0.00
CBQ-EA-29	17.7 ± 0.31	0.0 ± 0.00
CBQ-EBa-1	0.0 ± 0.00	0.0 ± 0.00
CBQ-EB-5	0.0 ± 0.00	0.0 ± 0.00
CBQ-EBa-22	0.0 ± 0.00	44.3 ± 1.40
CBQ-EBe-3	15.0 ± 0.12	0.0 ± 0.00
CBQ-EBe-15	0.0 ± 0.00	0.0 ± 0.00
CBQ-EBe-16	0.0 ± 0.00	0.0 ± 0.00
CBQ-EBe-19	16.1 ± 0.49	0.0 ± 0.00
CBQ-EBe-20	0.0 ± 0.00	0.0 ± 0.00
CBQ-EC-5	20.0 ± 0.78	53.2 ± 0.98
HSD <sub>0.05</sub>	6.7	8.5

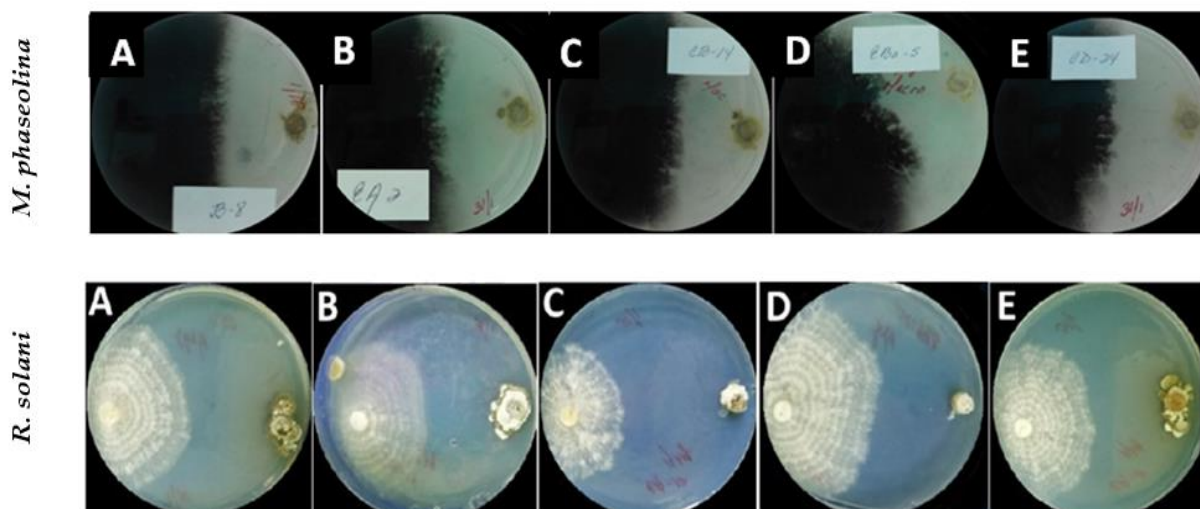
<sup>a</sup>Mycelial Growth Inhibition (MGI; %) for *Macrophomina phaseolina* isolate CCIBP-Mp1 and *Rhizoctonia solani* isolate CCIBP-Rh1 were obtained by dual culture assays on PDA at 30 °C for 7 days in darkness. Data represent the average of twelve Petri dishes for each BCA or control ± the standard error of the means.

<sup>b</sup>For each pathogen, significant differences between treatment means of MGI are given by a critical value for means comparison [HSD<sub>0.05</sub> = 6.7 and 8.5% for *M. phaseolina* and *R. solani*, respectively] according to Tukey's honestly significant difference (HSD) tests at *P* = 0.05.

### 3.4.2. Qualitative evaluation of enzyme activities of actinobacterial strains

There were significant differences between the 31 actinobacterial strains evaluated for their chitinolytic, cellulolytic or proteolytic activity (*P* < 0.0001) (Table 3.3). Twenty out of the 31 strains evaluated showed chitinolytic halo, which ranged from 25.3 ± 0.96 to 33.5 ± 1.91 mm for CBQ-CD-24 to CBQ-EBa-5, respectively. Concerning the cellulolytic activity, the cellulolytic halo ranged between 90.0 ± 0.41 (CBQ-B-8; -CB-14; -ECa-24; -ESFe-12; -Ni32; -Plat-2; -Plat-3; -Plat-4; and -WP-14) and 36.3 ± 0.75 mm (CBQ-EA-3). Only three out of the 30 strains evaluated did not show cellulolytic halo (CBQ-EB-27; -EC-18; -OSS-4).

Finally, 21 out of the 31 strains evaluated showed proteolytic halo, which ranged from  $51.5 \pm 1.50$  to  $27.0 \pm 0.71$  mm for CBQ-EA-12 to CBQ-ECa-24, respectively (Table 3.3).



**Figure 3.1.** Antagonistic effect of *Streptomyces* strains against *Macrophomina phaseolina* isolate CCIBPMp1 (top row photos) and *Rhizoctonia solani* isolate CCIBP-Rh1 (bottom row photos) growing in dual culture on PDA at 7 days after inoculation and incubated at 28 °C in the dark. *Streptomyces* strains evaluated were: (A) CBQ-B-8, (B) CBQ-EA-2, (C) CBQ-CB-14, (D) CBQ-EBa-5, and (E) CBQCD-24 (top row photos); and (A) CBQ-B-8, (B) CBQ-CB-14, (C) CBQ-EA-12, (D) CBQ-EBa-21, and (E) CBQ-EA-2 (bottom row photos).

**Table 3.3.** Chitinolytic, cellulolytic and proteolytic activity of the 31 actinobacterial strains selected for these experiments.

Actinobacterial Strain	Chitinolytic Halo (mm) <sup>a</sup>	Cellulolytic Halo (mm) <sup>b</sup>	Proteolytic Halo (mm) <sup>c</sup>
CBQ-B-8	32.8 ± 0.96 ab	90.0 ± 0.41 a	41.3 ± 0.48 abcd
CBQ-CB-3	0.0 ± 0.0 c	49.3 ± 0.48 ab	0.0 ± 0.0 e
CBQ-CB-14	32.5 ± 2.65 ab	90.0 ± 0.41 a	50.8 ± 2.53 abc
CBQ-CD-24	25.3 ± 0.96 b	63.0 ± 2.38 ab	34.5 ± 0.50 abcd
CBQ-EA-2	34.0 ± 1.41 a	86.3 ± 0.48 ab	44.8 ± 1.65 abcd
CBQ-EA-3	31.3 ± 0.96 ab	36.3 ± 0.75 b	31.8 ± 0.75 bcd
CBQ-CB-4	0.0 ± 0.0 c	86.3 ± 0.48 ab	0.0 ± 0.0 e
CBQ-EA-12	31.3 ± 3.20 ab	80.0 ± 0.71 ab	51.5 ± 1.50 a
CBQ-EBa-5	33.5 ± 1.91 a	85.3 ± 1.18 ab	42.3 ± 0.25 abcd
CBQ-EB-27	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 e
CBQ-EBa-21	29.8 ± 1.26 ab	68.5 ± 1.19 ab	47.8 ± 1.4 abc
CBQ-EC-3	0.0 ± 0.0 c	40.0 ± 0.71 ab	0.0 ± 0.0 e
CBQ-EC-18	29.3 ± 0.96 ab	0.0 ± 0.0 c	0.0 ± 0.0 e
CBQ-ECa-24	0.0 ± 0.0 c	90.0 ± 0.41 a	27.0 ± 0.71 d
CBQ-ESFe-4	24.8 ± 0.50 b	82.3 ± 2.59 ab	0.0 ± 0.0 e
CBQ-ESFe-5	0.0 ± 0.0 c	88.8 ± 0.75 ab	41.3 ± 2.39 abcd
CBQ-ESFe-10	31.3 ± 0.96 ab	81.8 ± 1.80 ab	0.0 ± 0.0 e
CBQ-ESFe-11	32.3 ± 2.90 ab	84.5 ± 1.55 ab	0.0 ± 0.0 e
CBQ-ESFe-12	29.0 ± 1.41 ab	90.0 ± 0.41 a	49.0 ± 0.71 ab
CBQ-J-4	27.0 ± 1.41 ab	45.0 ± 1.41 ab	27.3 ± 0.48 d
CBQ-Mg-6	0.0 ± 0.0 c	62.0 ± 0.71 ab	44.8 ± 0.48 abcd
CBQ-Ni-24	27.3 ± 0.96 ab	88.8 ± 0.75 ab	31.8 ± 0.25 cd
CBQ-Ni-32	29.8 ± 0.50 ab	90.0 ± 0.41 a	37.3 ± 0.48 abcd
CBQ-OSS-3	33.0 ± 4.8 ab	67.0 ± 0.71 ab	39.3 ± 0.48 abcd
CBQ-OSS-4	0.0 ± 0.0 c	0.0 ± 0.0 c	44.0 ± 0.71 abcd
CBQ-Plat-2	29.8 ± 0.50 ab	90.0 ± 0.41 a	44.3 ± 0.75 abcd
CBQ-Plat-3	0.0 ± 0.0 c	90.0 ± 0.41 a	32.8 ± 0.25 abcd
CBQ-Plat-4	31.3 ± 0.96 ab	90.0 ± 0.41 a	33.5 ± 0.50 abcd
CBQ-SFe-5	28.3 ± 3.36 ab	47.3 ± 0.75 ab	0.0 ± 0.0 e
CBQ-Wni-21	0.0 ± 0.0 c	42.3 ± 0.48 ab	36.5 ± 0.95 abcd
CBQ-WP-14	29.8 ± 0.50 ab	90.0 ± 0.41 a	44.0 ± 0.71 abc

<sup>a,b,c</sup> Halo develop (mm) for each actinobacterial strain grown onto chitin agar medium Colloidal, Yeast Extract Malt Extract Agar (ISP<sub>2</sub>) plates with cellulose (1%, w/v), and ISP<sub>2</sub> agar with 1% skimmed milk, respectively, at 28–30 °C in darkness for seven days. For each strain, data represent the average of twelve Petri dishes ± the standard error of the means. In each column, means followed by a common letter do not differ significantly according to Dunn's multiple comparisons for proportions test at *P* = 0.05.

### 3.4.3. Phenotypic characterization

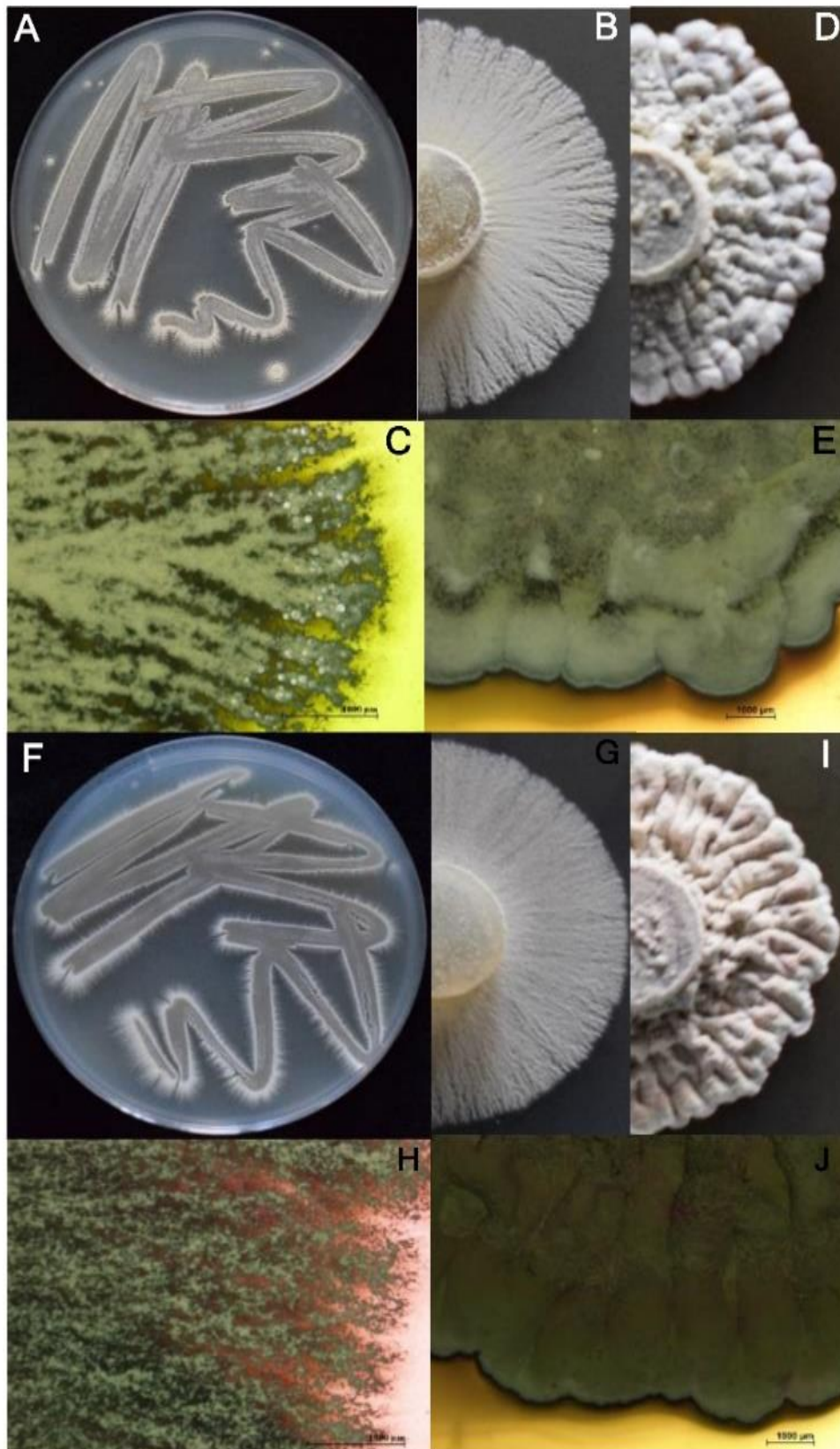
The macroscopic features of the 11 representative actinobacterial strains selected for this experiment are show in Table 3.4. In general, the colonies were mostly white in color, circular in shape, convex in elevation, with an entire edge, hard consistency and variable pigment production (Figure 3.2). Microscopic observation of Gram-stained bacterial cells showed stable branched mycelium bearing aerial hyphae, which

differentiate into short or long spore chains. Microscopic characterization using the microculture technique revealed details of aerial and vegetative mycelium, mycelial fragmentation and clustering of spores. A spiral arrangement of spores was observed on most of the microculture slides of each sample. In addition, all the strains were characterized as Gram-positive suggesting that they belong to the genus *Streptomyces*.

**Table 3.4.** Macroscopic characteristics of colonies of 11 actinobacterial strains (*Streptomyces* spp.) grown on Casein Starch Agar at 28°C in darkness for 10 days\*.

Actinobacterial Strain Code	Gram Stain <sup>a</sup>	Aerial Mycelium <sup>b</sup>	Color	Shape	Elevation	Edge	Consistency	Pigment
CBQ-B-8	+	+	White	Circular	Convex	Full	Hard	Yellow
CBQ-CB-14	+	+	White	Circular	Convex	Full	Hard	Yellow
CBQ-CD-24	+	+	White	Irregular	Convex	Whole	Hard	Beige
CBQ-EA-2	+	+	White	Circular	Convex	Lobed	Hard	Yellow
CBQ-EA-12	+	+	White	Circular	Pulvini	Whole	Hard	Brown
CBQ-EBa-5	+	+	Yellow	Irregular	Convex	Lobular	Hard	Yellow
CBQ-EBa-21	+	+	White	Irregular	Pulvini	Whole	Hard	Orange
CBQ-ESFe-4	+	+	Yellow	Circular	Convex	Lobed	Hard	Beige
CBQ-J-4	+	+	White	Circular	Convex	Whole	Hard	Beige
CBQ-OSS-3	+	+	Yellow	Irregular	Convex	Lobular	Hard	Yellow
CBQ-Plat-2	+	+	White	Circular	Convex	Whole	Hard	Yellow

<sup>a</sup>(+): actinobacteria G+. <sup>b</sup>(+): Presence of aerial mycelium. \* The phenotypic characteristics of the colonies of the actinobacterial strains were selected according with (Shirling and Gottlieb, 1966; Bergey, 2005).



**Figure 3.2.** Two-weeks-old colonies of *Streptomyces* strains CBQ-B-8 (A–E), and CBQ-EA-2 (F–J) growing on ACA medium (A–C, F–H) and on PDA medium (D, E, I, J) at 28°C in the dark.

## 3.4.4. Biochemical characterization and assimilation of carbon sources

None of the eleven strains under study were positive for indole production and the Voges Proskauer test. Strains CBQ-J-4, -OSS-3, -EA-2 and -EBa-5, were positive for casein hydrolysis; and the latter two strains were also able to be positive for the methyl red test, in addition to strains CBQ-B-8, -CB-14, -EBa-21 and -Plat-2. Only the strains CBQ-EA-12 and -ESFe-4 did not hydrolyse gelatine. The strains CBQ-OSS-3 and -Plat-2 did not hydrolyse starch (Table 3.5).

**Table 5.** Biochemical test results of the 11 actinobacterial strains selected for this experiment.

Biochemical Parameters *	Actinobacterial Strain Code										
	CBQ-B-8	CBQ-CB-14	CBQ-CD-24	CBQ-EA-2	CBQ-EA-12	CBQ-EBa-5	CBQ-EBa-21	CBQ-ESFe-4	CBQ-J-4	CBQ-OSS-3	CBQ-Plat-2
Catalase production	+	+	+	+	+	+	+	+	+	+	+
Lactose Fermentation	+	+	+	+	-	-	-	+	-	+	-
Glucose Fermentation	+	+	+	+	+	D	-	-	+	D	+
Mannitol Fermentation	+	+	-	+	-	+	-	+	-	-	-
Dextrose Fermentation	-	-	+	+	D	-	D	+	-	-	+
Fructose Fermentation	+	+	+	+	+	+	+	+	-	-	+
Maltose Fermentation	+	+	+	+	+	+	+	D	+	+	-
Sucrose Fermentation	+	+	+	+	+	+	+	D	+	-	-
Xylose Fermentation	+	+	-	+	+	-	+	+	-	+	+
Raffinose Fermentation	+	+	+	D	-	-	+	+	+	+	D
Casein Hydrolysis	-	-	-	+	-	+	-	-	+	+	-
Citrate Utilization	+	+	+	+	+	+	+	+	+	+	+
Urea Hydrolysis	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+
Indole production	-	-	-	-	-	-	-	-	-	-	-
Methyl red	+	+	-	+	-	+	+	-	-	-	+
Voges Proskauer	-	-	-	-	-	-	-	-	-	-	-
Gelatin hydrolysis	+	+	+	+	-	+	+	-	+	+	+
Starch hydrolysis	+	+	+	+	+	+	+	+	+	-	-

\* (+): Positive reaction; (-): Negative reaction; (D): Dubious.

On the other hand, all the evaluated strains were positive for catalase citrate utilization, nitrate reduction and urea hydrolysis. Variability between strains was also observed for the assimilation and utilization of carbohydrates (Table 3.5).

## 3.4.5. Molecular characterization

BLASTn searches on GenBank showed that the 16S rDNA sequences of the strains CBQ-EA-2 and CBQ-B-8 had 99.71 and 99.93% identity with strains of

*Streptomyces* sp. HBUM206419 (MT540570) and MP47-91 (EU263063), respectively. The sequences logged in GenBank and Blast results of the two representative actinobacterial strains selected for their highest effectiveness *in vitro* in this study are shown in Table 3.6.

**Table 3.6.** Identification by sequencing the 16S rDNA gene of the two actinobacterial strains selected for molecular characterization with their corresponding GenBank accession numbers and data of Blast results obtained from GenBank.

Species	Isolate	Genbank Accession <sup>a</sup>	Blast Accession <sup>b</sup>	Query Length	Gaps <sup>c</sup>	Identities <sup>d</sup>	Maximum Identity (%)
<i>Streptomyces</i> sp.	CBQ-EA-2	OM417233	MT540570	1437	3/1390	1386/1390	99.71
<i>Streptomyces</i> sp.	CBQ-B-8	OM417234	EU263063	1491	1/1491	1490/1491	99.93

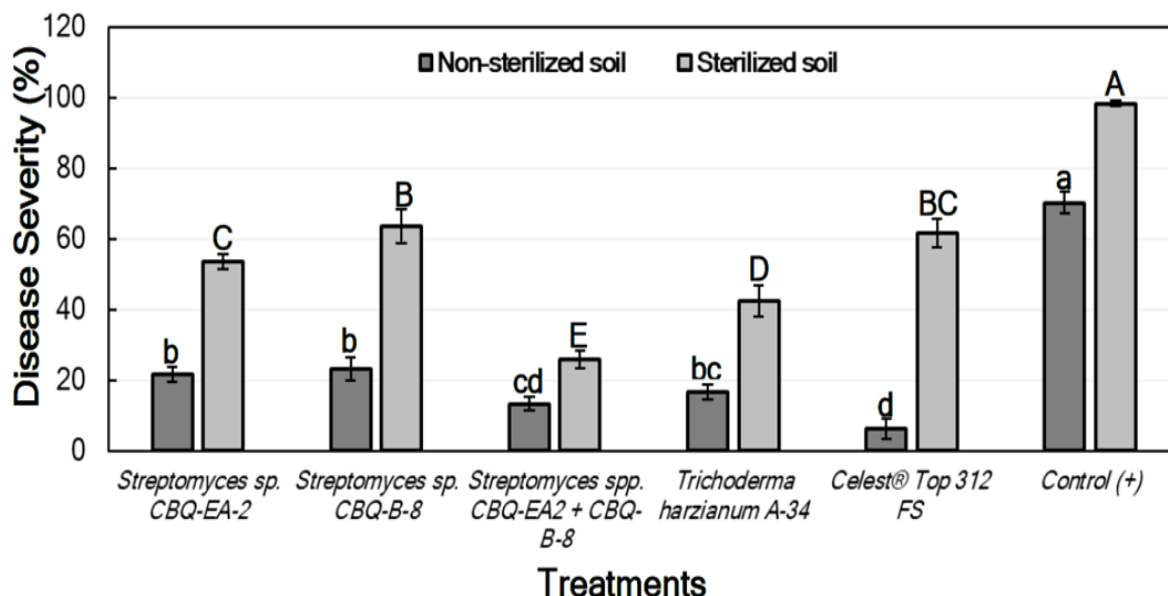
<sup>a</sup> Corresponding GenBank accession numbers of our isolates. <sup>b</sup> GenBank accession numbers blasted with the isolates obtained in this study. <sup>c</sup> Number of spaces introduced into the alignment to compensate for insertions and deletions in our sequence relative to blasted sequences. <sup>d</sup> Number of nucleotides of our sequences/Number of nucleotides of blasted sequences.

#### 3.4.6. Effect of actinobacterial strains against *Macrophomina phaseolina* and *Rhizoctonia solani* infections in planta

Because significant differences between sterilized and non-sterilized soils, treatments, and their interaction ( $P \leq 0.0001$  in all cases) were observed on their effect on total Disease Severity (DS) (for seedlings inoculated with *M. phaseolina*) and on DSstem and DSroot (for seedlings inoculated with *R. solani*), individual ANOVA per each type of soil was conducted to evaluate the effect of treatment on DS of each tissue.

##### 3.4.6.1. Effect of treatments against *Macrophomina phaseolina* in planta

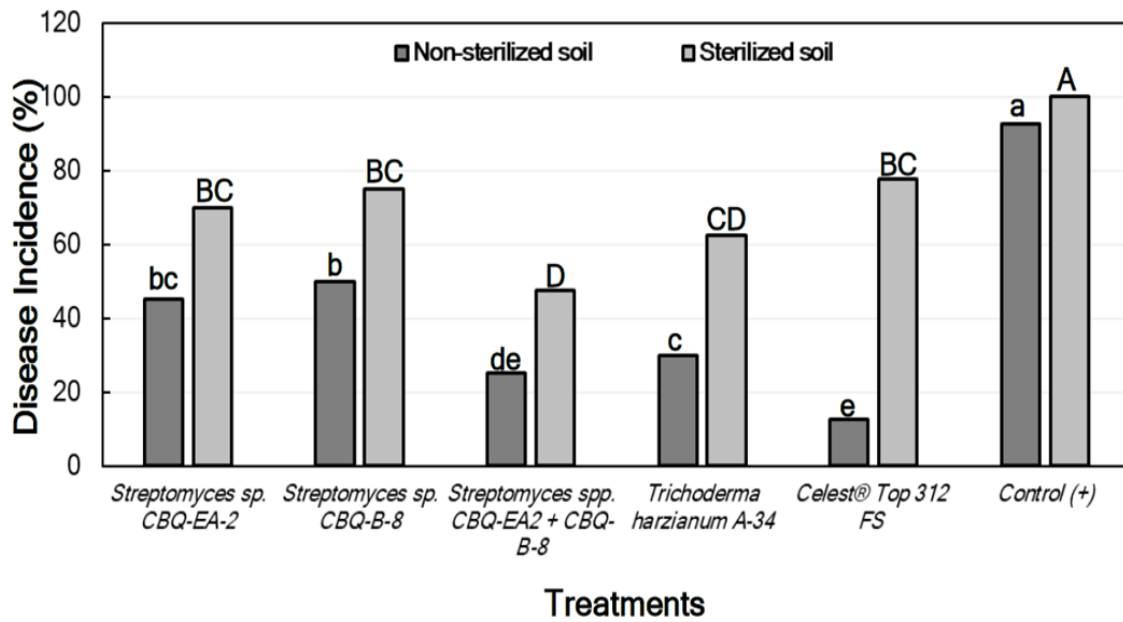
For the treatments conducted with seedlings grown in non-sterilized soil, significant differences between treatments were observed for their effect on DS ( $P \leq 0.0001$ ). DS ranged from  $21.7 \pm 2.1$  to  $6.4 \pm 2.8\%$  for seedlings treated with *Streptomyces* sp. CBQ-EA-2 and Celest® Top 312 FS, respectively, with all treatments showing a significant effect on the disease progress in comparison with the non-treated and inoculated seedlings (positive control; DS =  $70.3 \pm 3.1\%$ ) (Figure 3.3).



**Figure 3.3.** Disease severity (%) in *Phaseolus vulgaris* cv. Quivicán seedlings treated with biological or chemical compounds and inoculated with *Macrophomina phaseolina* isolate CCIBP-Mp1 at 35 days growing on non-sterilized or sterilized soil. Each column represents the mean of 40 seedlings per soil and treatment combination. Columns with a common uppercase or lowercase letter do not differ significantly according to Fisher's protected LSD test ( $P = 0.05$ ) for treatments on non-sterilized or sterilized soil, respectively. Vertical bars are the standard errors of the means.

Concerning the treatments conducted with seedlings grown in sterilized soil, significant differences between treatments were also observed for their effect on DS ( $p \leq 0.0001$ ). In this case, all treatments also resulted in significant effectiveness compared to the positive control (DS =  $98.3 \pm 0.7\%$ ). DS among treated seedlings ranged from  $63.6 \pm 4.9$  to  $25.8 \pm 2.5$  for treatments with *Streptomyces sp.* CBQ-B-8 and *Streptomyces sp.* CBQ-EA-2+ CBQ-B-8, respectively (Figure 3.3).

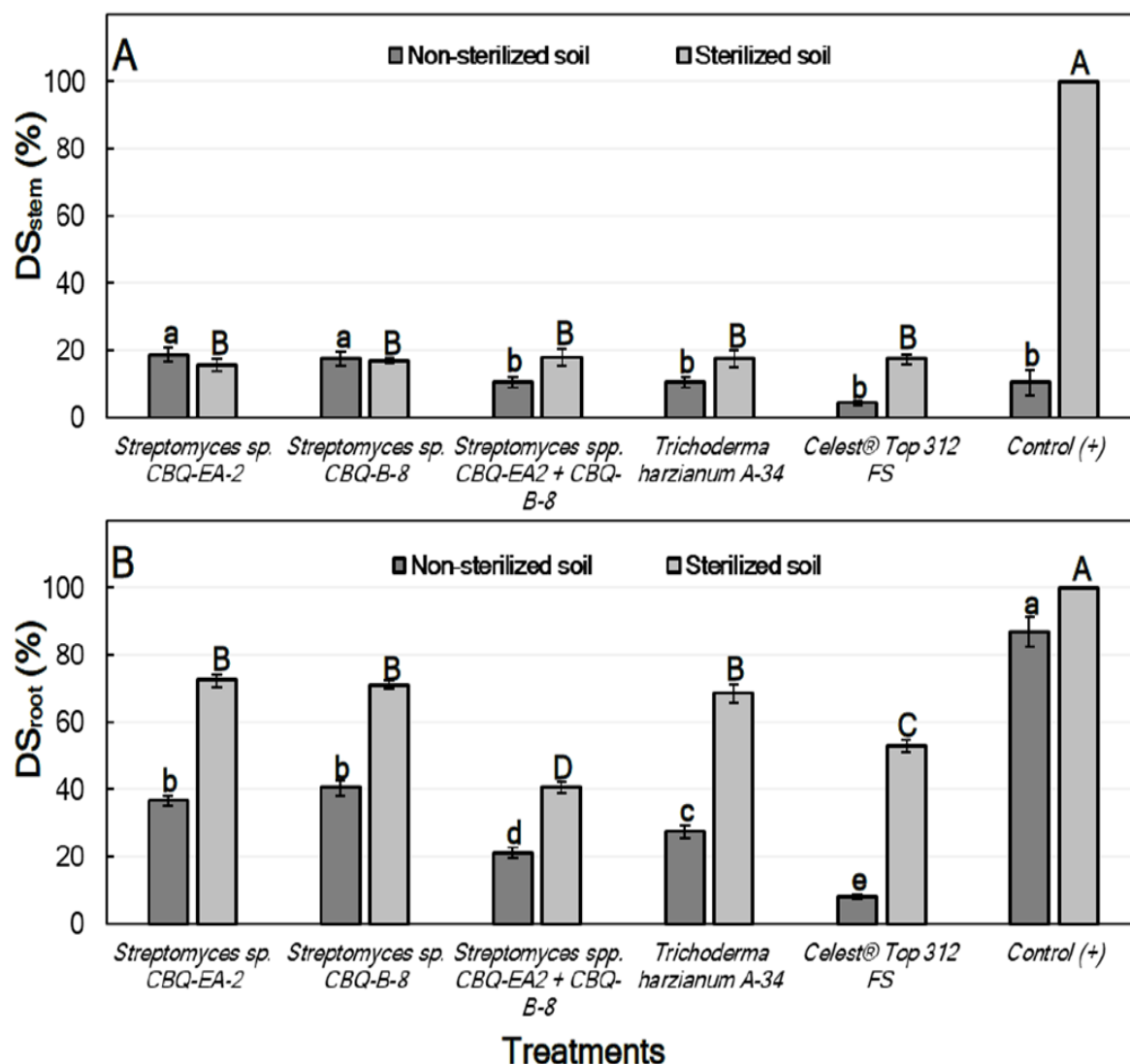
The Disease Incidence (DI) was markedly lower in treated seedlings grown in non-sterile soil than those grown in sterile soil. In both cases, not only there were significant differences in DI between treatments, but also significant differences were observed between all the treatments and the positive control ( $P \leq 0.0001$  in all cases), the latter always showing the highest DI values. In all cases, the treatments with *Streptomyces sp.* CBQ-EA-2 + CBQ-B-8, *T. harzianum* A-34, or Celest® Top 312 FS showed the lowest DI values (Figure 3.4). No seedling mortality was not observed in any case, except for the positive control grown in sterile soil which presented 100% mortality.



**Figure 3.4.** Disease incidence (DI, %) in *Phaseolus vulgaris* cv. Quivicán seedlings treated with biological or chemical compounds and inoculated with *Macrophomina phaseolina* isolate CCIBP-Mp1 at 35 days growing on non-sterilized or sterilized soil. Each column represents the mean of 40 seedlings per soil and treatment combination. Columns with a common uppercase or lowercase letter do not differ significantly according to Dunn’s multiple comparisons for proportions test ( $P = 0.05$ ) for treatments on non-sterilized or sterilized soil, respectively.

#### 3.4.6.2. Effect of treatments against *Rhizoctonia solani* in Planta

For treatments conducted with seedlings grown in non-sterilized soil, significant differences between treatments were observed for their effect on both DSstem ( $P = 0.0015$ ) and DSroot ( $P \leq 0.0001$ ). In all cases, DSstem was lower than DSroot, ranging from  $18.7 \pm 2.21$  to  $4.4 \pm 0.77\%$  for seedlings treated with *Streptomyces* sp. CBQ-EA-2 and Celest® Top 312 FS, respectively. But no important differences were observed for their effect on DSstem compared to the positive control (DSstem =  $10.6 \pm 3.78\%$ ). However, all the treatments showed significantly higher effectiveness on DSroot compared to the positive control (DSroot =  $86.8 \pm 4.38\%$ ). Treatments with *Streptomyces* sp. CBQ-EA-2 (DSroot =  $36.9 \pm 1.53\%$ ) or CBQ-B-8 (DSroot =  $40.6 \pm 2.21\%$ ) were the least effective, while the treatment that combined the two strains was highly effective (DSroot =  $21.2 \pm 1.53\%$ ) showing results similar to those observed for *T. harzianum* A-34 (DSroot =  $27.5 \pm 2.07\%$ ) (Figure 3.5).

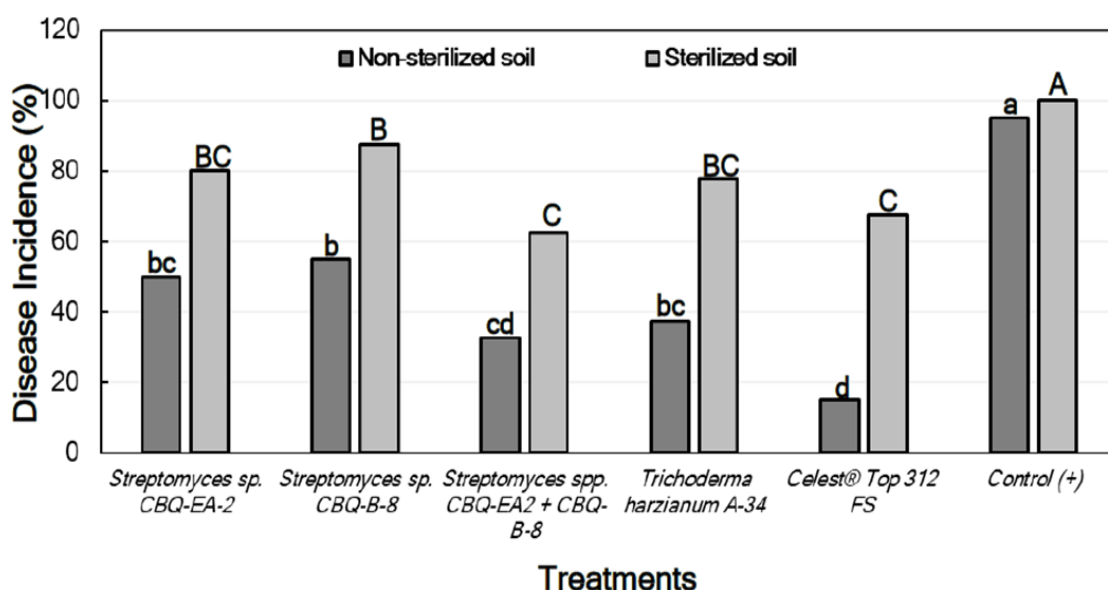


**Figure 3.5.** Disease severity (%) in stem (A; DS<sub>stem</sub>) and roots (B; DS<sub>root</sub>) of *Phaseolus vulgaris* of cv. Quivicán seedlings treated with biological or chemical compounds and inoculated with *Rhizoctonia solani* isolate CCIBP-Rh1 at 28 days growing on non-sterilized or sterilized soil. Each column represents the mean of 40 seedlings per soil and treatment combination. Columns with a common uppercase or lowercase letter do not differ significantly according to Fisher's protected LSD test ( $P = 0.05$ ) for treatments on non-sterilized or sterilized soil, respectively. Vertical bars are the standard errors of the means.

Regarding the treatments conducted with seedlings grown on sterilized soil, significant differences were also observed between treatments for their effect on both DS<sub>stem</sub> ( $P \leq 0.0001$ ) and DS<sub>root</sub> ( $P \leq 0.0001$ ). In this case, all the treatments were highly effective compared to the positive control (DS<sub>stem</sub> = 100%), but no significant differences in effectiveness between treatments were observed. DS<sub>stem</sub> ranged from  $18.1 \pm 2.50$  to  $15.6 \pm 1.71\%$  for treatments with *Streptomyces* sp. CBQ-EA-2 + CBQ-B-8, and

with *Streptomyces* sp. CBQ-EA-2, respectively. On the other hand, all the treatments also showed significantly lower DSroot values compared to the positive control (DSroot = 100%), but significant differences were also observed between treatments for their effect against the disease. The most effective treatment was *Streptomyces* sp. CBQ-EA-2+ CBQ-B-8 (DSroot = 40.6 ± 2.21%), and the least effective were *Streptomyces* sp. CBQ-EA-2 (DSroot = 72.5 ± 1.82%), *Streptomyces* sp. CBQ-B-8 (DSroot = 71.3 ± 1.17%) and *T. harzianum* A-34 (DSroot = 68.7 ± 2.62%) (Figure 3.5).

A pattern similar to that observed for seedlings inoculated with *M. phaseolina* was found for the effect of the treatments on the DI of seedlings inoculated with *R. solani*, with significant differences in DI being between all treatments and the positive control ( $P \leq 0.0001$  in all cases). In all cases, the treatment with *Streptomyces* sp. CBQ-EA-2 + CBQ-B-8 showed significantly less DI than the treatments with *Streptomyces* sp. CBQ-EA-2 or *Streptomyces* sp. CBQ-B-8, and also showed DI values similar to those observed for *T. harzianum* A-34 and Celest® Top 312 FS (Figure 3.6).



**Figure 3.6.** Disease incidence (DI, %) in *Phaseolus vulgaris* cv. Quivicán seedlings treated with biological or chemical compounds and inoculated with *Rhizoctonia solani* isolate CCIBP-Rh1 at 28 days growing on non-sterilized or sterilized soil. Each column represents the mean of 40 seedlings per soil and treatment combination; and columns with a common uppercase or lowercase letter do not differ significantly according to Zar's multiple comparisons for proportions test ( $P = 0.05$ ) for treatments on non-sterilized or sterilized soil, respectively.

Finally, for both lots of plants growing in non-sterilized and sterilized soil, the linear correlation analysis showed that there was not significant linear correlation between DSstem and DSroot (non-sterilized soil:  $r = 0.2869$ ;  $P = 0.5815$ ; sterilized soil:

$r = 0.7739$ ;  $P = 0.0709$ ), and DSstem and DI (non-sterilized soil:  $r = 0.3826$ ;  $P = 0.4541$ ; sterilized soil:  $r = 0.7409$ ;  $P = 0.0920$ ). Nevertheless, a significant positive linear correlation was observed between DSroot and DI in both non-sterilized soil ( $r = 0.9944$ ;  $P = 0.0001$ ) and sterilized soil ( $r = 0.9710$ ;  $P = 0.0013$ ).

### 3.5 DISCUSSION

Actinobacteria (*Streptomyces* spp. mainly) have been reported as potential BCAs against soil-borne pathogens of legumes during the last decade (Vurukonda *et al.*, 2021). However, the use of actinobacteria as BCAs in the frame of the integrated management of the major diseases of common bean caused by soil-borne pathogens in Cuba has not been explored yet. Therefore, this study aimed to characterize a collection of 60 actinobacterial strains from Cuba based on their *in vitro* effectiveness against the two main soil-borne pathogens of common bean in Cuba, as well as on their phenotypic and biochemical characteristics.

All the selected actinobacteria formed a smooth surface colony in CAS, becoming white to beige, hard and compact with age, varying in pigmentation, powdery or velvety appearance as a result of the formation of short and long chains of spores, with typical of wet soil (Figure 3.2). In the totality of the microcultures a spiral arrangement of the spores was observed. Similar results were obtained by (Ayuningrum and Jati, 2021), whom reported that isolates of actinobacterias forming powdery colonies with well-developed aerial hyphae divided into spore chains were termed *Streptomyces*-like actinomycete bacteria.

This fact together with the concordance of the morphological characters of our strains with those described by (Bergey *et al.*, 2005), for the *Streptomyces* genus, indicate that all of our actinobacteria strains belong to this genus. In addition, our *Streptomyces* strains showed high levels of cellulolytic and proteolytic activity. Our results are also in concordance with those previously obtained by several authors, who reported the ability of *Streptomyces* strains to produce high levels cellulase and protease (Manigundan *et al.*, 2020). For instance, 62% of our *Streptomyces* strains revealed a high cellulolytic capacity with a halo between 80 to 90 mm in diameter, and 90% of them developed a halo with considerable extension around the colony, which denotes an important cellulolytic hydrolysis. Similar results were recently obtained by (Rani *et al.*, 2021), who reported that the 67.5 and 60.0% of the *Streptomyces* isolates of their collection showed cellulolytic and proteolytic activity, respectively. Furthermore, the 66.7% of our

*Streptomyces* strains showed chitinolytic capacity, highlighting the CBQ-EBa-5 strain, with a 35.5 mm clearance halo surrounding the colony. These results are also in agreement with those obtained by (Liu *et al.*, 2016), who showed that *S. hydrogenans* (SSD60) and *S. spororaveus* (SDL15) had strong chitinolytic activity, and the 24% of the *Streptomyces* strains of their collection (n = 94) developed a clear halo surrounding the colony when evaluating their chitinolytic activity. Altogether, it not only confirms that our strains are well identified as *Streptomyces*, but also suggests that the actinobacteria form one of the most important microbial communities in soil rehabilitation and conservation, as they are largely responsible for their ability to produce extracellular cellulolytic, chitinolytic and proteolytic enzymes.

Actinobacteria represent a source of biologically active secondary metabolites, including enzymes (Selim *et al.*, 2021). In this study, we achieved specific qualitative metabolic characterization such as enzymatic, biochemical, morphological and antagonistic of at least 11 strains, which is the main criterion for determining their environmental role and their action in biogeochemical cycles. The challenge of our future research has its origins in this study, so evaluating the *in vitro* antagonistic activity of our strains showed that many of them disseminate secondary metabolites in the same culture medium in which they inhibit the growth of *M. phaseolina* and *R. solani*. After having evaluated the enzymatic activities, we could infer that the production of chitinases has a positive effect in this regard, since chitin is one of the major components of the fungal cell wall. In addition, actinobacteria combine with other soil microorganisms in their natural environment to decompose resistant plant debris, such as cellulose, as well as animal debris to maintain the biotic balance of the soil by cooperating with the nutrient cycle (Bhatti *et al.*, 2017). Although we were able to identify well all of our actinobacterial strains as *Streptomyces* spp. based on their phenotypic and biochemical characters, the identity of the two representative strains that showed that highest effectiveness on MGI *in vitro* in this study (CBQ-EA-2, and -B-8) was confirmed by sequencing the 16S rRNA gene using the universal primers 27f and 1492r for eubacteria. The consensus sequences obtained were blasted in GenBank and they match with more than 99% of maximum identity with reference sequences from *Streptomyces* spp. According to (Law *et al.*, 2018), the 16S rRNA gene has been extensively studied with proven sensitivity for taxonomic and phylogenetic identification of most bacteria including actinobacteria such as *Streptomyces* spp.

Regarding the *in vitro* efficacy of our 60 *Streptomyces* potential strains against *M. phaseolina* and *R. solani*, it varied depending on the soil-borne pathogen tested. It is worth mentioning that 40 and 25 out of the 60 actinobacterial strains inhibited the mycelial growth of *M. phaseolina* and *R. solani*, respectively. Among the most effective strains, 18 of them showed a common effect against both pathogens, with the CQB-EA2, and -CD-24 being among the strains that showed greater efficacy in inhibiting mycelial growth of the two pathogens. Our results are similar than those described by (Dalal *et al.*, 2014), who evaluated *in vitro* the antagonistic activity of 15 strains of actinobacteria against various soil-borne soybean pathogens. These authors reported that the 15 strains showed some effectiveness in inhibiting the mycelial growth of *R. solani*, and six of the 15 strains were also able to inhibit mycelial growth of *M. phaseolina* (Bhatti *et al.*, 2017). Similarly, Singh *et al.* (2016) evaluated the antifungal activity of 80 strains of actinobacteria against *C. truncatum*, *F. oxysporum*, *M. phaseolina*, and *S. rolfsii*, highlighting the greater efficacy of *Streptomyces* sp. strain ACITM-1 on inhibition of mycelial growth of all pathogens. In addition to these, several *Streptomyces* sp. strains has also been reported for their high efficacy in inhibiting the mycelial growth of soil-borne pathogenic fungi, such as *R. solani* (Fatmawati *et al.*, 2020), *R. bataticola* (Khendkar *et al.*, 2018), *M. phaseolina* (Manigundan *et al.*, 2020), *F. oxysporum*, *Alternaria* sp., and *Magnaporthe oryzae* (Manigundan *et al.*, 2020).

Finally, seed treatments with *Streptomyces* sp. CBQ-EA-2 and -B-8 were evaluated separately and in combination against infections by *M. phaseolina* and *R. solani* in inoculated seedlings of common bean under semi-controlled conditions. In general, the treatments conducted using a mix of the two *Streptomyces* sp. strains (CBQ-EA-2 + -B-8) showed a significant greater effectiveness against both pathogens compared to treatments performed with the two strains alone. In addition, the effectiveness of the two combined *Streptomyces* strains in controlling the disease was similar to that observed for the other comparative treatments such as *T. harzianum* A-34 or the chemical (Celest® Top312FS). Interestingly, the DS was higher in seedlings grown in sterilized soils than in those grown in non-sterilized soils, also varying the effectiveness of the different treatments with the soil used. It suggests that the microbiota of the soil is in active and positive interaction with the plant and the pathogen, making difficult the pathogen infection and development. Further research to evaluate the effect of the microbiota of

the soils used in this study on the biology of both *M. phaseolina* and *R. solani* should be conducted to determine the potential plant-soilpathogen interactions.

Our results are in concordance with those reported by Yadav *et al.*, 2014, who showed that *Streptomyces* sp. S160 reduced the incidence of charcoal rot caused by *M. phaseolina* under greenhouse conditions in chickpea by 33.3% relative to the control. Similarly, Alekhya *et al.* (2016) found that *Streptomyces* sp. (BCA-546 and CAI-8) significantly reduced charcoal rot in sorghum caused by *M. phaseolina* under semi-controlled conditions. On the other hand, our results are also in correspondence with those reported by (Korayem *et al.* (2020) who evaluated the biological activity of *S. parvulus* strain 10d against *R. solani* on green beans in a semi-controlled trial with sterilized and non-sterilized soil. These authors showed that seedlings plants treated with a spore suspension of *S. parvulus* strain 10 d showed the highest survival rate (88%) and the lowest DSroot (28%) in the whole of the experiment, showing much better results than those observed for seedlings treated with specific chemicals such as Rhizolex® (Yadav *et al.*, 2014). Similarly, Fatmawati *et al.* (2020) evaluated 10 strains of actinobacteria against *R. solani* on soybean seeds under controlled conditions, with *Streptomyces* spp. strain ASR53 showing the best results in suppressing damping-off disease by 68% and 91% in sterile soil and non-sterile soil, respectively.

This study represents the first report evaluating the effect actinobacteria against the main soil-borne pathogenic fungi of common bean in Cuba. It also shows that *Streptomyces* spp. should be considered as possible biocontrol alternatives against soil-borne pathogens, not only for their effectiveness in disease control, but also for their role in soil preservation which is highly recommended in the frame of sustainable agriculture. Due to the conclusions of this study are based on experiments under controlled conditions, the most effective *Streptomyces* strains of this study may be evaluated against the disease under natural field conditions in the future. Altogether will help us to develop potential BCAs for the control of *M. phaseolina* and *R. solani* associated with stem and root-rot diseases of common bean in Cuba.

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## *Chapter 4*





## **Biocontrol of root rot complex disease of *Phaseolus vulgaris* by *Streptomyces* sp. strains in the field**

### **ABSTRACT**

Soil-borne pathogens associated with root rot complex diseases cause major yield losses in common bean (*Phaseolus vulgaris* L.) worldwide. The lack of active ingredients available against these pathogens makes disease control difficult. Seed treatments with *Streptomyces* spp. have been described as a potential ecofriendly strategy against this complex disease. Here, we evaluate the effect of *Streptomyces* sp. strains CBQ-EA2 and CBQ-B-8 as biological control agents (BCAs) against root rot complex disease of *P. vulgaris*, mainly caused by *Macrophomina phaseolina* and *Rhizoctonia solani* under natural field conditions in Cuba. To this end, seed treatments with *Streptomyces* sp. strains CBQ-EA2 and CBQ-B-8, using single or mixed applications, were examined. Seed treatments with the BCA *Trichoderma harzianum* Rifai A-34 and the chemical Celest® Top 312 FS were included for comparison. In all experimental fields, treatments with *Streptomyces* spp. had significantly higher efficacy in reducing disease incidence (DI) and disease severity (DS) than the chemical Celest® Top 312 FS, and a similar effect as that by the BCA *T. harzianum* A-34. Most of the treatments evaluated showed higher germination, quality and yield of legumes than the nontreated control. Seed treatments using the combination of the two actinobacterial strains (*Streptomyces* sp. CBQ-EA-2+CBQ-B-8) showed higher effectiveness in reducing DI and DS, and enhancing germination, yield and quality of legumes compared with the nontreated control, with a similar effect as that with the BCA *T. harzianum* A-34 and the chemical Celest® Top 312 FS. Overall, microbial consortia deserve further research in the future to develop potential biological products for crop protection.

**KEYWORDS:** Actinobacteria, biological control, common bean, root diseases, soil-borne pathogens.

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#### 4.1. INTRODUCTION

Among legumes with edible seeds, the common bean (*Phaseolus vulgaris* L.) is one of the most consumed legumes worldwide due to its important components of proteins, carbohydrates, vitamins, vegetable fiber and minerals (Kiptoo *et al.*, 2016). Indeed, the cultivated surface of *P. vulgaris* worldwide is 15 million hectares, with an annual production of 31 million metric tons (FAO, 2021). In particular, common bean together with rice (*Oryza sativa* L.) comprises the basis of the Cuban diet, with both crops being an important source of income for small-scale producers. Annual production of common bean in Cuba is 136,570 t, with yields ranging from 1.1 to 1.4 t ha<sup>-1</sup> (FAO, 2021).

Common bean production in tropical countries is severely affected by multiple diseases associated with soil-borne fungal pathogens, among which *Fusarium* spp., *Macrophomina phaseolina* (Tassi) Goid, or *Rhizoctonia solani* Kühn (Schmidt *et al.*, 2019) are highlighted. These pathogens cause important economic losses in common bean that can reach 80 to 100% yield losses not only because they are able to survive for a long period of time in the soil, but also due to the lack of effective management strategies (Chavarro-Mesa *et al.*, 2020; Díaz-Díaz *et al.*, 2022).

To date, there is not only a lack of effective and authorized active ingredients to control root rot diseases of bean but also an urgent demand for the implementation of eco-friendly farming systems. For these reasons, searching for biological alternatives to control these bean diseases by beneficial microorganisms may be considered one of the most promising alternatives for their integrated management (Pandey *et al.*, 2021; Díaz-Díaz *et al.*, 2022). In this way, a broad diversity of microorganisms including bacteria such as *Agrobacterium*, *Bacillus*, *Burkholderia*, *Pseudomonas* and *Rhizobium*, and fungi such as *Trichoderma* have already been evaluated under controlled conditions for their effectiveness against soil-borne pathogens such as those associated with root rot diseases of common bean (Eke *et al.*, 2019; Bedine *et al.*, 2020).

Interestingly, some of them have also been evaluated in the field with successful results. For example, Volpiano *et al.* (2018) evaluated *Rhizobium* strains against *Sclerotium rolfsii* Sacc. on common bean in the field, with the strains SEMIA 439 and 4088 reducing the disease incidence (DI) by 18.3 and 14.5% in comparison with the nontreated control, naturally infected by *S. rolfsii*. Eke *et al.* (2019) also showed that the combination of *Trichoderma harzianum* T8 and the AMF2 mycorrhizal consortium

achieves significant suppression of *F. solani* Fs4 root rot in common bean under greenhouse conditions.

In addition to the biological control agents (BCAs) described above, actinobacteria (mainly *Streptomyces* spp.) are currently considered another potential alternative for the biocontrol of plant diseases. Indeed, actinobacteria may be one of the main tactics in integrated crop management since they have a wide diversity of beneficial effects, including the following: *i*) increased availability of main plant mineral nutrients through production of extracellular enzymes, phytohormones siderophores and solubilization of phosphorus and potassium; *ii*) release of enzymes such as cellulases, xylanases, glucanases, lipases, chitinases and proteases; and *iii*) antagonistic activity against phytopathogens (Díaz-Díaz *et al.*, 2022; Mitra *et al.*, 2022).

In this regard, *Streptomyces* spp. result in the highest effectiveness as BCAs against soil-borne pathogens such as fungal species belonging to the genera *Rhizoctonia*, *Phytophthora*, *Fusarium*, and *Pythium* in legumes as well as other herbaceous crops (Vurukonda *et al.*, 2021). Interestingly, Díaz-Díaz *et al.* (2022) recently evaluated the effectiveness of 60 strains of *Streptomyces* against *R. solani* and *M. phaseolina* by *in vitro* dual culture assays, and 19 of these 60 strains were able to significantly inhibit mycelial growth of both fungi. According to their *in vitro* results, Díaz-Díaz *et al.* (2022) selected *Streptomyces* sp. strains CBQ-EA-2 and CBQ-B-8 as representative of the most effective strains. Subsequently, their effectiveness was evaluated *in planta* (*Phaseolus vulgaris* cv. Quivicán) under greenhouse conditions against *M. phaseolina* and *R. solani* with applications of the two *Streptomyces* sp. strains alone or in combination. Their effectiveness was also compared with two well-known effective controls, the BCA *T. harzianum* A-34 and the chemical Celest® Top 312 FS (Syngenta®; Basilea, Switzerland), with *Streptomyces* treatments showing effectiveness similar to that observed for both control measures (Díaz-Díaz *et al.*, 2022). However, the effectiveness of these two *Streptomyces* sp. strains has not yet been demonstrated under field conditions. Thus, the main objective of this study was to evaluate the biocontrol effect of *Streptomyces* sp. strains CBQ-EA-2 and CBQ-B-8, applied alone or in combination, against root rot diseases of common bean associated with *R. solani* and *M. phaseolina*, under natural field conditions. Additionally, treatments with the BCA *T. harzianum* strain A-34 and the chemical Celest® Top 312 FS were included for comparative purposes. By

this study, we validate our previous results under controlled conditions to contribute to the development of integrated disease management alternatives against fungal diseases.

## **4.2. MATERIALS AND METHODS**

### *4.2.1. Experimental field conditions and soil characteristics*

The experiments were carried out under commercial production conditions in two geographic areas of the Credit and Services Cooperative (CCS) "8 de octubre" (*Experimental Field 1*; UTM coordinates: 22°43'90.5"N-79°90'45.4"W), and the Basic Unit of Cooperative Production (UBPC) "Jesús Menéndez" (*Experimental Fields 2 and 3*; UTM coordinates: 22°30'02"N-79°54'45"W), both belonging to the Integral Agricultural Enterprise Valle del Yabú (Villa Clara, Cuba). *Experimental Field 1* was established on October 28<sup>th</sup>, 2018; *Experimental Fields 2 and 3* were established on November 6<sup>th</sup>, 2019. All the fields were previously cultivated with maize (*Zea mays* L.).

Prior to establishing the experiments, the physical and chemical characteristics of the soil of each experimental field were analyzed. To this end, soil samples were taken from each block at a depth of 20 cm in each experimental field, homogenized and processed (Berihun *et al.*, 2017). The analyses were carried out in the soil laboratory of the Centro de Investigaciones Agropecuarias (CIAP) of the Facultad de Ciencias Agropecuarias of the Universidad Central Marta Abreu de Las Villas (Cuba) UCLV. Microbiological analysis of the soil was carried out in the microbiology laboratory of the Centro de Bioactivos Químicos (CBQ) of UCLV.

Chemical analyses of the soil were determined as follows: pH (KCl) was estimated by means of the method described by Hesse (1971) using a soil solution ratio of 1:2.5; organic matter was determined as described by Walkley and Black (1934), by oxidation with potassium dichromate and concentrated sulfuric acid; and assimilable phosphorus was estimated following the method described by Olsen *et al.* (1954), and the results are expressed in mg of P per 100 g soil<sup>-1</sup>. All chemical analyses were carried out according to MINAG Ramal 279 Standard (NRAG 279, 1980). For physical analysis of the soil, stable aggregates and structure factors were determined following the methods described by Henin *et al.* (1958).

In addition, the microbiota of the soil samples was determined using the plate count methodology to estimate colony forming units (CFUs) by the serial dilution method

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(Dhingra and Sinclair, 1995). The following culture media were used: *i*) for bacteria, tryptone soy agar (TSA; BioCen, Bejucal, Mayabeque, Cuba), at  $10^{-8}$  dilution (Vieira and Nahas, 2005); *ii*) for fungi, Rose Bengal dichloro-chloramphenicol agar (DRBC; bacteriological agar 15 g, bacteriological peptone 5 g, chloramphenicol 0.1 g, dextrose 10 g, magnesium sulfate 0.5 g, potassium phosphate 1 g, Rose Bengal 0.05 g; for 1000 mL of distilled water) at  $10^{-5}$  dilution (Moubasher *et al.*, 2018); *iii*) for actinobacteria, casein starch agar (50 mg mL<sup>-1</sup> cycloheximide) (ACA; soluble starch 10 g, casein 0.3 g, KNO<sub>2</sub> 2 g, NaCl 2 g, K<sub>2</sub>HPO<sub>4</sub> 2 g, MgSO<sub>4</sub>-7H<sub>2</sub>O 0.05 g, CaCO<sub>3</sub> 0.02 g, FeSO<sub>4</sub>-7H<sub>2</sub>O 0.01 g, agar 18 g and distilled water 1 L, pH 7.2) at  $10^{-5}$  dilution (Vieira and Nahas, 2005); *iv*) for azotobacter, Ashby's agar (5 g glucose, 5 g mannitol, 0.1 g CaCl<sub>2</sub>-2H<sub>2</sub>O, 0.1 g MgSO<sub>4</sub>-7H<sub>2</sub>O, 5 mg Na<sub>2</sub>MoO<sub>4</sub>-2H<sub>2</sub>O, 0.9 g K<sub>2</sub>HPO<sub>4</sub>, 0.1 g KH<sub>2</sub>PO<sub>4</sub>, 0.01 g FeSO<sub>4</sub>-7H<sub>2</sub>O, 5 g CaCO<sub>3</sub>, 15 g agar in 1 L distilled water; pH 7.3) at  $10^{-4}$  dilution (Shahid *et al.*, 2019); and *v*) for phosphate solubilizing bacteria, NBRIP (National Botanical Research Institute Phosphate Medium; 10 g glucose, 5 g Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>, 5 g MgCl<sub>2</sub>-6H<sub>2</sub>O, 0.025 g of MgSO<sub>4</sub>-7H<sub>2</sub>O, 0.2 g of KCl, 0.1 g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and 0.05 g of bromothymol blue) (Mohamed *et al.*, 2019).

Finally, the following climatic variables were recovered monthly over the period of the experiments: average temperature (°C), rainfall (mm) and relative humidity (RH; %) All data were obtained from the meteorological station Yabú (78343; UTM coordinates: 22°27'44.8"N-79°59'29.0"W), located in the municipality of Santa Clara (Cuba).

### 4.2.2. Plant material

Common bean (*P. vulgaris* L.) seeds of cv. Quivicán (white testa) registered in the official list of commercial cultivars (MINAG, 2016) of the Seed-based Business Unit (UBS), Villa Clara (Cuba) were used. According to the Quality Certificate, the germination percentage was 95%, and this was verified before conducting experiments. For this purpose, seeds were superficially disinfected by serial washing by immersing them first in a 70% ethanol solution for 5 min, followed by a 1.5% sodium hypochlorite solution (Cl at 5 g L<sup>-1</sup>) for 15 min, with three washes in distilled water for 20 min and drying on sterile paper towels. Subsequently, the seeds were plated in a humid chamber at 100% RH at 30 °C in the dark until germination.

#### 4.2.3. Biological control agents and inoculum preparation

The actinobacteria strains *Streptomyces* sp. CBQ-EA-2 and CBQ-B-8 were selected for this study due to their high effectiveness *in vitro* against *M. phaseolina* and *R. solani* previously reported by Díaz-Díaz *et al.* (2022). The inocula of the two *Streptomyces* sp. strains for seed treatments (*see below*) was prepared in tryptone soy broth medium (TSB, BioCen, Bejucal, Mayabeque, Cuba) and adjusted to  $10^8$  CFUs mL<sup>-1</sup>, as described by Díaz-Díaz *et al.* (2022).

Additionally, the BCA *T. harzianum* strain A-34 was included for comparison (Instituto de Investigaciones de Sanidad Vegetal; INISAV, Havana, Cuba; Stefanova *et al.*, 2014). The inoculum of *T. harzianum* A-34 for seed treatment (*see below*) was prepared in potato dextrose broth (PDB; BioCen, Bejucal, Cuba) and adjusted to  $10^8$  conidia mL<sup>-1</sup>, as described by Díaz-Díaz *et al.* (2022).

#### 4.2.4. Seed treatments and growth conditions

The following five seed treatments were evaluated: *i*) *Streptomyces* sp. CBQ-EA-2 at  $10^8$  CFUs mL<sup>-1</sup>; *ii*) *Streptomyces* sp. CBQ-B-8 at  $10^8$  CFUs mL<sup>-1</sup>; *iii*) a mixture of *Streptomyces* sp. strains CBQ-EA-2 and CBQ-B-8 at  $10^8$  CFUs mL<sup>-1</sup> global concentration; *iv*) *T. harzianum* strain A-34 ( $10^8$  conidia mL<sup>-1</sup>); and the chemical Celest® (water suspension of 192 mL of active ingredient per kg of seeds). The last two treatments were included for comparative purposes. In addition, seeds coated with noninoculated zeolite were included as nontreated control seeds.

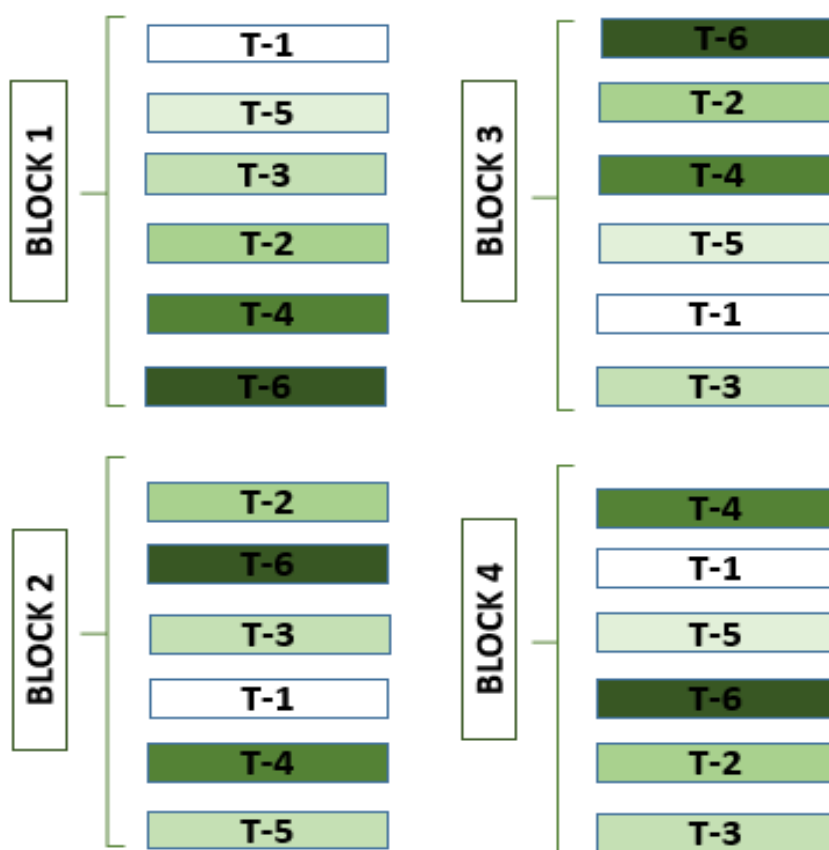
Seeds were treated by coating them with a mixture of sterilized zeolite and each of the treatments described above at the following proportion: 5 g of zeolite and 5.5 mL of the final CFUs or conidia concentration for each BCA or the dose for the chemical described above, with a seed/zeolite ratio of 7:1 (weight:weight) (Bunt *et al.* 2016). Zeolite was obtained from the quarry located in San Juan de los Yeras, municipality of Ranchuelo, Villa Clara (Cuba).

At 14 days after germination of cotyledons (DAGC), plants from the seeds treated with actinobacteria and *Trichoderma* were sprayed in the afternoon with a spore suspension at  $1.5 \times 10^6$  CFUs mL<sup>-1</sup> of the same BCA that was initially applied to the seeds; plants from seeds treated with Celest® and control plants were sprayed with water.

These foliar treatments were repeated biweekly until 44 DAGC. No additional chemical or biological products were applied.

#### 4.2.5. Experimental design

In all experimental fields, treatments were arranged in a randomized complete block design with 4 blocks and five treatments plus the control randomly distributed in each block (Fig. 4.1). For each block and treatment, the elementary plot consisted of four furrows 3 m long by 2.1 m wide, resulting in a total surface area of 6.3 m<sup>2</sup> (Faure *et al.*, 2014), with a plant spacing of 0.70 × 0.05 m and a plant density of 280,000 plants per ha<sup>-1</sup> (20 seeds per linear meter).



**Fig. 4.1.** Diagram of the experimental design used in all fields, in which the treatments were arranged in a randomized complete block design. The following treatments were evaluated: T1) *Streptomyces* sp. CBQ-EA-2; T2) *Streptomyces* sp. CBQ-B-8; T3) *Streptomyces* spp. CBQ-EA2 + CBQ-B-8; T4) *Trichoderma harzianum* A-34; T5) Celest® Top 312 FS; and T6) nontreated control (positive control).

#### 4.2.6. Assessment of disease-related parameters

In each experimental field, 20 plants per block and treatment were examined every 20 days from 10- to 69 DAGC to evaluate the presence of symptoms associated with natural infections of pathogens that cause root rot diseases. The percentage of germinated seeds (%) was assessed at 10 days after sowing (DAS), and the DI (% of affected plants), and the disease severity (DS; %) were estimated at 29, 49 and 69 DAGC. The DS was assessed by means of the following severity rating scale: 1 = no visible disease symptoms; 3 = slight discoloration without necrotic lesions or with 10% of hypocotyl and root tissues showing lesions; 5 = 25% of hypocotyl and root tissues showing lesions, with strong discoloration but with tissues remaining turgent; 7 = 50% of the hypocotyl and root tissues showing lesions, including soft, rot and severe reduction of the root system; and 9 =  $\geq 75\%$  of the hypocotyl and root tissues affected, showing severe rot and reduction of the root system (Navarrete *et al.*, 2009). Subsequently, a DS index was calculated for each evaluation moment by means of the following formula:

$$DS (\%) = \left[ \sum_{i=1}^9 n_i (st_i) / (N \times K) \right] \times 100$$

in which  $n_i$  = number of seedlings in DS development stage  $i$ ,  $st_i$  = value of the DS stage (1-9),  $N$  = total number of plants assessed, and  $K$  = largest scale level (9) (Townsend and Heuberger, 1943). Data of the DS index for each treatment and block were averaged, and data was used to calculate the area under the disease progress curve (AUDPC) using the following formula:

$$AUDPC = \sum_{i=1}^n [(DSindex_{i+1} + DSindex_i) / 2] \times (t_{i+1} \times t_i)$$

Where the DS index is the estimated DS index at each evaluation moment ( $i$ ),  $t_i$  is the time (days) at the evaluation moment, and  $n$  is the total number of evaluations (Campbell and Madden, 1990). AUDPC is expressed as a relative percentage (RAUDPC; %) to the disease parameter values of the plants treated with only water (nontreated control plants).

In each experimental field, ten plants per treatment showing symptoms of wilting, leaf chlorosis, stunting or yellowing and/or stem lesions were surveyed at random during the study period, carefully removed and transferred to the CBQ microbiology laboratory for fungal isolation. To this end, symptomatic roots were washed under running tap water, surface disinfected by dipping into a 10% (vol:vol) solution of commercial bleach (Cl at  $50 \text{ g L}^{-1}$ ) for 2 min, and then rinsed twice with sterile distilled water (Lee *et al.*, 2019).

Subsequently, four replicated fragments ( $\approx 1 \text{ mm}^3$ ) of infected root tissues per plant were separated and plated onto potato dextrose agar (PDA; BioCen, Bejucal, Mayabeque, Cuba) supplemented with  $30 \text{ mg L}^{-1}$  streptomycin sulfate (Globe Chemicals, Edo. México, México) to avoid bacterial contamination and incubated at  $22 \text{ }^\circ\text{C}$  in the dark for 10 days. All samples were single-spored using a serial dilution method (Dhingra and Sinclair, 1995) and identified by morphology (Watanabe, 2010).

#### 4.2.7. Assessment of yield and quality of legumes

At the end of the crop cycle, the total weight of seeds per treatment was obtained for each experimental field when seeds had 14% moisture content. Then, the total weight of the seeds in each replicate per treatment was estimated, and yield is expressed as tons per  $\text{ha}^{-1}$ . In addition, 30 plants per block and treatment were randomly selected to determine the number of legumes per plant (NLP), number of seeds per plant (NSP), and number of seeds per legume (NSL). Finally, from these same plants, the fresh weight of legumes per plant (g) and seeds per plant (g) was determined using a digital analytical balance (Kern FCB 30K1, KERN & SOHN, Balingen, Germany).

#### 4.2.8. Data analysis

Data for experimental field were evaluated separately. Before conducting analyses of variance (ANOVA), data were tested for normality and homogeneity of variances in all cases. Logarithmic transformation of the data was conducted when necessary. One-way ANOVA was conducted with DI, RAUDPC, germination (%), weight of seed per plant (g), yield ( $\text{t ha}^{-1}$ ), NLP, NSP or NSL as dependent variables and treatment as independent variables. Mean values were compared according to Fisher's protected LSD test at  $P = 0.05$  (Steel and Torrie, 1985). Pearson correlation coefficients ( $r$ ) between the DI or RAUDPC and germination (%), weight of seed per plant (g), yield ( $\text{t ha}^{-1}$ ), NLP, NSP or NSL were calculated using the average values of all variables for each treatment evaluated ( $n = 6$ ). All data analyses were conducted using Statistix 10 (Analytical Software, 2013).

### 4.3. RESULTS

#### 4.3.1. Experimental field conditions and soil characteristics

The physical and chemical characteristics of the soils used in this study are shown in (Table 4.1). Based on the chemical parameters evaluated, the pH- $\text{H}_2\text{O}$  ranged between 7.0 and 7.7, the phosphorus contents between 35.0 and 45.0  $\text{mg } 100 \text{ g soil}^{-1}$ , and the

potassium contents from 25.0 and 40.0 mg 100 g soil<sup>-1</sup>. Regarding physical parameters, the content of organic matter ranged between 2.1 and 2.3% indicating soil degradation. According to all these parameters, the soil of the three experimental fields was classified as Calcisol type.

**Table 4.1.** Chemical and physical characteristic of the soil of the three experimental fields used in this study.

Chemical parameters	Experimental Field		
	Field 1	Field 2	Field 3
pH-KCL	7.0	7.03	7.6
pH-H <sub>2</sub> O	7.6	7.7	7.0
P <sub>2</sub> O <sub>5</sub> (mg per 100 g soil <sup>-1</sup> )	45.0	40.0	35.0
K <sub>2</sub> O (mg per 100 g soil <sup>-1</sup> )	40.0	30.0	25.0
Physical parameters			
Clay (%)	50.0	50.0	50.0
Organic matter (%)	2.3	2.1	2.0
Permeability (log 10K)	2.3	2.4	2.2
Porosity (%)	50.0	60.0	50.0
Stable aggregates (%)	59.0	62.1	58.2
Structure Factor (%)	68.4	68.2	67.9
Soil subtype	Calcisol		

Soil microbiological analysis revealed the occurrence of actinobacteria, bacteria and fungi in the soil at different concentrations depending on the experimental field. The soil from experimental Field 1 showed the highest concentration of actinobacteria ( $1.43 \times 10^7$  CFU mL<sup>-1</sup>) and bacteria ( $1.90 \times 10^9$  CFU mL<sup>-1</sup>), whereas the highest concentration of fungi was found in the soil from the experimental Field 2 ( $7.20 \times 10^7$  CFU mL<sup>-1</sup>). In addition, the presence of *Azotobacter*, a genus of growth-promoting bacteria able to fix nitrogen and induce plant growth, were detected, with the soil from experimental Field 2 showing the highest concentration ( $6.75 \times 10^5$  CFU mL<sup>-1</sup>). Finally, phosphate solubilizing bacteria were detected, with the soil from experimental Field 2 also showing the highest level ( $5.7 \times 10^6$  CFU mL<sup>-1</sup>) (Table 4.2).

**Table 4.2.** Microbiological analysis to determine the microbiota (CFU mL<sup>-1</sup>) in the soils of the experimental fields used in this study.

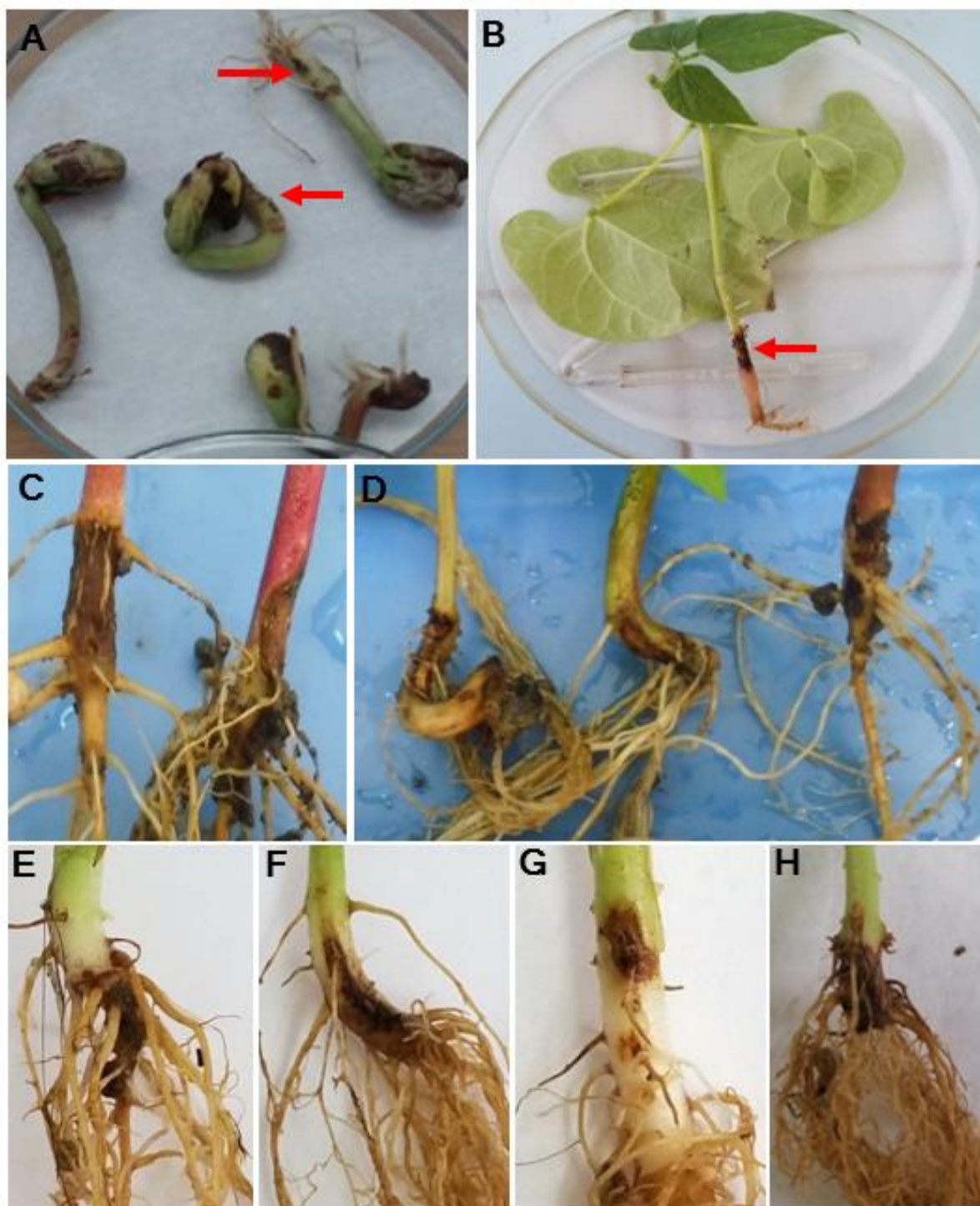
Microbiota analyzed <sup>a</sup>	Microbiological soil analysis (CFU mL <sup>-1</sup> )		
	Field 1	Field 2	Field 3
Actinobacteria	1.43 × 10 <sup>7</sup>	1.39 × 10 <sup>6</sup>	5.80 × 10 <sup>6</sup>
Azotobacter	1.36 × 10 <sup>5</sup>	6.75 × 10 <sup>5</sup>	1.36 × 10 <sup>5</sup>
Bacteria	1.90 × 10 <sup>9</sup>	1.39 × 10 <sup>6</sup>	2.26 × 10 <sup>7</sup>
Fungi	4.85 × 10 <sup>6</sup>	7.20 × 10 <sup>7</sup>	2.28 × 10 <sup>7</sup>
Phosphate Solubilizing Bacteria	2.10 × 10 <sup>6</sup>	5.70 × 10 <sup>6</sup>	2.24 × 10 <sup>6</sup>

<sup>a</sup> The microbiota was analyzed using casein starch agar (CSA), diluted to 10<sup>-5</sup> for actinobacteria (Vieira and Nahas, 2005); Ashby's agar at a 10<sup>-4</sup> dilution for azoto-bacteria (Shahid *et al.*, 2019); tryptone soy agar (TSA) at a 10<sup>-8</sup> dilution for bacteria (Vieira and Nahas, 2005); Rose Bengal dichloran agar with chloramphenicol (DRBC) at 10<sup>-5</sup> for fungi (Moubasher *et al.*, 2018); and NBRIP at a 10<sup>-8</sup> dilution for phosphate solubilizing bacteria (Mohamed *et al.*, 2019).

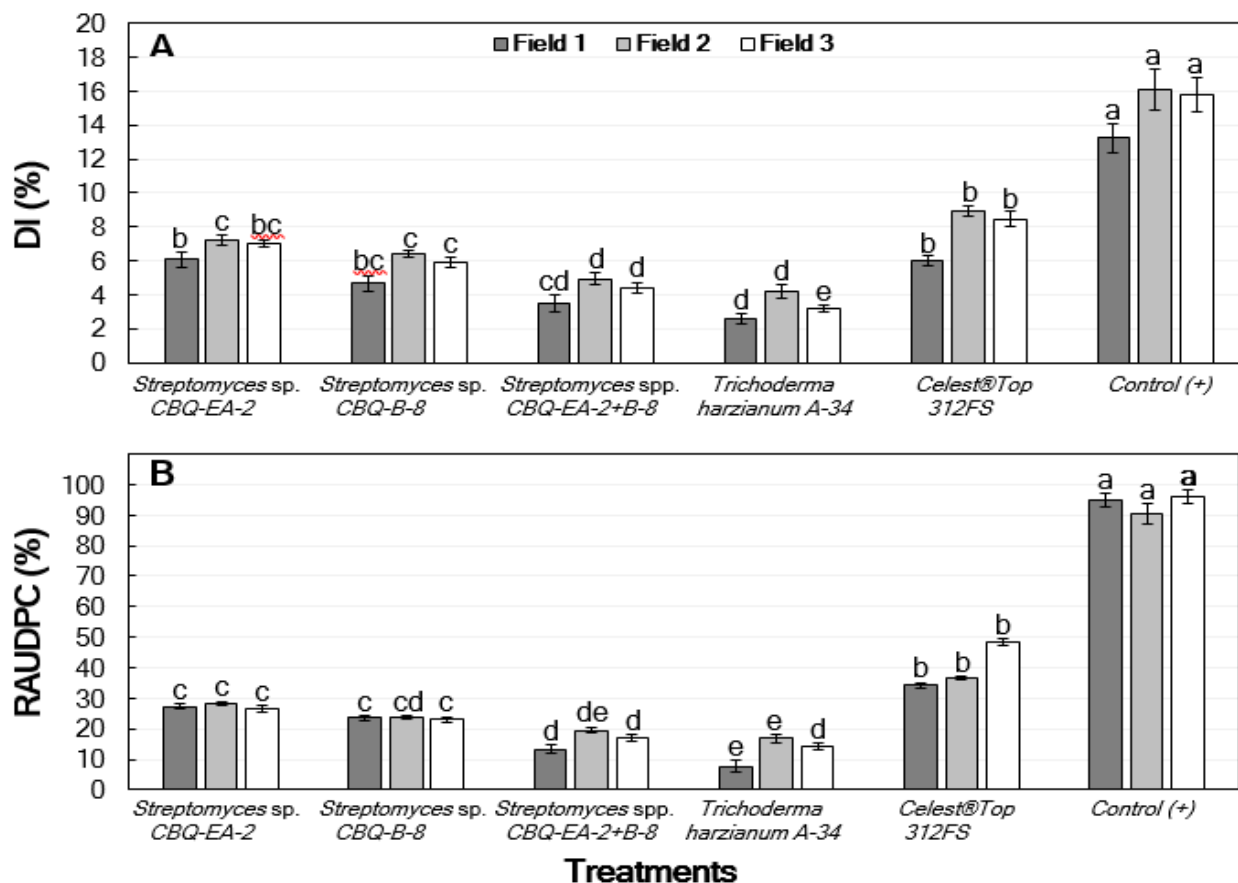
The climatic data for the experimental periods are shown in Supplementary Table 4.1.

#### 4.3.2. Effect of biological treatments against root rot complex disease of common bean

The results regarding the effectiveness of treatments on DI (%) and DS (RAUDPC; %) of root rot diseases of *Phaseolus vulgaris* in the three experimental fields are shown in Figs. 4.2 and 4.3. In all experimental fields, significant differences ( $P < 0.05$ ) for both DI and DS data were observed between treatments in comparison with the nontreated controls. All treatments resulted in high effectiveness against disease in the field. In general, treatments with *Streptomyces* sp. strain CBQ-EA-2 or *Streptomyces* sp. strain CBQ-B-8 alone showed significantly lower effect on the reduction of DI and DS than treatments combining the two strains (*Streptomyces* sp. CBQ-EA-2+ CBQ-B-8), regardless of the experimental field. As an exception, DI and DS were not significantly different between treatments with *Streptomyces* sp. strain CBQ-B-8 alone and the mixture with the two actinobacteria in Field 1 and Field 2, respectively. In addition, three treatments with *Streptomyces* spp. resulted in significantly higher effectiveness than those conducted with the chemical Celest® Top 312 FS when considering both DI and DS, with the exception of the treatment with *Streptomyces* sp. CBQ-EA-2 in Fields 1 and 2, and treatment with *Streptomyces* sp. CBQ-B-8 in Field 1 for DI. In contrast, most of the treatments with *Streptomyces* had a significantly lower effect than the BCA *T. harzianum* A-34 (Fig. 4.3).



**Figure 4.2.** A-B, Preemergence (A) and postemergence (B) symptoms of root and stem rot (red arrows) caused by *Macrophomina phaseolina* in nontreated seedlings of *Phaseolus vulgaris* cv. Quivicán (positive control); C-D, Postemergence root rot and necrotic lesions in the roots and basal stem rot caused by *Rhizoctonia solani* in nontreated seedlings of *P. vulgaris* cv. Quivicán (positive control); E-H, Disease severity at the end of the experiment in plants of *P. vulgaris* cv. Quivicán treated with *Streptomyces* sp. CBQ-EA-2, Celest® Top 312 FS, and *Trichoderma harzianum* strain A-34 and in nontreated plants (positive control), respectively.



**Figure 4.3. A**, Disease incidence (DI; %), and **B**, Disease severity (Relative area under the disease progress curve, RAUDPC; %) of root rot in *Phaseolus vulgaris* cv. Quivicán plants treated with biological or chemical compounds under field conditions at 86 days after sowing. For each experimental field (Field 1, 2 and 3), columns represent the mean of 80 seedlings per treatment, and columns with a letter do not differ significantly according to Fisher's protected LSD test at  $P = 0.05$ . The untransformed data was used in all cases, with the exception of DI log-transformed data that were used in the Field 2 and Field 3. Vertical bars are the standard errors of the means.

For plants selected for reisolation, a total of 225 fungal colonies were collected and, identified as *Fusarium* spp., *M. phaseolina*, *R. solani* and *Sclerotium* sp. This result confirmed that natural root rot infections occurred in all the experimental fields.

#### 4.3.3. Effect of biological treatments on yield and quality of legumes

The effect of treatments on the seed germination, yield and quality of *P. vulgaris* legumes in the three experimental fields is shown in Table 4.3. In all experimental fields, significant differences ( $P < 0.05$ ) between treatments were observed for all dependent variables evaluated. In general, most of the treatments led to higher germination, quality and yield of legumes than in the nontreated control in the three experimental fields.

**Table 4.3.** Effect of treatments on germination, yield and quality of the legumes of *Phaseolus vulgaris* of cv. Quivicán plants treated with biological or chemical compounds under field conditions at 86 days after seedling.

Treatments	Germination (%) <sup>a,d</sup>			Weight (g of seeds plant <sup>-1</sup> ) <sup>b,d</sup>		
	Field 1	Field 2	Field 3	Field 1	Field 2	Field 3
<i>Streptomyces</i> sp. CBQ-EA-2	86.5 ± 0.15 e	86.2 ± 0.37 e	86.3 ± 1.03 d	8.3 ± 0.06 c	7.0 ± 0.11 bc	7.7 ± 0.14 b
<i>Streptomyces</i> sp. CBQ-B-8	87.4 ± 0.15 d	88.2 ± 0.51 d	87.2 ± 0.86 cd	8.4 ± 0.12 c	7.0 ± 0.06 bc	7.8 ± 0.23 b
<i>Streptomyces</i> spp. CBQ-EA2 + CBQ-B-8	89.4 ± 0.28 c	89.7 ± 0.51 c	89.3 ± 0.64 bc	8.9 ± 0.14 bc	8.0 ± 0.02 ab	8.3 ± 0.21 ab
<i>Trichoderma harzianum</i> A-34	90.8 ± 0.38 b	90.9 ± 0.51 b	90.2 ± 0.17 ab	9.5 ± 0.26 a	8.0 ± 0.28 a	8.5 ± 0.22 a
Celest® Top 312 FS	93.8 ± 0.17 a	92.7 ± 0.51 a	92.1 ± 0.92 a	9.1 ± 0.30 ab	8.0 ± 0.07 ab	8.2 ± 0.31 ab
Control (+)	83.3 ± 0.34 f	82.0 ± 0.92 f	82.4 ± 1.10 e	6.8 ± 0.14 d	6.2 ± 0.73 c	6.5 ± 0.11 c
Treatments	Yield (t ha <sup>-1</sup> ) <sup>b,d</sup>			Number of legumes per plant (NLP) <sup>c,d</sup>		
	Field 1	Field 2	Field 3	Field 1	Field 2	Field 3
<i>Streptomyces</i> sp. CBQ-EA-2	1.9 ± 0.02 c	1.6 ± 0.03 b	1.8 ± 0.02 b	9.7 ± 0.17 c	9.5 ± 0.11 d	10.1 ± 0.10 b
<i>Streptomyces</i> sp. CBQ-B-8	2.0 ± 0.03 c	1.6 ± 0.02 b	1.8 ± 0.05 b	10.8 ± 0.41 b	9.9 ± 0.04 c	10.5 ± 0.17 b
<i>Streptomyces</i> spp. CBQ-EA2 + CBQ-B-8	2.1 ± 0.03 b	1.9 ± 0.02 a	2.0 ± 0.05 a	12.3 ± 0.56 a	11.1 ± 0.12 b	12.0 ± 0.04 a
<i>Trichoderma harzianum</i> A-34	2.3 ± 0.06 a	1.9 ± 0.07 a	2.0 ± 0.05 a	12.6 ± 0.15 a	11.4 ± 0.17 ab	12.1 ± 0.14 a
Celest® Top 312 FS	2.3 ± 0.08 a	2.0 ± 0.01 a	2.0 ± 0.08 a	12.2 ± 0.26 a	11.6 ± 0.19 a	12.2 ± 0.17 a
Control (+)	1.5 ± 0.03 d	1.4 ± 0.15 c	1.4 ± 0.03 c	9.2 ± 0.23 c	8.8 ± 0.10 e	9.5 ± 0.18 c
Treatments	Number of seeds per plant (NSP) <sup>c,d</sup>			Number of seeds per legume (NSL) <sup>c,d</sup>		
	Field 1	Field 2	Field 3	Field 1	Field 2	Field 3
<i>Streptomyces</i> sp. CBQ-EA-2	44.0 ± 4.72 b	33.6 ± 1.83 abc	43.0 ± 4.05 b	4.5 ± 0.08 b	4.4 ± 0.14 ab	4.4 ± 0.09 c
<i>Streptomyces</i> sp. CBQ-B-8	43.5 ± 2.80 b	32.7 ± 1.78 bc	40.2 ± 0.80 bc	4.5 ± 0.10 b	4.6 ± 0.14 a	4.4 ± 0.10 c
<i>Streptomyces</i> spp. CBQ-EA2 + CBQ-B-8	54.7 ± 2.44 a	40.4 ± 6.77 ab	53.1 ± 2.43 a	5.3 ± 0.23 a	4.5 ± 0.15 a	5.3 ± 0.21 b
<i>Trichoderma harzianum</i> A-34	56.4 ± 1.41 a	41.9 ± 1.26 a	54.6 ± 1.41 a	5.6 ± 0.17 a	4.6 ± 0.10 a	5.8 ± 0.03 a
Celest® Top 312 FS	54.5 ± 1.41 a	42.5 ± 1.21 a	53.7 ± 0.63 a	5.2 ± 0.19 a	4.6 ± 0.07 a	5.2 ± 0.14 b
Control (+)	35.5 ± 2.89 b	30.2 ± 4.40 c	33.7 ± 2.89 c	4.0 ± 0.06 c	4.0 ± 0.09 b	4.0 ± 0.03 d

<sup>a</sup>Germination: (seeds germinated/total seeds planted) × 100; Data of germinated seeds was assessed at 10 days after sowing.

<sup>b</sup>The total weight of seeds of each plant per treatment was obtained at 14 % moisture content; and the total weight of the plants in each replicate per treatment was estimated to express the yield as tones ha<sup>-1</sup>.

<sup>c</sup>NLP, NSP and NSL represents the mean of 30 plants randomly selected per block ( $n = 4$ ) and treatment ( $n = 6$ ).

<sup>d</sup>Means in a column followed with a common letter do not differ significantly according to Fisher's protected LSD test at  $P = 0.05$  applied to untransformed data in all cases, with the exception of yield log-transformed data that were used in the experimental Field 1.

## Chapter 4

When evaluating germination percentage, treatments with *Streptomyces* sp. alone or in combination resulted in significantly higher germination than in nontreated control, but they had a significantly lower effect than the BCA *T. harzianum* A-34 and the chemical Celest® Top 312 FS in Fields 1 and 2. Similar results were observed in Field 3, but in this case the combined treatment with *Streptomyces* sp. CBQ-EA-2+CBQ-B-8 did not differ significantly from BCA *T. harzianum* A-34 treatment.

Regarding the effect of treatment on crop yield, treatments with *Streptomyces* sp. alone showed significantly higher yield than the nontreated control, but significantly lower yield than the remaining treatments. However, treatment with *Streptomyces* sp. CBQ-EA-2+CBQ-B-8 had a significantly higher effect on yield compared to those with *Streptomyces* sp. alone in the three fields, the same effect as the BCA *T. harzianum* A-34 and the chemical Celest® Top 312 FS in Fields 2 and 3, and an intermediate effect in Field 1.

For the effect on NSP, treatments with *Streptomyces* sp. CBQ-EA2 or CBQ-B-8 did not differ significantly compared to the nontreated control in Fields 1 and 2, or in the three fields, respectively. Nevertheless, treatment with *Streptomyces* sp. CBQ-EA-2+CBQ-B-8 showed a significantly higher effect on NSP than the nontreated control in the three fields, with an effect similar to that observed for the BCA *T. harzianum* A-34 and the chemical Celest® Top 312 FS.

Regarding the effect of treatment on the weight of seeds, treatments with *Streptomyces* sp. alone showed a significantly higher effect than the nontreated control in the Fields 1 and 2 but did not differ significantly from the nontreated control in Field 2. The effect of treatment with *Streptomyces* sp. CBQ-EA-2+CBQ-B-8 did not differ significantly with the effect of treatment with *Streptomyces* sp. alone, but they had a significantly higher effect than the nontreated control in all fields. In addition, treatment with *Streptomyces* sp. CBQ-EA-2+CBQ-B-8 showed a similar effect as that of the BCA *T. harzianum* A-34 and the chemical Celest® Top 312 FS in Fields 2 and 3 and a similar effect as Celest® Top 312 FS in Field 1.

Concerning the effect of treatment on NLP, treatments with *Streptomyces* sp. CBQ-EA-2+CBQ-B-8, the BCA *T. harzianum* A-34 and the chemical Celest® Top 312 FS showed the highest effect, followed by *Streptomyces* sp. CBQ-B-8; treatment with *Streptomyces* sp. CBQ-EA-2 did not differ significantly compared to the nontreated

control in Field 1. In Fields 2 and 3, all treatments with *Streptomyces* sp. alone or in combination as well as with the BCA *T. harzianum* A-34 and the chemical Celest® Top 312 FS showed a significantly higher effect on NLP than the nontreated control. In Field 3, treatment with *Streptomyces* sp. CBQ-EA-2+CBQ-B-8 showed an effect similar to that observed for the BCA *T. harzianum* A-34 and the chemical Celest® Top 312 FS, and a significantly higher effect than that observed for treatments with *Streptomyces* sp. alone.

Finally, for the effect of treatment on NSL, treatments with *Streptomyces* sp. CBQ-EA-2 did not differ significantly compared to the nontreated control in Field 2, where the remaining treatments showed a significantly higher effect compared to the nontreated control but no differences between them were observed. In Field 1, treatment with *Streptomyces* sp. CBQ-EA-2+CBQ-B-8 had a similar effect as the BCA *T. harzianum* A-34 and the chemical Celest® Top 312 FS, and a significantly higher effect than that observed for treatments with *Streptomyces* sp. alone. In Field 3, treatment with *Streptomyces* sp. CBQ-EA-2+CBQ-B-8 had a similar effect as the chemical Celest® Top 312 FS and a significantly higher effect than that observed for treatments with *Streptomyces* sp. alone.

Linear correlation analysis showed a negative significant linear correlation between the weight of seeds per plant and DI ( $r = -0.8882$ ;  $P = 0.0180$ ) or RAUDPC ( $r = -0.8633$ ;  $P = 0.0267$ ); and between yield and DI ( $r = -0.8359$ ;  $P = 0.0382$ ) or RAUDPC ( $r = -0.8093$ ;  $P = 0.0511$ ), though there was a higher level of significance in the latter case. Finally, there was a positive significant linear correlation between DI and DS average data ( $r = 0.9928$ ;  $P = 0.0001$ ) (Table 4.4), and between seed germination and most parameters related to the yield or quality of fruits ( $P < 0.05$ ).

**Table 4.4.** Pearson correlation coefficients ( $r$ ) between Disease Incidence or RAUDPC and germination data, or quality of the legumes parameters analyzed using the averaged data of the three experimental fields.

Global Average <sup>a</sup>	DI (%)		RAUDPC (%)	
	$r$	$P$	$r$	$P$
Germination (%)	-0.6972	0.1236	-0.6742	0.1419
Weight (g seeds plant <sup>-1</sup> )	-0.8882	0.0180*	-0.8633	0.0267*
Yield (tones ha <sup>-1</sup> )	-0.8359	0.0382*	-0.8093	0.0511
NLP	-0.7093	0.1145	-0.6536	0.1592
NSP	-0.7382	0.0938	-0.6949	0.1254
NSL	-0.8052	0.0532	-0.7537	0.0835
RAUDPC (%)	0.9928	0.0001*	-	-

<sup>a</sup>NLP: Number of legumes per plant; NSP: Number of seeds per plant; NSL: Number of seeds per legume; \*Significant differences at  $P < 0.05$ .

#### 4.4. DISCUSSION

Actinobacteria are a heterogeneous group of aerobic, filamentous and gram-positive bacteria belonging to Actinomycetaceae, with the genus *Streptomyces* being broadly widespread and represented in nature by the largest number of species (Díaz-Díaz *et al.*, 2022). Their potential as BCAs against soil-borne pathogens such as *Rhizoctonia*, *Phytophthora*, *Fusarium*, and *Pythium* spp. in legumes and other herbaceous crops has been demonstrated in recent decades (Vurukonda *et al.*, 2021; Díaz-Díaz *et al.*, 2022).

Indeed, Díaz-Díaz *et al.* (2022) recently characterized 60 actinobacterial strains for their effectiveness against *M. phaseolina* and *R. solani* by means of dual culture *in vitro* and, *in planta* experiments under controlled conditions. These authors showed that forty and twenty-five of the 60 actinobacteria strains are able to inhibit the mycelial growth of *M. phaseolina* and *R. solani*, respectively, with 18 of them showing a common effect against both pathogens. Among them, *Streptomyces* sp. strains CBQ-EA-2 and CBQ-B-8 were selected as representatives of the most effective strains *in vitro* and tested *in planta*, with treatments combining the two strains achieving high effectiveness in reducing the DS *P. vulgaris* inoculated with *M. phaseolina* and *R. solani*. This effectiveness was similar to treatments with *T. harzianum* strain A-34 and the chemical Celest® Top 312 FS (Díaz-Díaz *et al.*, 2022).

The present study showed that in three experimental fields, combined treatment of *Streptomyces* spp. CBQ-EA-2+CBQ-B-8, had a significantly higher efficacy in reducing DI and SD than *Streptomyces* sp. strains separately and the control treatment. These results, together with previous research by Díaz-Díaz *et al.*, (2022), indicate that the possible mechanism of action of *Streptomyces* spp. CBQ-EA-2+CBQ-B-8 is the combination of hydrolytic enzymatic action with antibiotic production (antibiosis), as reinforced in this microbial consortium. The antagonistic activity of *Streptomyces* against plant pathogens may be closely related to the production of antimicrobials, such as bioactive secondary metabolites, and extracellular production of hydrolytic enzymes, which promote fungal cell lysis, enhance nutrient uptake, and promote plant growth among other biological functions (Song *et al.*, 2020; Pacios-Michelena *et al.*, 2021). According to Wonglom *et al.* *Streptomyces* strains have a broad antibiotic spectrum and a variety of extracellular enzymes that act on other microorganisms, with emphasis on fungal cell wall degradation as a possible mechanism of action (Wonglom *et al.*, 2019).

In concordance with our results, several authors have reported the efficiency of combined treatments with *Streptomyces* strains. El-Tarabily *et al.*, (2009) showed that the

combination of endophytic actinobacteria such as *Actinoplanes campanulatus*, *Micromonospora chalcea* and *Streptomyces spiralis* suppress damping-off and crown and root rot of cucumber with higher effectiveness than the fungicide metalaxyl. Recently, Nimnoi and Ruanpanun, (2020) showed that treatments combining *Streptomyces* spp. strains KPS-A032 and KPS-E004 significantly promote plant growth and suppression of root-knot nematodes (*Meloidogyne incognita*) in chili. Finally, Abbasi *et al.*, (2020) reported that treatments combining *S. rochei* IT20 + *S. vinaceusdrappus* SS14 of chili inoculated with *Phytophthora capsici* significantly reduce disease symptoms by 40-60% compared to the control. Due to the interesting results obtained in previous work, the aim of the present study was to validate the effectiveness of both *Streptomyces* sp. strains CBQ-EA-2 and -CBQ-B-8 against root rot diseases of *P. vulgaris* in the field. In this case, we consider the disease to be a root rot complex disease associated mainly with *M. phaseolina* and *R. solani* due to the difficulty of separating the effect of each pathogenic fungus in the field. In the three experimental fields, treatments combining the two *Streptomyces* strains (CBQ-EA-2 + CBQ-B-8) were able to significantly reduce the DI and DS compared with the nontreated control, with a similar effect as treatments with the BCA *T. harzianum* A-34 and the chemical Celest® Top 312 FS. Nevertheless, the effectiveness of treatments using single applications of *Streptomyces* sp. CBQ-EA-2 or CBQ-B-8 was significantly less compared with the remaining treatments. These results confirmed the observations by Díaz-Díaz *et al.* (2022) in experiments conducted *in planta* in greenhouse testing these same treatments.

Our results are also in agreement with those obtained by several authors who tested the effectiveness of *Streptomyces* spp. against soil-borne pathogens in the field (Xue *et al.*, 2013; Palazzini *et al.*; 2017; El-Shatoury *et al.*, 2020; Gebily *et al.*, 2021). For instance, Xue *et al.* (2013) evaluated the effectiveness of *Streptomyces cyaneofuscatus* Kudrina & Pridham, *S. flavotricini* Pridham, *S. kanamyceticu* Okami & Umezawa, and *S. rochei* Berger against *Verticillium* wilt (*Verticillium dahliae* Kleb.) in cotton (*Gossypium hirsutum* L.) under field conditions, showing that treatments with *Streptomyces* species were able to significantly reduce the DS compared with the nontreated control. The efficacy of *Streptomyces* sp. strains in controlling *Fusarium graminearum* Schwabe in wheat (*Triticum aestivum* L.) was assessed by Palazzini *et al.* (2017), who treated wheat ears with *Streptomyces* sp. RC 87B during anthesis for two continuous years under field conditions. These authors showed that *Streptomyces* sp. RC 87B significantly reduced the DS compared with the nontreated control naturally infected

with *F. graminearum*, with DS ranging from 26.1 to 37.2% in 2014 and from 33.7 to 55.7% in 2015 for treated and nontreated plants, respectively. They also applied *Streptomyces* sp. RC 87B on crop stubble, reducing *F. graminearum* inoculum by 82% at 45 days, and 100% at 90 days (Palazzini *et al.*, 2017). In addition, El-Shatoury *et al.* (2020) evaluated the effectiveness of the strains *S. flavoviridis* SF 1 and *S. variabilis* SF 2 against *Botrytis cinerea* Pers. on broad bean seeds (*Vicia fabae* L.). These authors also demonstrated that treatments combining the two strains significantly reduced the DS and DI compared with the treatments with the *Streptomyces* strains alone or with the nontreated control. Finally, studies conducted in the field by Gebily *et al.* (2021), showed that treatments with *S. griseus* Krainsky, *S. rochei* and *S. sampsonii* Millard and Burr, applied alone or in combination, against *Sclerotinia sclerotiorum* (Lib.) de Bary on common bean significantly reduced the DS compared with the nontreated control.

In parallel, it is worth mentioning that the treatments conducted in this study were able to significantly increase the yield and quality of legumes compared with nontreated plants regardless of whether both *Streptomyces* sp. strains (CBQ-EA-2 and CBQ-B-8) were applied alone or in combination. The fact that *Streptomyces* strains are able to improve these parameters has been previously demonstrated. Indeed, Gopalakrishnan *et al.* (2015) showed that treatments with *Streptomyces* sp. strains CAI-17, CAI-68, CAI-78, KAI-26, or KAI-27 by means of single applications significantly increased the crop yield to chickpea (*Cicer arietinum* L.) compared with the nontreated control. In addition, the study described above by Gebily *et al.* (2021) found that treatments with *S. griseus*, *S. rochei*, and *S. sampsonii* were able to significantly increase the yield in common bean compared with the nontreated control.

This study represents the validation of our previous results under controlled conditions, demonstrating that the two *Streptomyces* strains used are potential BCAs against root rot complex disease of common bean. The results obtained not only confirm that *Streptomyces* sp. may be considered a BCA against soil-borne pathogens, but also as promoting the growth of microorganism due to the increased effectiveness in improving quality and crop yield. Therefore, this work constitutes an important step toward developing potential BCAs against root rot disease commonly associated with *M. phaseolina* and *R. solani*.

## ACKNOWLEDGMENTS

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**Table 4.1:** Climatological data from climatic Station 78343 (Santa Clara, Villa Clara, Cuba) during the experimental periods 2018/19 and 2019/20.

<i>Experimental period 2018/19</i>							
Month/Year	Temperature (°C)			Relative Humidity (%)			Accumulated rainfall (mm)
	Maximum	Average	Minimum	Maximum	Average	Minimum	
October/2018	31.2	25.5	21.9	98	85	56	62.6
November/2018	30.4	24.2	19.5	98	82	53	12.6
December/2018	28.4	22.8	18.9	97	82	56	33.8
January/2019	27.8	21.4	16.8	98	81	51	46.5
February/2019	29.5	22.6	17.4	98	80	49	23.9
<i>Experimental period 2019/20</i>							
Month/Year	Temperature (°C)			Relative Humidity (%)			Accumulated rainfall (mm)
	Maximum	Average	Minimum	Maximum	Average	Minimum	
October/2019	30.7	25.8	22.9	98	86	63	136.6
November/2019	29.3	23.4	19.0	98	84	56	8.1
December/2019	28.8	22.7	18.4	98	87	59	50.4
January/2020	27.6	21.7	17.9	96	81	54	22
February/2020	29.3	23.2	18.9	96	79	49	8.2



## *Chapter 5*





## ***Streptomyces* spp. strains as potential biological control agents against Verticillium wilt of olive**

### **ABSTRACT**

The hemibiotrophic fungus *Verticillium dahliae* is considered the main phytopathogen affecting olive (*Olea europaea*) in Mediterranean-type climate regions causing Verticillium wilt. The lack of effective chemical products against this pathogen makes necessary search towards to find alternative control measures such as biological control. Thus, the main goal of this work was to evaluate the effect of six *Streptomyces* spp. strains as biological control agents (BCAs) against Verticillium wilt of olive. For this purpose, their effect was evaluated *in vitro* on mycelial growth development and conidia germination of *V. dahliae* (isolates V-004 and V-323) as well as on microsclerotia viability using naturally infested soils. In addition, their effect on the disease development was evaluated by means of bioassays in olive plants inoculated with *V. dahliae* isolate V-323. In all the experiments, the reference BCAs *F. oxysporum* FO12 and *A. pullulans* AP08 were included for comparative purposes. The six *Streptomyces* sp. strains and the BCAs *F. oxysporum* FO12 and *A. pullulans* AP08 showed a significant effect on mycelial growth inhibition for both *V. dahliae* isolates V-004 and V-323 compared to the positive control. The reference BCA FO12 was the most effective on mycelial growth inhibition followed by AP08 for the two *V. dahliae* isolates; while the *Streptomyces* sp. strains showed a moderate effect for both *V. dahliae* isolates. Among the *Streptomyces* strains evaluated, CBQ-EA-2 was the most effective inhibiting mycelial growth of *V. dahliae*. To date, the experiments in planta have not yet completed, and only the methodology is shown in this chapter.

**KEYWORDS:** Actinomycetes, biocontrol, *Olea europaea*, soil-borne pathogen, *Verticillium dahliae*

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## 5.1. Introduction

The hemibiotrophic soil-borne fungus *Verticillium dahliae* Kleb. is considered among the main pathogens of the cultivated olive (*Olea europaea* subsp. *europaea* L.). It is the causal agent of Verticillium wilt of olive (VWO) in the main olive growing regions across the Mediterranean basin, including Spain. The disease has important effects on fruit yield reduction as well as in the plant viability (Montes-Osuna and Mercado-Blanco, 2020). In Andalusia region, where represents the major percentage of national cultivated olive surface with 1.673.071 ha (MAPA, 2021), two pathotypes of *V. dahliae* have been identified causing VWO; i) the non-defoliating pathotype (ND) that affect with low levels of severity without defoliation; and the defoliating pathotype (D) that cause the most severe symptomatology in affected olives including defoliation, xylem discoloration and tree death (López-Escudero and Mercado-Blanco, 2011). The pathogen produces resistance structures called microsclerotia (MS), which allow the pathogen survival in the soil for long periods of time. These structures are the primary inoculum source that infect the olive through the roots as consequence of biochemical stimuli induced by the exudates excreted by the roots (Montes-Osuna and Mercado-Blanco, 2020; López-Moral *et al.*, 2023).

It is well known the difficulty to control VWO due the interaction of several factors such as the ability of the pathogen to produce MS in the soil, the lack of effective chemical treatments that are able to arrive to the xylem, the inadequate agronomic practices, or the use of homogeneous olive genotypes populations that induce the pathogen to overcome the genetic resistance among others (López-Escudero and Mercado-Blanco, 2011; Montes-Osuna and Mercado-Blanco 2020; López-Moral *et al.*, 2021). Considering this scenario, the need to establish an integrated management strategy to control the disease based on eco-friendly control methods including cultural practices, genetic resistance and biocontrol is an urgent demand in our society.

Regarding the biological control of VWO, important advances have been done during the last decade. A broad diversity of natural compounds such as plant extracts (Varo *et al.*, 2017), organic amendments (Varo-Suárez *et al.*, 2018, Mulero-Aparicio *et al.*, 2020b), biostimulants and biofertilizers (López-Moral *et al.*, 2021) and beneficial microorganisms including bacteria, i.e., *Bacillus amyloliquefaciens* (López-Moral *et al.*, 2021, 2022b) *Bacillus velezensis* (Castro *et al.*, 2020), *Pseudomonas fluorescens* (Mercado-Blanco *et al.*, 2004; Mansoori *et al.*, 2013); and fungi, i.e., *Aureobasidium*

*pullulans* AP08 (López-Moral *et al.*, 2021, 2022b), non-pathogenic *Fusarium oxysporum* FO12 (Mulero-Aparicio *et al.*, 2019; ), *Trichoderma* spp. (Carrero-Carrón *et al.*, 2018; Reghmit *et al.*, 2021), have been evaluated against VWO under controlled conditions. Some of these compounds have resulted as potential biological control agents (BCAs) against VWO under controlled conditions. However, only a few of them have been already evaluated in the field with successful results in reducing the disease severity (DS), with the non-pathogenic *F. oxysporum* strain FO12, a *Thymus* extract or a grapemarc compost being the most effective compounds (Mulero-Aparicio *et al.*, 2020a).

Due to the difficulty to obtain effective BCAs, the search for new biological alternatives is necessary. In this way, *Streptomyces* species (Actinomycetes: Streptomycetales) are considered soil-borne bacteria that colonise the rhizosphere promoting plant growth (Monteiro *et al.*, 2017; Olanrewaju and Babalola, 2019) and they are able to produce antibiotic substances that inhibit the growth of plant pathogenic fungi (Li *et al.*, 2020). Several *Streptomyces* species has been described as plant growth promoters as well as for their effectiveness against plant diseases.

The effect of *Streptomyces* species against *V. dahliae* viability has been already reported. The strains *S. plicatus* 101 (Shahidi y Aghighi 2005), *Streptomyces* sp. StB-3, StB-6, StB-11 and StB-12 (Bubici *et al.*, 2013), *S. cyaneofuscatus* (strains ZY153 and AA-2), *S. flavotricini* Z-13, *S. kanamyceticus* B-49 and *S. rochei* X-4 inhibited the mycelial growth of *V. dahliae* by means of *in vitro* activity of cell-free filtrates of the *Streptomyces* strains. In addition, Xue *et al.* (2013) observed microscopically that *Streptomyces* hyphae coiled around the hyphae of *V. dahliae*; and demonstrated that the *S. cyaneofuscatus* (ZY-153), *S. kanamyceticu* (B-49), *S. rochei* (X-4) and *S. flavotricini* (Z-13) strains reduced significantly the disease severity (DS) in cotton plants under both greenhouse and field conditions compared to the nontreated controls (Xue *et al.*, 2013). Other studies expand the pathogenic fungi on which the *Streptomyces* strains have had an effect. Errakhi *et al.* (2009) evaluated several *Streptomyces* sp. strains (J-2, B-5, B-11, B-33, B-40, B-42, B-62, D-29, D-35 and D-75) against *Sclerotium rolfsii*, *Botrytis cinerea*, *F. oxysporum* and *V. dahliae* *in vitro* by dual confrontation or by culture filtrate, and *in vivo* in *Beta vulgaris*. Yang *et al.* (2019) tested *S. corchorusii* strain AUH-1 against *Botryosphaeria dothidea*, *F. oxysporum* f. sp. *cucumerinum*, *F. oxysporum* f. sp. *niveum*, *F. oxysporum* f. sp. *vasinfectum*, *Rhizoctonia solani* and *V. dahliae* *in vitro* by dual confrontation. Finally, Carlucci *et al.*, (2022) showed that the strain *S. albidoflavus*

CARA17 inhibited the mycelial growth of *S. rolfsii*, *F. oxysporum*, *Sclerotinia sclerotiorum* and *V. dahliae*, and *in vivo* in *Phoeniculum vulgare*.

Likewise, no studies on the potential efficacy of *Streptomyces* against VWO have been reported. Therefore, the main goal of this study was to evaluate the effectiveness of six *Streptomyces* strains that previously have been characterized as potential BCAs of soilborne fungal pathogens such as *Macrophomina phaseolina* and *Rhizoctonia solani* associated with stem and root rot in common bean (*Phaseolus vulgaris*) under both controlled (Díaz-Díaz *et al.*, 2022) and field conditions (Díaz-Díaz *et al.*, 2023). To this end, the effect of six *Streptomyces* strains on mycelial growth, conidia germination and MS inhibition of *V. dahliae* was evaluated by means of dual cultures or by sensitivity tests *in vitro*. Subsequently, their effectiveness on the disease development was also evaluated by means of bioassays in olive plants inoculated with the pathogen under semicontrolled conditions. In parallel, the *Streptomyces* strains were characterized molecularly.

## **5.2. Materials and Methods**

### **5.2.1. Fungal isolates and culture conditions**

Two isolates of *V. dahliae* were used for this study; the highly virulent isolate V-323, classified as D pathotype (López-Moral *et al.*, 2022), and the slightly virulent isolate V-004, classified as ND pathotype (Blanco-López *et al.*, 1989). These isolates are maintained in the fungal collection of the Department of Agronomy at the University of Cordoba (UCO, Spain).

Prior to carrying out the studies described below, fresh colonies of the two *V. dahliae* isolates were obtained from the collection and grown on Potato Dextrose Agar (PDA; Difco® Laboratories, MD, United States) acidified with lactic acid [APDA; 2.5% (vol/vol) at 2.5 ml/litre of medium] and incubated at 24°C in the dark for 10 days. Fresh colonies were transferred to PDA, incubated as described before and then used as inoculum source.

### 5.2.2. Biological control agents and inoculum preparation

Six *Streptomyces* strains (CBQ-EBa-21, CBQ-EA-12, CBQ-CD-24, CBQ-CB-14, CBQ-EA-2 and CBQ-B-8) belonging to the collection of the Microbiology Laboratory of the CBQ of the Central University "Marta Abreu" of Las Villas (Cuba), and previously characterized by Díaz-Díaz *et al.* (2022) were tested in this study. They are preserved at -80 °C in 20% glycerol. Prior to conduct the experiments, the strains were grown on casein-starch agar (CSA; pH = 7.0) at 30 °C for 7 days in the dark. For further experiments, inoculum was prepared in liquid medium as described by Díaz-Díaz *et al.* (2022).

Additionally, the non-pathogenic strain *Fusarium oxysporum* FO12 (Mulero-Aparicio *et al.*, 2019) and *Aureobasidium pullulans* AP08 (López-Moral *et al.*, 2021) were included for comparative purposes since they were previously characterized as potential BCAs against VWO. Both isolates belong to the fungal collection of the Department of Agronomy (UCO). Growth conditions and inoculum preparation for *F. oxysporum* FO12 and *A. pullulans* AP08 were conducted as described by Mulero-Aparicio *et al.* (2019) and López-Moral *et al.* (2021), respectively.

### 5.2.3. Molecular characterization of *Streptomyces* strains

#### 5.2.3.1. DNA extraction, PCR analysis and sequencing.

The six *Streptomyces* strains were grown on CSA as described above. DNA was extracted from the resulting pellet using the PureLink™ Genomic DNA Mini Kit reagent (Invitrogen, Waltham, MA, USA), following the manufacturer's instructions. The 16S and 23S rRNA gene were amplified by PCR using the primer pairs 27F and 1492R (Lane, 1991), and 1463 and 1719 (Stackebrandt *et al.*, 1991), respectively. Each reaction mixture contained each primer at 20 µM, dNTPs at 10 µM, 5 µL of 10X MgSO<sub>4</sub> and buffer, dimethyl sulfoxide (5%), 1 µg of genomic DNA and 1 unit of taq DNA polymerase, for a final volume of 50 µL. PCR steps included an initial denaturation at 94 °C for 3 min, followed by 30 cycles at 94 °C for 30 s, 47 °C for 33 s and 72 °C for 90 s and a final extension step at 72 °C for 7 min. PCR products were run through 1% agarose gel electrophoresis stained with RedSafe™ dye (iNtRONBiotechnology), followed by purification using the PureLink™ kit (Invitrogen, Waltham, MA, USA) and determination of amplicon quality by spectrophotometry (NanoDrop 2000, ThermoScientific; Waltham, MA, USA). Sequencing was carried out on the ABI310

Prism automated sequencer (Applied Biosystems; Waltham, MA, USA), and the resulting sequences were compared with those in the GenBank database using the BLAST (Basic Local Alignment Search Tool) algorithm to identify closely related sequences.

#### 5.2.3.2. Phylogenetic Analysis

Consensus sequences were obtained from DNA sequences generated with forward and reverse primers using SeqMan software (DNASTART Lasergen SeqMan version 7.0.0, Madison, WI) and were compiled into a single FASTA file format. Two separate phylogenetic analyses were conducted to identify the *Streptomyces* strains by means of 16S or 23S rRNA gene sequences. A total of 31 or 32 taxa were included for the phylogenetic analysis with 16S or 23S rRNA genes, respectively, from which 6 taxa were our *Streptomyces* strains and the remaining taxa were GenBank reference sequences selected for their high similarity with our query sequences using MegaBLAST (Table 5.1). *Streptomyces caviscabies* strain 8 and *S. scabies* strain 87.79 were used as outgroup for the 16s and 23S phylogenesis, respectively (Table 5.1). In both cases, GenBank sequences were added to the sequences obtained and aligned by Clustal W. Subsequently, maximum parsimony (MP), analyses by the tree-bisection-regrafting algorithm with search level one in which the initial trees were obtained by the random addition of sequences (10 replicates). Adding sequences, alignments, and MP analyses were performed using MEGA version 11 software (Kumar *et al.*, 2016). Gaps and missing data were treated as complete deletions. The robustness of the topology was evaluated by 2,000 bootstrap replications (Felsenstein, 1985). Measurements for the MP analyses, namely, tree length (TL), consistency index (CI), retention index (RI), homoplasy index (HI), and rescaled consistency index (RC) were calculated for the resulting tree. The sequences derived in this study have not yet lodged at GenBank since this is a preliminary draft of this study.

**Table 5.1.** *Streptomyces* strains used in the phylogenetic analysis and their corresponding GenBank accession numbers.

Actinomycetes species	Strain	Host	Origin	Date	16S	23S
<i>Streptomyces albidoflavus</i>	UST040711-291	- <sup>z</sup>	- <sup>z</sup>	2009	FJ591130	- <sup>z</sup>
	CCOS 2040	Grasslans soil	Switzerland	2009	- <sup>z</sup>	OX371414
	SM254	Deep subsurface	USA	2010	-	CP014485
	RSU1	Mangrove soil	India	2012	KP698738	
	IHBA_9992	Suraj Tal Lake Water		2015	KR085950	-
	TN10	Termite nest	India	2017	MH021968	-
	IMB16-111	Marine sediment	China	2017	MG190667	-
	UYFA156	-	-	2019	CP040466	-
	J1074	-	-	-	-	DS999645
<i>Streptomyces albogriseolu</i>	LBX-2	Soil	China	2012	-	CP042594
<i>Streptomyces aquilus</i>	GGCR-6	<i>Xanthium sibiricum</i>	China	2017	-	CP034463
<i>Streptomyces argenteolus</i>	CGMCC 4.1693	-	-	-	EU048540	-
<i>Streptomyces aurantiacus</i>	JCM 4677	-	-	-	NR041436	AP023440
<i>Streptomyces aureoverticillatus</i>	HN6	Soil	China	2015	-	CP048641
<i>Streptomyces avermitilis</i>	MA-4680	-	-	-	-	NR076331
<i>Streptomyces calvus</i>	DSM 41452	-	-	-	-	CP022310
<i>Streptomyces caviscabies</i>	8	<i>Solanum tuberosum</i>	-	2016	KU743151	-
<i>Streptomyces champavatii</i>	173855	-	-	2008	EU570590	-
<i>Streptomyces coeruleorubidus</i>	ATCC 13740	Soil	-	-	-	CP023694
<i>Streptomyces cyanogenus</i>	S136	Soil/Goa mountain	India	1985	-	CP071839
<i>Streptomyces daghestanicus</i>	ICN1054	Marine	India	2010	KX775313	-
<i>Streptomyces deccanensis</i>	KCTC 19241	-	China	2019	-	CP092431
<i>Streptomyces dengpaensis</i>	XZHG99	Soil	China	-	NR179842	CP026654
<i>Streptomyces exfoliatus</i>	3-11	Yalu River	North Korea	2014	KJ571038	-
	USC006	Fresh water	Australia		KX358629	-
<i>Streptomyces fodineus</i>	TW1S1	Soil	South Korea	2015	-	CP017248
<i>Streptomyces griseus</i>	NBRC 13350	Soil	-	-	-	NC010572
	MS098	-	-	2009	GU169072	-
<i>Streptomyces hydrogenans</i>	IMB16-184	Marine sediment	China	2017	MG190777	-
	IMB16-142	Marine sediment	China	2017	MG190741	-
<i>Streptomyces koyangensi</i>	VK-A60T	Soil	South Korea	2005	-	CP031742
	SCSIO 5802	Deep sea sediment	China	2016	-	CP049945
	B003	Sediment	-	2019	MG188671	-
<i>Streptomyces krainskii</i>	RSU51	Mangrove soil	India	2012	KP698745	-
<i>Streptomyces lividans</i>	RSU26	Mangrove soil	India	2012	KP698743	-
	XQ42	<i>Euphausia superba</i>	China	2012	KU291358	-
	T38	Soil	China	2016	KU317912	-
<i>Streptomyces nodosus</i>	ATCC 14899	Soil	-	-	-	CP023747
<i>Streptomyces phaeoluteigriseus</i>	Qhu-M197	Soil	China	2019	-	CP099468
<i>Streptomyces rutgersensis</i>	NBH77	Soil	Antarctica	2017	-	CP045705
<i>Streptomyces violascens</i>	KLBMP 5501	<i>Ginkgo biloba</i> L.	-	2015	KP636799	-
	IMB16-161	Marine sediment	China	2017	MG190757	-
	IMB16-135	Marine sediment	China	2017	MG190734	-
	ATCC 27968	Marine soil	China	2017	-	CP029377
	EGI125	-	-	2019	MN704434	-
<i>Streptomyces scabiei</i>	87.79	-	-	-	-	AB041112
<i>Streptomyces sampsonii</i>	KJ40	Soil	South Korea	2005	CP016824	CP031742
	GACK10	Soil	-	2015	KP970678	-
<i>Streptomyces seoulensis</i>	HEK131	Soil	Japan	2014	-	AP025667
<i>Streptomyces somaliensis</i>	IMB16-131	Marine sediment	China	2017	MG190730	-
<i>Streptomyces sudanensis</i>	SD 504	Mycetoma patient	Sudan	-	EF515876	CP049945

<sup>z</sup>:- non determined/non available

#### 5.2.4. Effect of *Streptomyces* spp. on *Verticillium dahliae* viability

##### 5.2.4.1. Effect on mycelial growth by dual culture assay

The antagonism of the six *Streptomyces* strains (CBQ-EBa-21, -EA-12, -CD-24, -CB-14, -EA-2 y -B-8) was evaluated against the two *V. dahliae* isolates (V-323 and V-004) by means of a dual culture assay. The two BCAs FO12 and AP08 were also included in this study. A mycelial agar plug (7.5 mm diameter) was removed from the edge of a 7-day-old actively growing colony of the pathogen and seeded in the centre of a Petri dish (9.0 cm diameter) filled with PDA. Subsequently, two mycelial plugs (7.5 mm diameter) obtained from the margin of 7-day-old colonies of each *Streptomyces* strain, FO12 or AP08 were then placed on the same PDA plate, but on both sides of the pathogen no beyond than 3 cm from the *V. dahliae* plug. PDA Petri dishes seeded only with each *V. dahliae* strain were included as positive control. For each *V. dahliae* isolate, there were three replicated Petri dishes per BCA ( $n = 6$ ) or control arranged in a completely randomized design.

The Petri dishes were incubated at 28°C for 14 days. The largest and smallest diameter of *V. dahliae* colonies were measured at 14 days after confrontation. The mean data were converted to obtain the mycelial growth rate (MGR, mm day<sup>-1</sup>), and the mycelial growth inhibition percentage [mycelial growth inhibition, MGI (%)] was calculated as:

$$\text{MGI} = [1 - (\text{MGR}_{\text{dc}}/\text{MGR}_{\text{control}})] \times 100$$

where “MGR<sub>dc</sub>” is the MGR of *V. dahliae* in dual cultures with BCAs, and “MGR<sub>control</sub>” is the MGR of *V. dahliae* alone (López-Moral *et al.*, 2021).

##### 5.2.4.2. Effect on conidia viability

Conidial suspensions of *Streptomyces* strains were adjusted at  $2 \times 10^8$  conidia mL<sup>-1</sup>. In addition, a conidial suspension mixing the *Streptomyces* strains CBQ-EA-2 and CBQ-B-8 was also prepared and adjusted to a final concentration of  $2 \times 10^8$  conidia mL<sup>-1</sup> of each strain. Conidial suspensions of FO12 or AP08 were adjusted at  $2 \times 10^6$  conidia mL<sup>-1</sup> based on previous studies (Mulero-Aparicio *et al.*, 2019; López-Moral *et al.*, 2021). In parallel, conidial suspensions of both *V. dahliae* isolates V-004 and V-323 were adjusted at  $8 \times 10^5$  conidia mL<sup>-1</sup>. All the conidial suspensions were adjusted based on haematocytometer counts. Then, 250 µL of ACBs + 250 µL of the *V. dahliae* isolate V-

004 or V-323 were added to a 1.5 mL Eppendorf tube; 250  $\mu$ L of 100% sterile distilled water (SDW) + 250  $\mu$ L of each isolate of *V. dahliae* were mixed and used as a positive control. A 5- $\mu$ L drop of conidial suspension of *V. dahliae* isolate V-004 or V-323 ( $2 \times 10^5$  conidia  $\text{mL}^{-1}$ ) was mixed with another 5- $\mu$ L drop of conidial suspension of each BCA ( $2 \times 10^8$  esporas  $\text{mL}^{-1}$ ) on a microscope coverslip (20  $\times$  20 mm). The coverslips were then placed in Petri dishes with water agar on their base, which were used as humidity chamber. After the incubation period has elapsed, 5  $\mu$ L drop of 0.01% acid fuchsine in lactoglycerol (1:2:1 lactic acid:glycerol:water) was added to each coverslip to stop conidial germination, and they were mounted on a slide. The percentage of germinated conidia was determined by observing 50 conidia randomly selected on each coverslip and counting the germinated and non-germinated conidia. There were three replicated coverslips per treatment combination [(2 *V. dahliae* isolates  $\times$  8 BCAs) + 2 positive controls = 18 coverslips]. The inhibition of conidial germination (RGI; %) was estimated with respect to the control according to the following formula:

$$\text{RGI (\%)} = [(\text{Ge control} \times \text{Ge BCAs}) / \text{Ge control}]$$

where Ge control = percentage of conidia germinated after incubation in SDW, and Ge BCAs = percentage of conidia germinated after incubation with BCAs.

#### 5.2.4.3. Effect on microsclerotia viability

To conduct this experiment, a natural infested soil was collected from an olive orchard affected by VWO located in Villanueva de la Reina (Jaen province, Andalusia region, southern Spain; Geographic UTM coordinates 38. 0140.220, -3,9100.390). The soil was manually sieved using a 0.8 mm diameter sieve to remove organic debris and large particles (Trapero *et al.*, 2013).

Sterile PVC pots (100 mL vol.) were used, and five holes of 2-mm in diameter each were done in the base to facilitate percolation. In each pot, 60 g of soil were added and immediately irrigated with 30 mL of the conidial suspension of each treatment. For this experiment, the six *Streptomyces* strains, the mix of *Streptomyces* strains CBQ-EA-2 and CBQ-B-8, and the BCAs FO12 and AP08 were used. Conidial suspensions were prepared and adjusted as described before but at  $10^8$  conidia  $\text{mL}^{-1}$  or  $10^6$  conidia  $\text{mL}^{-1}$  for *Streptomyces* or fungal BCAs (FO12; AP08), respectively. In addition, pots with soil irrigated with SDW were included as positive control. After treatment percolation, the pots were hermetically closed and incubated for 24 h at room temperature, after which

the samples were placed in individual aluminium trays and air-dried at room temperature for 10-14 days (López-Moral *et al.*, 2022a). A completely randomized design with three replicated pots per treatment or control was used [9 biological treatments + 1 positive control) × 3 replicated PVC ppots = 30 PVC pots in total].

Inoculum density of *V. dahliae* was expressed as MS per g soil for each treated soil sample. It was assessed by the wet sieving method (Huisman and Ashworth, 1974) using 10 Petri dishes with modified sodium polypectate agar (MSPA) (Butterfield and DeVay, 1977) according to the protocol described by Varo *et al.* (2016). Subsequently, the reduction in inoculum density was estimated with respect to the control and expressed as MS inhibition (MSI, %).

### **5.2.5. Effect of *Streptomyces* spp. on the development of *Verticillium* wilt in olive plants**

#### *5.2.5.1. Plant material and growth conditions*

Six-month-old healthy olive potted plants of cv. Picual (highly susceptible to *V. dahliae*; López-Escudero *et al.*, 2004) were obtained from a commercial nursery. Prior to conduct the experiments, plants were preconditioned in environmentally controlled chambers at  $23 \pm 2^\circ\text{C}$  with a 14:10-h (light:dark) photoperiod of white fluorescent light (10.000 lux) and 60% Relative Humidity (RH) for 1 month before start the experiments, and they irrigated with water three times a week (López-Moral *et al.*, 2021).

#### *5.2.5.2. Biological control agents*

For this experiment, the six *Streptomyces* strains, a mix of *Streptomyces* strains CBQ-EA-2 and CBQ-B-8, and the BCAs FO12 and AP08 were used. Conidial suspensions were prepared and adjusted as described before, but at  $10^8$  conidia  $\text{mL}^{-1}$  or  $10^6$  conidia  $\text{mL}^{-1}$  for *Streptomyces* or fungal BCAs (FO12; AP08), respectively. The biological treatments were applied by irrigating the olive plants with 250 mL of the conidial suspensions at different times (*see below*).

#### *5.2.5.2. V. dahliae* isolate and inoculum preparation

The *V. dahliae* isolate PV-323 was used for the *in planta* bioassay. The fungus was previously grown in PDA as described above. The inoculum was prepared in 2 L Erlenmeyer flasks filled with 1 kg of a homogeneous mixture consisting of sand, maize flour and distilled water (9:1:2, weight:weight:volume). Then, they were double-sterilized

in two consecutive days at 120°C for 50 min (1st day) and at 120°C for 20 min (2nd day), with the flasks being shaken manually between sterilization treatments. Subsequently, the flask was seeded with 50 mycelial plugs of 7.5 cm diameter of *V. dahliae* isolate V-323 overgrown on PDA as described before. The inoculated flasks were incubated at 24°C in the dark for 4 weeks, and shaken manually every 7 days to promote homogeneous colonization of the substrate. After the incubation period, the inoculum density of the colonized mixture was assessed by the serial dilution method on PDA and expressed as colony forming units (CFU) (theoretical inoculum density of the final substrate =  $10^7$  CFU  $g^{-1}$ ; Mulero-Aparicio *et al.*, 2019).

#### 5.2.5.3. Biological treatments and *V. dahliae* inoculation

Conidial suspensions of each biological treatment were adjusted as described above, and they were applied by irrigating the olive plants with 250 mL of the conidial suspension in three different moments, at 7 and 2 days before inoculation with *V. dahliae*, and at 10 days after inoculation. At the inoculation time, olive plants were transplanted to 1 L PVC pots previously disinfested in a 10% sodium hypochlorite solution, and filled with a 20% (weight/weight) mixture of the colonized corn meal-sand and sterile peat moss, obtaining a theoretical inoculum density of the final substrate of  $10^7$  CFU  $g^{-1}$  (Mulero-Aparicio *et al.*, 2019). Additionally, olive plants transplanted into 1 L PVC pots filled with a 20% (weight/weight) mixture of sterile corn meal-sand and sterile peat moss were used as negative controls. All plants were incubated in a growth chamber at 20°C in the dark and 100% RH for 7 days. Subsequently, light and humidity parameters were progressively modified over 1 week until reaching 23°C, a 12-h photoperiod of fluorescent light [10,000 lux] and 70% RH, which were maintained until the end of the experiment. Plants were irrigated three times per week.

A randomized complete block design (three blocks) was used with nine biological treatments and two controls (positive and negative) as independent variables and five replicated olive plants per treatment and block ( $11 \times 3 \times 5 = 165$  olive plants).

#### 5.2.5.4. Disease severity assessment

DS was assessed according to the percentage of affected plant tissues (leaves and shoots) with symptoms of chlorosis, necrosis and/or defoliation. The plants were evaluated weekly for 16 weeks by means of a 0-16 severity rating scale. This scale is subdivided into four categories (0-25, 26-50, 51-75 and 76-100% of affected tissue) with

four values per category (0.25, 0.5, 0.75 and 1.0) (Varo *et al.*, 2018). DS data were used to calculate the relative area under the disease progress curve (RAUDPC) by means of the trapezoidal integration method (Campbell and Madden, 1990). Furthermore, the final DS, the disease incidence (DI; % of affected plants) and mortality (% of dead plants) were estimated at the end of the experiment as the percentage of symptomatic or dead plants, respectively. Three symptomatic plants per treatment or control were randomly selected at the end of the experiment to confirm the infection of the pathogen by fungal isolation. Basal stems of the plants were washed under running tap water for 2 h. Subsequently, small fragments of the affected tissue were cut and surface sterilized by dipping them in a 10% solution of commercial bleach (Cl at 50 g L<sup>-1</sup>) for 1 min, air-dried on sterilized filter paper for 10 min, and plated onto APDA. Petri dishes were incubated as described before, and the frequency of isolation of *V. dahliae* was estimated as percentage of reisolation.

#### **5.2.6. Data analysis**

All the experiments were conducted twice, and data from the two repetitions of each experiment were combined after checking for homogeneity of the experimental error variances by the F test ( $P \geq 0.05$ ). Subsequently, in all cases, data were tested for normality, homogeneity of variances, and residual patterns. For dual culture assay, a factorial ANOVA was conducted with "MGI" as dependent variable, and "BCAs", fungal isolates and their interaction as independent variables. Because significant differences were observed for "BCAs" ( $P \leq 0.0001$ ) and fungal isolates ( $P = 0.0002$ ), but not for their interaction ( $P = 0.0919$ ), a global one-way ANOVA was conducted for the two fungal isolates with "MGI" as dependent variable and "BCAs" as independent variable. For microsclerotia viability, a one-way ANOVA was conducted with "MSI" as dependent variable and "BCAs" as independent variables. The experiments on the effect of "BCAs" on conidia germination and on the disease development *in planta* have not been yet completed, and they are not showed in this present draft. In any case, we expect to analyse as follow: for the experiment on conidia germination, a factorial ANOVA will be conducted with "RGI" as dependent variable, and "BCAs", fungal isolates and their interaction as independent variables; for the *in planta* experiment, two one way ANOVAs will be conducted with RAUDPC or DS as dependent variables and "BCAs" as the independent variable. In all cases, treatments that did not show symptoms wer not included in the análisis. In all cases, treatment means were compared according to

Fisher's protected HSD test at  $P = 0.05$  (Steel and Torrie, 1985). Data on the final DI (% of affected plants) and mortality (% of dead plants) will be also analyzed by multiple comparisons for proportions tests at  $P = 0.05$  (Zar, 2010). Additionally, the Pearson correlation coefficients ( $r$ ) between the MGI, RGI and MSI of *V. dahliae*, and the RAUDPC will be calculated using the average values of the three variables for each of the BCAs evaluated. Data from this study were analyzed using Statistix 10.0 software (Analytical Software, 2013).

## 5.3 Results and Discussion

### 5.3.1 Effect of *Streptomyces* spp. on *Verticillium dahliae* viability

#### 5.3.1.1. Effect on mycelial growth by dual culture assay

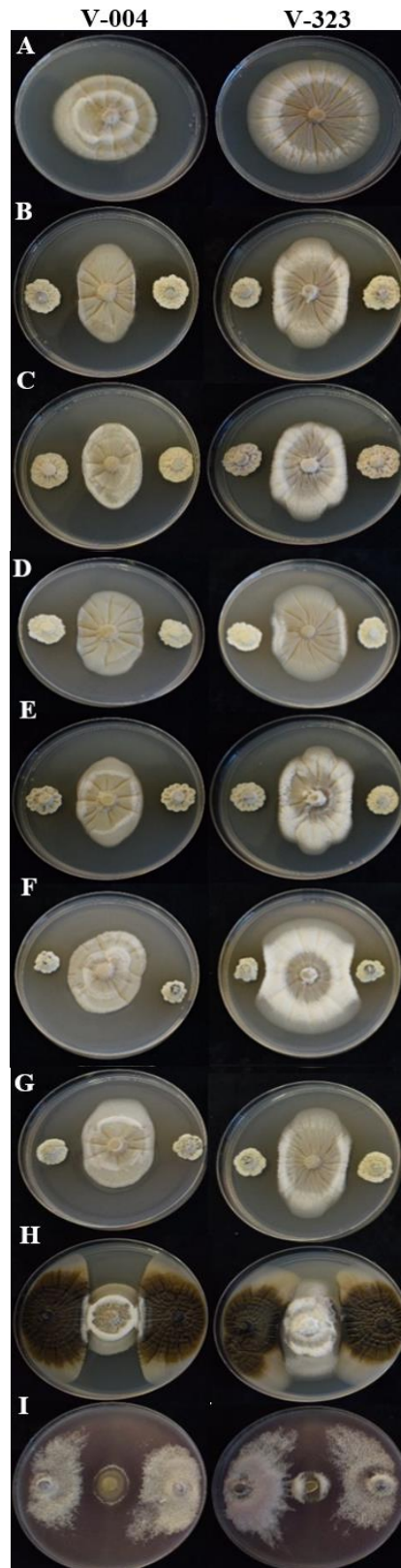
The six *Streptomyces* sp. strains and the two fungal BCAs F012 and AP08 showed a significant effect on MGI for both *V. dahliae* isolates V-004 and V-323 ( $P < 0.001$  in all cases) compared to the positive control. For all BCA treatments, *V. dahliae* isolates showed differences in mycelial growth, with V-232 growing more than V-004. The reference BCA *F. oxysporum* FO12 was the most effective on MGI followed by *A. pullulans* AP08; while the *Streptomyces* sp. strains showed a moderate effect. Among the *Streptomyces* strains, CBQ-EA-2, CBQ-B-8 and CBQ-CB-14 were the most effective, while the CBQ-EA-12 strain was the least effective, with MGI ranging from  $44.4 \pm 1.67$  to  $33.6 \pm 2.91\%$  for *Streptomyces* sp. CBQ-EA-2 and CBQ-EA-12, respectively (Table 5.2; Fig. 5.1). Several studies have demonstrated the potential of actinomycetes against a broad diversity of soil-borne fungal pathogens (Khair *et al.*, 2011; Dalal and Kulkarni, 2014; Kunova *et al.*, 2016; Dezfally *et al.*, 2018; Manigundan *et al.*, 2020; Rejón-Martínez *et al.*, 2022; Díaz-Díaz *et al.*, 2022, 2023). However, only few studies have reported the effect of *Streptomyces* spp. against *V. dahliae*. In this way, our results are in agreement with those obtained by Xue *et al.* (2013) since they showed that the strains *S. cyaneofuscatus* ZY-153, *S. kanamyceticu* B-49, *S. rochei* X-4 and *S. flavotricini* Z-13 had a significant effect on MGI of *V. dahliae* (D-J), with MGI ranging from 17.0 to 85.6 % for ZY-153 and B-49, respectively, compared to the positive control. Similarly, Bubici *et al.* (2013) showed that several *Streptomyces* spp. strains (StB-3, StB-6, StB-11 and StB-12) inhibited mycelial growth development of *V. dahliae*. Chen *et al.* (2021) also tested the effect of *S. globisporus* strains Act28 and Act7 on *V. dahliae*, with MGI from 70.6 and 80.4 %, respectively. Recently, Carlucci *et al.* (2022) demonstrated that *S.*

*albidoflavus* strain CARA17 inhibited mycelial growth of *V. dahliae* with the MGI values being 87.42, 77.15 and 71.30 % at 14, 21 and 28 days of incubation. Regarding the effect of the reference BCAs, the highest effect of FO12 agree with previous studies conducted by Mulero-Aparicio *et al.* (2019). However, the results obtained here for AP08 are in contrast with those showed by López-Moral *et al.* (2021), since they showed that AP08 did not had any significant effect on MGI compared to the positive control, although this BCA was highly effective reducing the DS in olive plants inoculated with *V. dahliae* isolate V-180. This contradictory effect could be associated with the virulence of the *V. dahliae* isolates since different isolates were tested in each study.

**Table 5.2.** Effect of biocontrol agents (BCAs) on mycelial growth of *Verticillium dahliae* in dual cultures *in vitro*.

BCA species	BCA strain	MGI (%) <sup>z</sup>
<i>Streptomyces</i> sp.	CBQ-B-8	42.8 ± 1.50 c
	CBQ-CB-14	42.3 ± 1.05 c
	CBQ-CD-24	37.3 ± 1.31 cd
	CBQ-EA-2	44.4 ± 1.67 c
	CBQ-EA-12	33.6 ± 2.91 d
	CBQ-EBA-21	39.0 ± 2.89 cd
<i>Aureobasidium pullulans</i>	AP08	52.1 ± 1.83 b
<i>Fusarium oxysporum</i>	FO12	76.4 ± 1.87 a

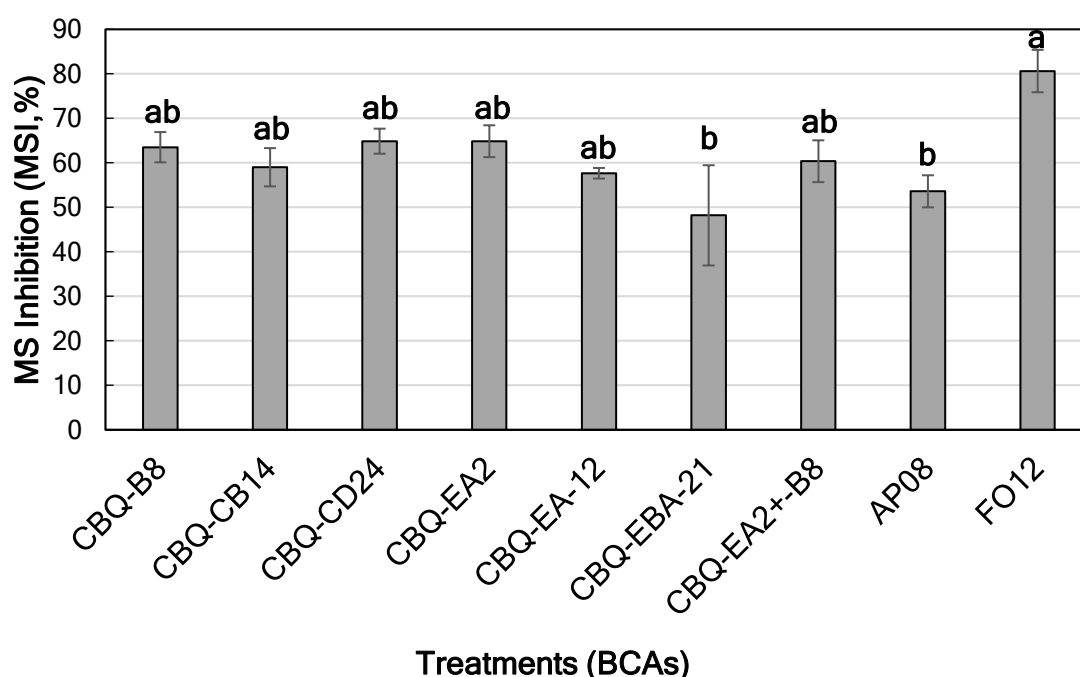
<sup>z</sup>Mycelial Growth Inhibition (MGI; %) was obtained after growing both the BCAs and *V. dahliae* (isolate V-004 or V-323) in dual cultures on PDA at 28°C for 14 days in the dark. Data represent the average of two 12 replicated Petri dishes for each BCA or control ± the standard error of the means, corresponding to two experiments, two *V. dahliae* isolates and three repetitions. Means followed by a common letter do not differ significantly according to Fisher protected HSD test at  $P = 0.05$ .



**Figure 5.1.** Antagonistic effect of BCAs against *Verticillium dahliae* isolates V-004 (left column) and V-323 (right column) in dual culture growing on PDA at 28°C for 14 days in the dark. The control or treatments are arranged in a row as follow: **A)** positive control (*V. dahliae* V-004 or V-323), **B-G,** *Streptomyces* spp. strains (**B)** CBQ-B-8, (**C)** CBQ-CB-14, (**D)** CBQ-CD-24, (**E)** CBQ -EA-2, (**F)** CBQ -EA-12, (**G)** CBQ -EBa-21, (**H)** *Aureobasidium pullulans* AP08, and (**I)** *Fusarium oxysporum* FO12.

### 5.3.1.2. Effect on Microsclerotia Viability

All the BCAs tested showed a significant effect on the inhibition of *V. dahliae* MS germination ( $P = 0.0218$ ) compared to the nontreated control. All the *Streptomyces* strains, including the treatment combining CBQ-EA-2 and CBQ-B8, showed similar effect, with MSI ranging between  $48.2 \pm 11.3$  and  $64.9 \pm 2.8$  or  $64.9 \pm 3.6$  % for *Streptomyces* sp. strains CBQ-EBA-21 and CBQ-CD-24 or CBQ-EA2, respectively. All they shared similarity with the reference BCA AP08 (MSI =  $53.6 \pm 3.6\%$ ); while *F. oxysporum* FO12 showed a significant highest effectiveness on MSI (MSI =  $80.6 \pm 4.8\%$ ) compared to the remaining treatments (Fig. 5.2). The effect of *Streptomyces* strains on the MS viability of *V. dahliae* has also been tested in previous studies. In this way, Xue *et al.* (2012) showed that culture filtrates of several *Streptomyces* sp. strains (B49, D184 and Act12) were able to inhibit the 100% of the germination of *V. dahliae* MS. Other authors showed that strains of *S. lividans* (Meschke and Schrempf, 2010) or *S. plicatus* (Aghighi *et al.*, 2004) were able to significantly reduce the viability of *V. dahliae* MS compared to the control.

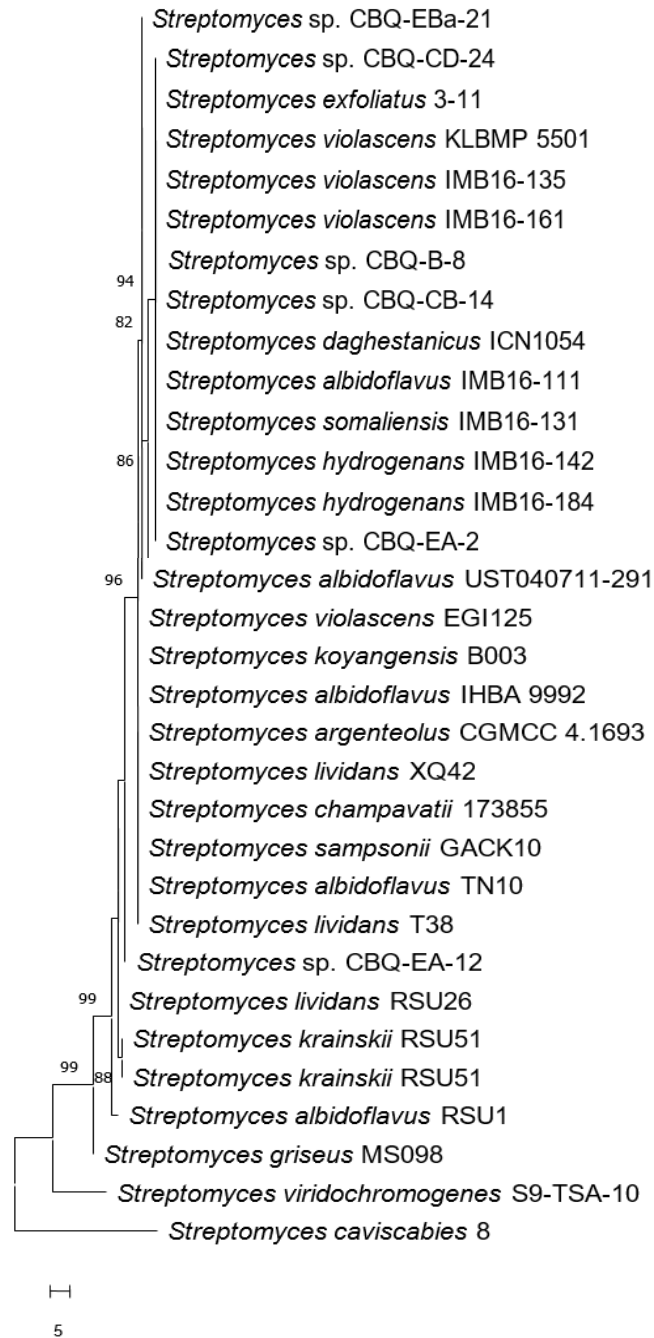


**Figure 5.2.** Effect of BCAs on the viability of MS [MS inhibition (MSI), %] of *Verticillium dahliae* in a naturally infested soil. Treatments with strains from *Streptomyces* were performed by irrigating the soil with 30 mL of a conidial suspension of each treatment adjusted at  $10^8$  conidia  $\text{ml}^{-1}$ . While *Fusarium oxysporum* FO12 and *Aureobasidium pullulans* AP08 treatment adjusted at  $10^6$  conidia  $\text{ml}^{-1}$ . Columns represent the average of six replicated plastic pots, and columns followed with a common letter do not differ significantly according to Tukey HSD test at  $P = 0.05$ . Vertical bars represent the standard error of the means.

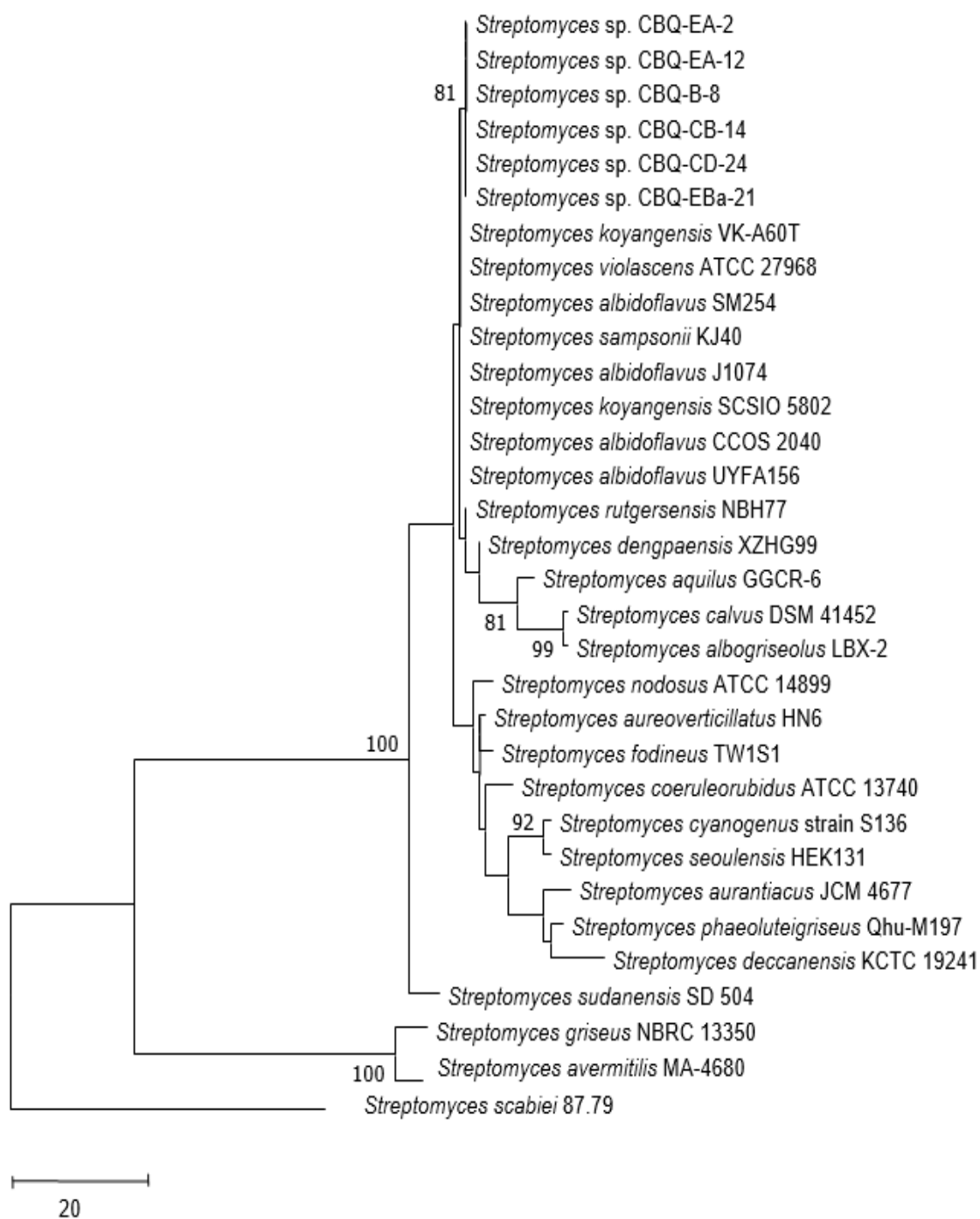
#### 5.4. Molecular characterization of *Streptomyces* strains

Amplification of the 16 and 23S rRNA gene of the six actinomycetes strains used in this study confirmed their identity as *Streptomyces* sp., with 90–99% similarity with the 16 and 23S rRNA sequence of relative close species in NCBI database. The results of the phylogenetic analysis of the 16 and 23S rRNA gene are shown in Figures 5.3 and 5.4. The final alignment of the 16S and 23S rRNA gene had 72 variable sites and 18 Parsimonious information sites; 198 Variable sites and 149 Parsimonious information, respectively.

Although several authors confirmed the identity of *Streptomyces* species by means of 16S or 23S rRNA sequencing (Cumsille *et al.*; 2017; Sarwar *et al.*, 2019; Chen *et al.*, 2021; Ayed *et al.*, 2022), we were not able to identify our *Streptomyces* strains beyond the genus level. In this way, several authors revealed that 16S gene is not sufficient to identify *Streptomyces* species (Guo *et al.*, 2008; Li *et al.*, 2023; Khadayat *et al.*, 2020; Komaki, 2022). These authors point out although 16S rRNA gene sequences are conventionally analysed in bacterial molecular identification, their resolution is not sufficient for species identification closely related. Thus, further research is needed towards identify the species of our *Streptomyces* strains. In this way, Lee and Wong (2009) suggested the BOX-PCR technique to identify *Streptomyces* species since the BOX A1R primer target and amplifies selectively the regions located BOX and can simultaneously survey many DNA regions scattered in the genome of bacteria. This is because the BOX repetitive sequences are interspersed throughout the bacterial genome and act on 154 base pair (bp)-box elements comprising 3 subunits (boxA 59bp, boxB 45bp, and boxC 50bp). Another molecular technique described in the literature that can discriminate closely related *Streptomyces* strains is the sequencing of protein-coding genes such as the gyrase B (*gyrB*) and tryptophan B (*trpB*) housekeeping genes (Rong and Huang, 2010). More recently, Khadayat *et al.* (2020) suggest the use of analysis of acid methyl esters, metabolite profiles and DNA-DNA hybridisation to improve the identity of *Streptomyces* species.



**Figure 5.3.** Evolutionary relationships of *Streptomyces* spp. based on the 16S rRNA gene sequences. The evolutionary history was inferred by Test Maximum Parsimony. One of five most parsimonious tree is showed (TL = 88; CI = 0.926; RI = 0.980; HI = 0.074; RC = 0.907). Bootstrap support values [MP, >70%] are shown at the nodes (2000 replications). *Streptomyces caviscabies* strain 8 was used as the outgroup. Evolutionary analyses were conducted in MEGA11 (Tamura *et al.*, 2021).



**Figure 5.4.** Evolutionary relationships of *Streptomyces* spp. based on the 23S rRNA gene sequences. The evolutionary history was inferred by Test Maximum Parsimony. One of five most parsimonious tree is showed (TL = 320; CI = 0,844; RI = 0,890; HI = 0,94; RC = 0,750). Bootstrap support values [MP, >70%] are shown at the nodes (2000 replications). *Streptomyces scabiei* strain 87.79 was used as the outgroup. Evolutionary analyses were conducted in MEGA 11 (Tamura *et al.*, 2021).

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## *Chapter 6*





## 6. GENERAL DISCUSSION

The present PhD Thesis was mainly conceived to characterize 60 actinomycetes strains belonging to the microbial collection of the Centro de Bioactivos Químicos of the Universidad Central "Marta Abreu" de Las Villas (Cuba). This characterization has been conducted based on their *in vitro* antagonistic activity by dual confrontation against *M. phaseolina* and *R. solani*, two of the main soil-borne fungal pathogens of common bean in Cuba. The most effective strains were also characterized according to their cellulolytic, chitinolytic and proteolytic extracellular enzymatic activity, as well as by their morphological and biochemical characters *in vitro*. In addition, the two most effective actinomycetes strains (CBQ-EA-2 and CBQ-B-8) against both *M. phaseolina* and *R. solani* were evaluated to determine their effect against the disease development on seedlings of common bean under semi-controlled conditions (*Chapter 3*). Due the high effectiveness observed for the seed treatment with both strains applied alone or in combination, the results *in planta* from *Chapter 3* were validated in the field establishing three experimental fields with soils naturally infested by *M. phaseolina* and *R. solani* (*Chapter 4*). Thus, these studies represent an advance in the search for biological, viable and eco-friendly alternatives for the environment, compared to the undesirable effects that conventional pesticides induce in the short and medium term, both for human health and for the soil itself. Several agricultural applications are attributed to actinomycetes, ranging from insecticides, fungicides and herbicides, as well as plant growth-promoting endophytes and as biocontrol agents for plant diseases, especially against soil-borne plant pathogenic fungi (Doolotkeldieva *et al.*, 2015; Gomes *et al.*, 2018; Gebily *et al.*, 2021; Rajeeswari *et al.*, 2021; Carlucci *et al.*, 2022).

The availability of actinomycetes strains isolated from the central region of Cuba, with demonstrated effect on the reduction of the DI and DS of root rot of common bean could constitute a biological alternative to be included in the programme for the management of diseases caused by these soil-borne pathogenic fungi in common bean, which has not been explored in Cuba until now. The first objective of this PhD Thesis was (*Chapter 2*) to carry out a comprehensive literature review on the use of actinomycetes as biocontrol agents against soil-borne fungal pathogens. Although several reviews about actinomycetes can find in the literature, we notice that no specific reviews about the biocontrol of soil-borne fungal pathogens by means of actinomycetes were available. So, we conceived this review towards to compile the main research studies

focused on the biocontrol of these pathogens by means of *Streptomyces* spp., the most frequent genus of actinomycetes used as BCAs. Through this review, we compiled a large number of research studies that demonstrate the potential of *Streptomyces* spp. strains as BCAs against a broad diversity of soil-borne fungal pathogens (Díaz-Díaz *et al.*, 2023). All this information was useful to hypothesize that our actinomycetes strains could be also potential BCAs against soil-borne pathogens that deserved to be tested against one of the most soil-borne disease of common bean in Cuba.

Based on phenotypic and biochemical characters, the 60 actinomycetes strains revealed typical characteristics of the genus *Streptomyces*, showing well-developed aerial mycelium, powdery or velvety appearance of colonies resulting from the formation of short and long, white to beige, hard and compact chains with age, similar to those observed by several authors (Shirling and Gottlieb, 1966; Bergey, 2005; Li *et al.*, 2016; Ayuningrum and Jati, 2021; Díaz-Díaz *et al.*, 2023). The identity of six representative strains (CBQ-EBa-21, CBQ-EA-12, CBQ-CD-24, CBQ-CB-14, CBQ-EA-2 and CBQ-B-8) from our collection was confirmed by sequencing the 16S and 23S rRNA genes (Chapters 3 and 5). The use of these two genes was not enough to identify the strains at species level and all they were identified as *Streptomyces* spp. Several *Streptomyces* species shared a high similarity (97.00-99.78%) with our strains in NCBI database. Although we can find many studies in the literature in which the authors are able to identify *Streptomyces* species only sequencing the 16S rRNA gene (Cumsille *et al.*; 2017; Sarwar *et al.*; 2019; Chen *et al.*; 2021; Ayed *et al.*; 2022), our results agree with Khadayat *et al.* (2020) since these authors indicate that the identity of *Streptomyces* species is not possible only by means of 16S and 23S rRNA sequences due to the close relationship between species. They suggest analysis of acid methyl esters, metabolite profiles and DNA-DNA hybridisation to improve the identity of *Streptomyces* species (Khadayat *et al.*, 2020).

Qualitative evaluation of extracellular enzyme activities showed significant differences between strains. Twenty of the 31 strains evaluated showed chitinolytic activity with a clearance zone around the colony ranging from 25.3 - 33.5 mm, cellulolytic activity ranged from 90.0 - 36.3 mm, while the proteolytic halo ranged from 51.5 - 27.0 mm (Chapter 3). Our results are in concordance with those showed by several authors who report the ability of *Streptomyces* spp. strains to produce high levels of extracellular

enzymes including cellulases, chitinases and proteases (Manigundan *et al.*, 2020; Selim *et al.*, 2021; Rani *et al.*, 2021).

*In planta* experiments under semi-controlled conditions aimed to determine the effect of seed treatments by *Streptomyces* sp. CBQ-EA-2 and CBQ-B-8, applied alone or in combination, against the disease development in seedlings of common bean of cv. Quivicán inoculated with *M. phaseolina* or *R. solani*, growing on both sterilized and natural soils. In general, there were significant differences between all treatments with respect to the nontreated control and between the two types of soils; for both DI and DS, the treatment combining the two *Streptomyces* sp. strains (CBQ-EA-2 + -B-8) showed a significantly higher effectiveness against both pathogens compared with the treatments using the strains applied alone, and similar effect to that observed in comparative commercial treatments such as the BCA *T. harzianum* A-34 or the chemical Celest® Top312FS. The DS was higher in plants grown in sterilized soils than in those grown in nonsterilized soils, with the efficacy of the different treatments also varying with the soil used (*Chapter 3*). Our results are in concordance with several research studies on such topic that can be found in the literature. Thus, Yadav *et al.* (2014) showed that *Streptomyces* sp. S160 reduce the DI of charcoal rot caused by *M. phaseolina* under greenhouse conditions in chickpea by 33.3% compared to the control. Alekhya *et al.* (2016) demonstrated that the strains *Streptomyces* sp. BCA-546 and CAI-8 significantly reduced the DI of charcoal rot in sorghum caused by *M. phaseolina* under semi-controlled conditions; while *S. parvulus* strain 10d reduced the DI of *R. solani* on green beans under semi-controlled conditions using sterilized and non-sterilized soil (Korayem *et al.*, 2020). Similarly, Fatmawati *et al.* (2020) evaluated 10 actinomycetes strains against *R. solani* on soybean seeds under controlled conditions, highlighting the disease suppressive activity of *Streptomyces* sp. strain ASR53 with 68% and 91% for sterile soil and nonsterile soil, respectively.

To validate the results *in planta* obtained from the *Chapter 3*, field experiments were carried out by establishing three experimental fields with soils naturally infested by *M. phaseolina* and *R. solani*, evaluating the same treatments described above. In the field, significant differences were observed for DI and DS between treatments compared to nontreated controls. The treatment combining *Streptomyces* spp. CBQ-EA-2+CBQ-B-8, significantly reduced DI and DS compared to the nontreated controls. Most of the *Streptomyces* treatments had a significantly lower effect than the BCA *T. harzianum* A-

34, but similar or superior to the chemical Celest® Top 312 FS. Several authors have also evaluated the efficacy of *Streptomyces* spp. against soil-borne fungal pathogens under field conditions obtaining similar results than those observed in our study (Xue *et al.*, 2013; Palazzini *et al.*; 2017; El-Shatoury *et al.*, 2020; Gebily *et al.*, 2021). El-Tarabily *et al.* (2009) showed that the combination of endophytic actinobacteria such as *Actinoplanes campanulatus*, *Micromonospora chalcea* and *S. spiralis* suppress damping-off and crown and root rot of cucumber with higher effectiveness than the fungicide metalaxyl. Similarly, the combination of *S. rochei* IT20 + *S. vinaceusdrappus* SS14 in the treatment of chilli inoculated with *P. capsici* significantly reduced DS by 40-60% compared to the control; moreover, these authors reported reduction of disease symptoms by 40-60% compared to the control, when chilli inoculated with *P. capsici* were treated with the combination of *S. rochei* IT20 + *S. vinaceusdrappus* SS14 (Abbasi *et al.*, 2020). Similarly, El-Shatoury *et al.* (2020) evaluated the efficacy of *S. flavoviridis* SF 1 and *S. variabilis* SF 2 strains against *Botrytis cinerea* on broad bean (*Vicia fabae* L.) seeds. These authors also showed that treatments combining the two strains significantly reduced DS and DI compared with the treatments using *Streptomyces* strains alone or with the nontreated control. Finally, studies conducted in the field by Gebily *et al.* (2021), showed that treatments with *S. griseus*, *S. rochei* and *S. sampsonii*, applied alone or in combination, against *S. sclerotiorum* on common bean significantly reduced the DS compared with the nontreated control.

As a culmination of the study carried out in the three experimental fields, the harvest yield and quality of the legumes were evaluated. It should be noted that all treatments, regardless of whether both *Streptomyces* sp. strains (CBQ-EA-2 and CBQ-B-8) were applied alone or in combination, were able to significantly increase the yield and quality of the legumes compared to the nontreated plants. Our results agree with those showed by several authors who reported that *Streptomyces* strains improve these agronomical parameters (Gopalakrishnan *et al.*, 2015; Htwe *et al.*, 2019; Gebily *et al.*, 2021; Gopalakrishnan *et al.*, 2021). For example, Gopalakrishnan *et al.*, (2015) showed that single applications of *Streptomyces* sp. CAI-17, CAI-68, CAI-78, KAI-26 or KAI-27 significantly increased chickpea (*Cicer arietinum* L.) crop yield compared to the nontreated control. Gebily *et al.* (2021) demonstrated that treatments with *S. griseus*, *S. rochei* or *S. sampsonii* significantly improve yield in common bean compared to the nontreated control.

In addition, as a complementary study of this PhD Thesis, the effect of six *Streptomyces* strains (CBQ-EBa-21, CBQ-EA-12, CBQ-CD-24, CBQ-CB-14, CBQ-EA-2 and CBQ-B-8) was evaluated against *V. dahliae*, the causal agent of Verticillium wilt of olive (Varo *et al.*, 2017; Trapero *et al.*, 2018; Mulero-Aparicio *et al.*, 2020; López-Moral *et al.*, 2022). The effect of actinomycetes strains was evaluated on the mycelial growth inhibition, and conidia and MS viability of *V. dahliae* by means of *in vitro* experiments as well as on the disease progress on olive plants. To conduct this study, two additional fungal BCAs, the non-pathogenic strain *F. oxysporum* FO12 and *A. pullulans* AP-08, were included for comparative purposes since they resulted highly effective in reducing VWO in previous studies (Mulero-Aparicio *et al.*, 2019; López-Moral *et al.*, 2021). All the BCAs tested i.e., the six actinomycetes, FO12 and AP-08, showed a significant effect on MGI of *V. dahliae* compared to the positive control. *Fusarium oxysporum* FO12 was the most effective on MGI, while the *Streptomyces* sp. strains showed a moderate effect. Similarly, Bubici *et al.* (2013) showed that several *Streptomyces* spp. strains (StB-3, StB-6, StB-11 and StB-12) inhibited mycelial growth development of *V. dahliae*. Chen *et al.* (2021) also tested the effect of *S. globisporus* strains Act7 and Act28 on *V. dahliae*, with MGI ranging between 70.6 and 80.4 %. Recently, Carlucci *et al.* (2022) showed that *S. albidoflavus* strain CARA17 inhibited the mycelial growth of *V. dahliae*, with MGI ranging from 87.42 to 71.30 %. Regarding the effect of *Streptomyces* on MS viability, our results agree with those obtained by Xue *et al.* (2012) because they showed that culture filtrates of several *Streptomyces* sp. strains inhibited the 100% of the germination of *V. dahliae* MS. In addition, Aghighi *et al.* (2004) also showed that *Streptomyces* spp. strains were able to significantly reduce the viability of *V. dahliae* MS compared to the control. Finally concerning the reference BCAs included in this study, the results on the effect of FO12 against *V. dahliae* were in concordance with those from previous studies conducted by Mulero-Aparicio *et al.* (2019). However, the results obtained for AP-08 were in contrast with those shown by López-Moral *et al.* (2021) since these authors revealed a low effect of AP-08 against the pathogen *in vitro* but a high effectiveness in reducing the disease severity in inoculated olive plants.

In summary, this PhD Thesis has been useful for qualitative characterization of the extracellular enzyme activities, the antagonism of the *Streptomyces* spp. strains as well as the *in vivo* studies against *M. phaseolina* and *R. solani* under semi-controlled

## *Chapter 6*

conditions. This research has allowed us to characterize promising *Streptomyces* strains as BCAs, and to have a biological alternative in the framework of the integrated management of the main common bean diseases caused by soil-borne pathogens in Cuba. In addition, the *Streptomyces* strains selected in this study have shown potential efficacy against other soil-borne plant pathogens, such as *Verticillium dahliae*, the causal agent of vascular wilt in olive trees, and other woody and herbaceous hosts.



## *Conclusions*







## CONCLUSIONS

1. The qualitative characterization of the extracellular enzyme activities, the antagonism of the *Streptomyces* spp. strains, as well as the *in vivo* studies against *M. phaseolina* and *R. solani* under semi-controlled conditions have allowed us to characterize promising strains as BCAs, and to have a biological alternative in the framework of the integrated management of the main common bean diseases caused by soil-borne pathogens in Cuba.
2. A total of 62% of the *Streptomyces* strains revealed a high cellulolytic capacity with a halo between 80 to 90 mm in diameter, and 90% of them developed a halo with considerable extension around the colony, which denotes an important cellulolytic hydrolysis.
3. The 66.7% of the *Streptomyces* strains showed chitinolytic capacity, highlighting the CBQ-EBa-5 strain, with a 35.5 mm clearance halo surrounding the colony.
4. Based on the phenotypic and biochemical characters, all the strains were identified as *Streptomyces* spp. The sequencing and phylogeny of the 16S and 23S rRNA genes were not enough to identify our *Streptomyces* strains at the species level, with further research being needed to determine their specific identification.
5. The *in vitro* efficacy of the 60 *Streptomyces* potential strains against *M. phaseolina* and *R. solani*, varied depending on the soil-borne pathogen tested. Forty and twenty-five out of the 60 actinobacterial strains inhibited the mycelial growth of *M. phaseolina* and *R. solani*, respectively. Among the most effective strains, 18 of them showed a common effect against both pathogens, with the CQB-EA2, and -CD-24 being among the strains that showed greater efficacy in inhibiting mycelial growth of the two pathogens.
6. The treatments conducted using a mix of two *Streptomyces* sp. strains (CBQ-EA-2 + CBQ-B-8) showed a significant greater effectiveness against both pathogens compared to treatments performed with the two strains separately. In addition, the effectiveness of the two combined *Streptomyces* strains in controlling the disease was similar to that observed for other comparative treatments, such as *T. harzianum* A-34 or the chemical (Celest® Top312FS).
7. The experiments conducted in the field corroborate the results obtained under controlled conditions. Therefore, treatments by coating common bean seeds with

the CBQ-EA-2 or CBQ-B-8 strains of *Streptomyces* sp., applied before sowing both separately and in combination, significantly reduced the DI and the DS of root rot disease associated with *M. phaseolina* and *R. solani* compared to untreated control plants. In addition, these treatments were able to improve the quality of legumes, significantly increasing crop yield.

8. Six selected *Streptomyces* sp. strains and two fungal BCAs *Fusarium oxysporum* FO12 and *Aureobasidium pullulans* AP08 used as reference showed a significant effect on the mycelial growth of *V. dahliae* compared to the untreated control, with FO12 being the most effective treatment, followed by AP08 or the *Streptomyces* strains that showed a moderate effect.
9. The six *Streptomyces* sp. strains and the two fungal BCAs FO12 and AP08 also showed a significant effect on the microsclerotia viability of *V. dahliae*, but in this case most of the *Streptomyces* strains showed similar effect than that observed for the reference strain FO12, including the treatment combining CBQ-EA-2 and CBQ-B8. The least effective treatments were AP08 and CBQ-EBA-21.

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*Scientific production during the PhD*





## Scientific production during the PhD

### PEER-REVIEWED JOURNAL PUBLICATIONS

1. **Díaz-Díaz, M.**, Bernal-Cabrera, A., Trapero, A., Medina-Marrero, R., Leiva-Mora, M., Agustí-Brisach, C. 2023. Actinomycetes as potential biocontrol agents against soil-borne plant pathogens: A review. *Plants*, X(X): XXX. (IF: 4.658; Q1).
2. **Díaz-Díaz, M.**, Bernal-Cabrera, A., Trapero, A., Medina-Marrero, R., Sifontes-Rodríguez, S., Cupull-Santana, R.D., García-Bernal, M., Agustí-Brisach, C. 2022. Characterization of actinobacterial strains as potential biocontrol agents against *Macrophomina phaseolina* and *Rhizoctonia solani*, the main soil-borne pathogens of *Phaseolus vulgaris* in Cuba. *Plants*, 11(5): 645. (IF: 4.658; Q1).
3. **Díaz-Díaz, M.**, Bernal-Cabrera, A., Trapero, A., Jiménez-González, A., Medina-Marrero, R., Cupull-Santana, R.D., Águila-Jiménez, E., Agustí-Brisach, C. 2023. Biocontrol of root rot complex disease of *Phaseolus vulgaris* by *Streptomyces* sp. strains in the field. *Crop Protection*, 165: 106164. (IF: 3.036; Q1).
4. Arencibia-Martínez, Y., Medina-Marrero, R., García-Bernal, M., Parra, M.G., Cupull-Santana, R.D., **Díaz-Díaz, M.**, Fexas, M.Á.S. 2019. Utilización de *Streptomyces* sp. RL8 como agente probiótico en pollos de la raza Leghorn. ene-jun: 107116. ISSN Versión Impresa 1816-0719, ISSN Versión en línea 19949073. *The Biologist*, 17. (IF: 0.056; Q4).
5. Mazón-Suástegui, J.M., Ojeda-Silvera, C.M., García-Bernal, M., Avilés-Quevedo, M.A., Abasolo-Pacheco, F., Batista-Sánchez, D., Tovar-Ramírez, Arcos-Ortega, F., Murillo-Amador, B., Nieto-Garibay, A., Ferrer-Sánchez, Y., Morelos-Castro, R.M., Alvarado-Mendoza, A., **Díaz-Díaz, M.**, Bonilla-Montalván, B. 2019. A Agricultural Homoeopathy: A New Insight into Organics. In Multifunctionality and Impacts of Organic Agriculture. *IntechOpen*. 157–174 DOI: <http://dx.doi.org/10.5772/intechopen.84482>. (IF: 0.016; Q4).

### INTERNATIONAL CONFERENCES PAPERS

1. II Convención Científica Internacional UCLV. "Efecto de cepas de actinomicetos sobre *Rhizoctonia Solani* Kühn y *Macrophomina Phaseolina* en frijol común (*Phaseolus Vulgaris* L.)". **Díaz-Díaz, M.**, Medina-Marrero, R., Bernal-Cabrera,

- A., Rodríguez-Soris, E., Mesa-Marcilla, J.P., Cupull-Santana, R.D., García-Bernal, M., Martínez-Arencia, Y., Águila-Jiménez, E., Casanova-González, M. Cayo Santa María, Cuba. 26-29 October 2021. Oral presentation.
2. Primer Simposio Internacional Protección de Plantas. "Selección de cepas de actinomicetos como agentes de biocontrol de *Colletotrichum gloeosporioides* en limón (*Citrus aurantifolia*)". **Díaz-Díaz, M.**, Bernal-Cabrera, A., Medina-Marrero, R., García-Bernal, M., Augusto-Henriques, D.A. On-line, Ecuador, 2020. Oral Presentation.
  3. IX Congreso Científico de Estudiantes de doctorados de la Universidad de Córdoba, "Actinomicetos antagonistas de *Macrophomina phaseolina* en el cultivo de judía en Villa Clara, Cuba". **Díaz-Díaz, M.**, Bernal-Cabrera, A., Trapero-Casas, A., Agustí-Brisach, C. On-line, España, 3- 6 de mayo, 2021.
  4. III Convención Científica Internacional UCLV. Simposio Internacional sobre Desarrollo Agropecuario Sostenible". "Efectividad *in vivo* de cepas de actinomicetos como agente de biocontrol contra *Rhizoctonia solani* en *Phaseolus vulgaris*". **Díaz-Díaz, M.**, Bernal-Cabrera, A., Trapero-Casas, A., Medina-Marrero, R., Cupull-Santana, R.D., García-Bernal, M., Águila-Jiménez, E., Casanova-González, M., Mederos-Hurtado de Mendoza, Y., Agustí-Brisach, C. Cayo Santa María, Cuba, 22-30 noviembre 2021, Oral presentation.
  5. XIV Simposio Internacional de Biotecnología Vegetal, 2022. "Caracterización de actinobacterias y su actividad antagónica *in vitro* frente a *Rhizoctonia solani* y *Macrophomina phaseolina*". **Díaz-Díaz, M.**, Bernal-Cabrera, A., Trapero-Casas, A., Agustí-Brisach, C., Medina-Marrero, R., Cupull-Santana, R.D., García-Bernal, M., Casanova-González, M. Cayo Santa María, Cuba, 3-6 de mayo de 2022. Poster.
  6. XX 'Congreso de la Sociedad Española de Fitopatología'. "Actinobacterias como potenciales agentes de biocontrol frente a *Macrophomina phaseolina* y *Rhizoctonia solani* asociados con la podredumbre de raíz y tallo de la judía común". **Díaz-Díaz, M.**, Bernal-Cabrera, A., Trapero-Casas, A., Agustí-Brisach, C., Medina-Marrero, R., Cupull-Santana, R.D., García-Bernal, M., Casanova-González, M. Valencia, España, 24- 26 october de 2022, Oral presentation.

## PARTICIPATION IN RESEARCH PROJECTS

NA223VC001-011. Estudios toxicológicos y ecotoxicológicos de bioproductos, productos químicos y residuales (Servicios profesionales). PI.: Osmani Marrero Chang. Participation as researcher. 01/09/2018-30/07/2022.

PT 223VC003-001. Obtención de cepas de origen microbiano para el manejo de insectos, plagas y enfermedades en el cultivo de frijol y garbanzo (Producción de alimentos). PI.: Ubaldo Álvarez Hernández; Ricardo Medina Marrero (CIAP/CBQ). Participation as researcher. 01/01/2021-30/12/2023.

## AWARDS

**Premio CITMA Provincial a la Investigación.** “Actinobacterias como agentes de biocontrol contra *Macrophomina phaseolina* y *Rhizoctonia solani* en frijol común”. **Díaz-Díaz, M.**, Bernal-Cabrera, A., Trapero, A., Medina-Marrero, R., Sifontes-Rodríguez, S., Cupull-Santana, R. D., García-Bernal, M., Agustí-Brisach, C. Villa Clara, Cuba, 2022.

**Mención.** Evento de las BTJ a nivel universitario (UCLV). "Evaluaciones ecotoxicológicas y toxicológicas *Trichoderma asperellum* cepa Ta. 13 (SevetriC)" Autores: Sotolongo-González, K., Marrero-Chang, O., Águila-Jiménez, E., Castañedo-Hernández, Z., Seijo-Wals, M., Meneses-Marcel, A., **Díaz-Díaz, M.**, Cupull-Santana, R.D., Medina-Marrero, R., Casanova-González, M., Martínez-Arencia, Y., Lago-Durán, C., Monteagudo, X., García-Moya, C., Benavides-Rodríguez, M., Contreras-Morffi, T., Mederos-Hurtado de Mendoza, Y., Ferrer-Cruz, J., García-Pérez, M., Sifontes-Rodríguez, S.

**Mención.** Viabilidad de las cepas de actinomicetos en la pelletización del frijol común (*Phaseolus vulgaris* L.). **Díaz-Díaz, M.**, Bernal-Cabrera, A., Medina-Marrero, R., Cupull-Santana, R.D., García-Bernal, M., Casanova-González, Águila-Jiménez, E. October 2020.

**Premio relevante.** Fórum Científico Técnico de base. Castañedo-Hernández, Z., Águila-Jiménez, E., Marrero-Chang, O., **Díaz-Díaz, M.**, Mollinedo-Diogo, N., Meneses-Marcel, A., Seijo-Wals, M., Sotolongo-González, K., Hernández-González, R., Cupull-Santana, R.D., Casanova-González, M., Sifontes-

Rodríguez, S., Medina-Marrero, R., Mederos-Hurtado de Mendoza, Y., García-Moya, C. "Evaluación Ecotoxicológico y Toxicológico de *Trichoderma asperelum* (SevetriC) en *Poecilia reticulata* y *Eisenia foetida*". July 2018.

**Premio relevante.** Fórum Científico Técnico de base. Evaluación toxicológica y ecotoxicológica de los bioproductos IHPLUS®, CBQ-AgroG® y CBQ-VTC®. Águila-Jiménez, E., Martínez-Díaz, A., Marrero-Chang, O., Sotolongo-González, K., Castañedo-Hernández, Z., Meneses-Marcel, A., Mollineda-Diogo, N., **Díaz-Díaz, M.**, Seijo- Wal, M., Chaviano-Montes de Oca, C., Sifontes-Rodríguez, S., Ramos-Méndez, R., Mederos-Hurtado de Mendoza, Y. October 2020.