USE OF SOME *STREPTOMYCES* SPECIES AS BIO-AGENTS TO CONTROL BEAN WHITE ROT DISEASE CAUSED BY *Sclerotinia sclerotiorum*

By

DOHA ALAAELDIN SAAD SOUFI ESMAIL GEBILY
B.Sc. Agric. Sci. (Plant Pathology), Fac. Agric., Fayoum Univ., 2010
M.Sc. Agric. Sci. (Plant Pathology), Fac. Agric., Fayoum Univ., 2015

THESIS

Submitted in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

In

Agricultural Sciences
(Plant Pathology)

Department of Plant Pathology
Faculty of Agriculture
Cairo University
EGYPT

2021

Format Reviewer Vice-Dean for Graduate Students and Research

Name of Candidate: Doha Alaa Eldin Saad Gebily **Degree**: Ph.D.
Title of Thesis: Use of some *Streptomyces* species as bio-agents to control bean white rot disease caused by *Sclerotinia sclerotiorum*
Supervisors: Dr. Gamal Amin Mohamed Ghanem
Dr.Mona Mahmoud Maher Ragab
Dr. Ayat Mahmuod El-Sayed
Dr.Nour El-Deen Kamel Soliman (**Late**) &Dr. Tawfik Hafez Abd El-Moity (**Late**)
Department: Plant Pathology
Approval: 27 /5/2021

ABSTRACT

White mold disease, caused by *Sclerotinia sclerotiorum* the devastating pathogen, attacks green bean (*Phaseolus vulgaris* L.) and several crops worldwide. The present investigation was conducted to introduce some antagonistic microorganisms as novel antifungal substances to be an alternative and secure method to effectively control the disease. Out of 24 isolates, three *Streptomyces* isolates were molecularly characterized. PCR amplification of the fungus pathogen and *Streptomyces* isolates 16S rDNA gene sequences exhibited amplicons of around 535bp and 1300bp, respectively. The characterized *Streptomyces* isolates were sequenced and submitted into Genebank under accession numbers *i.e., S. griseus* (MT210913 "DG5"), *S. rochei* (MN700192 "DG4") and *S. sampsonii* (MN700191 "DG1"). Phylogenetic tree of the nucleotide sequence analysis of the three *Streptomyces* spp. indicated that *S. griseus* MT210913 was closely related to *S. sampsonii* MN700191 (96%), secondly ranked by *S.rochei* MN700192 (93.1%). Afterward, the antifungal activity of *Streptomyces* spp. against *S. sclerotiorum* was evaluated *in vitro* and *in vivo* (in the greenhouse and field). *In vitro* tests, proved that the reduction percentages in mycelial growth of pathogen ranged between 31.4-60.17%, indicating that *S. rochie* gave the highest inhibition percent. Incorporations of *Streptomyces* spp culture filtrate components into culture media proved that *S. sampsonii* was more efficient as a bioagent in reducing mycelial growth pathogen by 84.50%. When the effectiveness of the bioagent volatile compounds was evaluated, the inhibition of the pathogen growth ranged between 54.50-72.54%, respectively, revealing that *S. rochei* was the highest inhibitor followed by *S. griseus*. Results of GC-Mass analysis revealed the presence of 44, 54 and 47 diverse secondary metabolites compounds produced by *S. sampsonii* DG1, *S. rochie* DG4, and *S. griseus* DG5, respectively. Examining parasitic activity of *Streptomyces* spp upon *S. sclerotiorum* was demonstrated by light and scanning electron (SEM) micrographs exhibited the interaction as deformation, contraction, and collapse in the mycelium of the pathogen. Viability and germination of pathogen sclerotia were reduced when they dipped into the Streptomyces culture broth for 10, 20, and 30 days. Application of the 3 *Streptomyces* spp. in the field proved a great potential to control the disease. The results suggested that the 3 *Streptomyces* spp. and their secondary metabolites can be biofertilizers as enhancers in plant growth and potential biocontrol agents for controlling bean white rot disease. Finally, the author suggests that the phenomenon of the mycoparsitism in case of Streptomyces could be named actinoparasitism.

Key words: *S. sclerotiorum*, *Streptomyces* spp., antifungal activity, green bean

CONTENTS

	Page
INTRODUCTION	1
REVIEW OF LITERATURE	5
1. White rot disease and its economic importance	6
a. Survey of white rot disease	11
b. Isolation and identification of the causal organism	12
c. Molecular identificationof the pathogen (*S. sclerotiorum*)	13
2. Actinomycetes (Actinobacteria)	14
a. Difination, classification and characteristics	14
b. Prevalence of Actinobacteria	17
c. Isolation and identification of Actinobacteria	18
d. Molecular characterization of Actinobacteria	19
e. Characterization of *Streptomyces* spp. bioactive secondary metabolites utilizing Gas chromatography-mass spectrometry (GC-Mass) analysis	23
f. Application of Actinobacteria to control plant diseases	29
1. Potential activities of Actinobacteria *in vitro*	30
2. Application of Actinobacteria *in vivo*	33
3. Postharvest studies	41
MATERIALS AND METHODS	45
1. Isolation and purification of the causal pathogen	45
2. Molecular identification of the pathogen	45
3. Isolation and purification of Streptomyces isolates	47
4. Molecular characterization of Streptomyces isolates	50
5. GC-Mass analysis of *Streptomyces* spp. secondary metabolites	52
a. Extraction of the secondary metabolites	52
b. Gas chromatography-mass spectrometry (GC-Mass analysis)	52
6. Antifungal bio-assays of *Streptomyces* spp *in vitro*	53
a. Dual culture technique	53
b. Mechanism of parasitism	54

Page

c. Impact of light and darkness conditions on the efficiency of *Streptomyces* spp. antifungal compounds on the pathogen mycelial growth suppression 56

d. Impact of filtration and heat treatment on the efficacy of *Streptomyces* spp. antifungal compounds on the pathogen mycelial growth suppression..... 57

e. Antifungal activity of volatile compounds produced by *Streptomyces* spp. 57

f. Impact of soaking pathogen sclerotia into culture broth of *Streptomyces* spp. on germination and mycelium growth….. 58

7. Greenhouse experiments..... 59

a. Reactions of green bean cultivars to the infection by *S. sclerotiorum* under greenhouse conditions..... 60

b. Impact of inoculum potentiality of *S. sclerotiorum* on green bean cv. Paulista white mold diseases incidence..... 60

c. Determine the efficacy of *Streptomyces* spp. *in vivo*..... 61

8. Field experiments..... 61

a. Influence of spraying *Streptomyces* spp. at different times on the percentages of disease incidence and plant parameters & components..... 63

b. Effect of spraying *Streptomyces* spp. at different concentrations on the percentages of disease incidence and plant parameters & components..... 64

c. Impactof spraying *Streptomyces* spp. at different numbers on the percentages of disease incidence and plant parameters & components..... 64

9. Post-harvest and Shelf-life experiments..... 64

a. Influence of applying *Streptomyces* spp. at different times on controlling the disease and extending shelf-life period of bean pods..... 66

b. Effect of applying *Streptomyces* spp. at different concentrations on controlling the disease and extending shelf-life period of bean pods..... 66

Page

c. Influence of spraying *Streptomyces* spp. at different numbers on controlling the disease and extending shelf-life period of bean pods..... 67

Statistical Analysis 67

RESULTS AND DISCUSSION..... 69

1. Isolation, purification and molecular characterization of the pathogen..... 70

2. Molecular characterization of the *Streptomyces* spp...... 76

3. GC-Mass analysis of *Streptomyces* spp. secondary metabolites 80

4. *In vitro* evaluation of antifungal activity of *Streptomyces* spp. 101

a. *In vitro* inhibitory potential of *Streptomyces* spp. against *S. sclerotiorum* 101

b. Examination of the parasitic mechanisms of *S. rochei* using slide technique..... 105

c. Impact of light and darkness conditions on antifungal activity in suppression of pathogen mycelium growth..... 107

d. Impact of filtration and heat treatment on antifungal activity of *Streptomyces* spp. in suppression of pathogen mycelium growth 111

e. Inhibitory effects of VOCs produced by *Streptomyces* spp. on mycelium growth of *S. sclerotiorum* 114

f. Impact of soaking pathogen sclerotia into culture broth of *Streptomyces* spp. on sclerotia germination and mycelium growth..... 119

5. Greenhouse experiments..... 121

a. Reactions of green bean cultivars to the infection by *S. sclerotiorum* under greenhouse conditions..... 121

b. Impact of inoculum potentiality of *S. sclerotiorum* on green bean cv. Paulista white mold diseases incidence..... 123

c. Evaluation of *Streptomyces* spp. antifungal activity against *S. sclerotiorum* under greenhouse conditions..... 124

6. Biological control of white mold of green bean disease by *Streptomyces* spp. under field conditions (*in vivo*) .. 126

a. Times of application experiments..... 127

Page

a.1. Influence of applying *Streptomyces* spp. at different times on the percentages of disease incidence under field conditions **127**

a.2. Impact of applying *Streptomyces* spp. at different times on green bean plant vigour under field conditions. **130**

a.3. Influence of applying *Streptomyces* spp. at various times on chemical components of the treated plants..... **132**

b. Impact of culture broth concentrations **136**

b.1. Impact of applying *Streptomyces* spp. at different concentrations on bean white rot disease incidence..... **136**

b.2. Impact of applying *Streptomyces* spp. at various concentrations of on green bean plant vigour **139**

b.3. Effect of applying *Streptomyces* spp. at different concentrations on chemical components of the treated plants. **141**

c. Number of applications..... **144**

c.1. Impact of applying *Streptomyces* spp. at different numbers on the percentage of disease incidence..... **144**

c.2. Influence of applying *Streptomyces* spp. at different numbers on green bean plant vigour..... **146**

c.3. Effect of applying *Streptomyces* spp. at different numbers on chemical components of the treated plants **149**

7. Post-harvest and shelf life. **152**

a. Influence of applying *Streptomyces* spp. at different times on shelf-life of bean pods at 5ºC and 20±2ºC..... **152**

b. Impact of applying *Streptomyces* spp. at different concentrations on shelf life of bean pods at 5ºC and 20±2ºC.... **157**

c. Effect of applying *Streptomyces* spp. at different numbers on shelf- life of bean pods at 5ºC and 20±2ºC..... **159**

CONCLUSION..... **164**

RECOMMENDATIONS..... **165**

SUMMARY..... **166**

REFERENCES **173**

ARABIC SUMMARY .....

LIST OF TABLES

No.	Title	Page
1.	Sources and locations of the *Streptomyces* isolates	**48**
2.	Composition of the used media to grow the pathogen and actinobacteria…….……..	**49**
3.	Sharing (%) of nucleotide sequences between the three *Streptomyces* spp. and others isolates from different geographical regions available in the GenBank……..	**78**
4.	GC-Mass analysis of the ethyl acetate extracts of *Streptomyces* spp. …….…….……..	**87**
5.	Inhibitory potential of *Streptomyces* spp. against fungus *S. sclerotiorum in vitro*……...…….……	**104**
6.	Impact of light and darkness conditions on antifungal activity efficiency in the suppression of pathogen mycelium growth …	**108**
7.	Suppression of pathogen mycelial growth by antifungal activity of *Streptomyces* spp. culture filtrate using Millipore membrane or heat treatment. ……...…….	**113**
8.	Inhibitory effects of VOCs produced by *Streptomyces* spp. on mycelium growth of *S. sclerotiorum in vitro*……	**117**
9.	Impact of soaking pathogen sclerotia into culture broth of *Streptomyces* spp. on sclerotia germination and mycelium growth.....	**120**
10.	Reactions of green bean cultivars to the infection by *S. sclerotiorum* under greenhouse conditions	**122**
11.	Impact of inoculum potentiality of *S. sclerotiorum* on green bean cv. Paulista white mold diseases incidence…….	**123**
12.	Evaluation of *Streptomyces* spp. on controlling *S. sclerotiorum* under greenhouse onditions…….	**125**
13.	Influence of applying *Streptomyces* spp. at different times on the percentages of disease incidence under field conditions….	**129**

No.	Title	Page
14.	Impact of applying *Streptomyces* spp. at different times on green bean plant vigour under field conditions.....	**133**
15.	Influence of applying *Streptomyces* spp. at various times on chemical components of the treated plants.....	**134**
16.	Impact of applying *Streptomyces* spp. at different concentrations on bean white rot disease incidence.....	**140**
17.	Impact of applying *Streptomyces* spp. at various concentrations of on green bean plant vigour	**143**
18.	Effect of applying *Streptomyces* spp. at different concentrations on chemical components in the treated plants.....	**145**
19.	Impact of applying *Streptomyces* spp. at different numbers on the percentage of disease incidence.....	**148**
20.	Influence of applying *Streptomyces* spp. at different numbers on green bean plant vigour.....	**150**
21.	Effect of applying *Streptomyces* spp. at different numbers on chemical components of the treated plants.....	**153**
22.	Influence of applying *Streptomyces* spp. at different times on shelf life of bean pods at 5ºC and 20±2ºC.....	**158**
23.	Impact of applying *Streptomyces* spp. at different concentrations on shelf life of bean pods at 5ºC and 20±2ºC.....	**159**
24.	Effect of applying *Streptomyces* spp. at different numbers on shelf life of bean pods at 5ºC and 20±2ºC.....	**162**

LIST OF FIGURES

No	Title	Page
1	Disease severity index for white rot of bean pods showed various degrees of severity as the scale from 0-10……..	**65**
2a.	Symptoms of naturally-infected bean plants cv. Paulista exhibited water-soaked, olive-green lesions, followed by white-cottony mold with collapsed tissues and sclerotia formation……..	**72**
2b.	Formation of mycelium on the collar region & above the ground ground level (leaves & pods) …….....	**72**
2c.	Sclerotia formed on the infected pod…….	**73**
2d.	Pure culture of *S. sclerotiorum* grown on Cook's medium	**73**
2e.	Multi apothecia produced from one sclerotium ……..……	**73**
3a.	Products of polymerase chain reaction amplification isolate using the universal primers ITS-1 and ITS-4. A product (amplicon) of 553 bp length was amplified in green bean isolate (Is). M= Leader and Is=isolated fungus. …..……..	**75**
3b.	Phylogenetic tree obtained from the alignment of nucleotide sequences of the Egyptian isolate and fifteen sequences from GenBank. All 15 sequences show their GenBank codes and countries of origin…….	**75**
4a.	Products of polymerase chain reaction amplification isolates using the two universal primers 63-forward and 1387-reverse. Products(amplicons) of 1300 bp length were amplified from the three isolates of Streptomyces (Is). M = Leader, 1,2 and 3 = *isolated S. sampsonii*, *S. rochei*, and *S. griseus*. …….	**78**
4b.	Phylogenetic tree based on 16S rRNA gene sequence of *S. rochei*, *S. griseus*, and *S. sampsonii* with the reference Streptomyces 16S rRNAgene available in the GenBank……...	**79**

No	Title	Page
5.a	Chemical structures of some secondary metabolites produced from GC-Mass analysis crude extract of *Streptomyces* spp. ……..…….……...	98
5.b	Relative abundance of *Streptomyces* spp…….……	100
6.	Inhibitory potential of *Streptomyces* spp. against fungus *S.sclerotiorum In vitro*.……..…	103
7.	Light micrograph shows the colonization of *S. rochei* on *S. sclerotiorum* mycelium. **a)**: Untreated mycelium of *S. sclerotiorum*; **b)**: Smaller and thinner *S. rochei* mycelium,broken and distorted of *S. sclerotiorum* mycelium and **c)**: *S. rochei* growing a long the mycelium of *S. sclerotiorum* ……..……...	109
8.	Scanning electron micrographs of the deformation of the fungal mycelium. **a)**: Untreated (control) mycelium of *S. sclerotiorum*, **b)**: Bioagent *S. rochei* induced pores in the mycelium of *S. sclerotiorum* and **c)**: *S. rochei* induced swollen, deformation, distortion and lysis of the pathogen mycelium as well as its grown inside the hyphae……	110
9.	Impact of light and darkness conditions on culture filtrates efficiency in the suppression of pathogen mycelial growth …….…….……..…..	111
10.	Suppression of *S. sclerotiorum* mycelial growth by antifungal activity of *Streptomyces* spp. culture filtrate using Millipore membrane or heat treatment……..	114
11.	Effect of volatile organic compounds produced by the three *Streptomyces* spp. on mycelium growth of *S. sclerotiorum.* …….…….….....	117
12.	Light micrograph shows that volatiles compounds of *S. rochei* induced mycelial growth abnormalities in *S. sclerotiorum*: (**a**) mycelium of *S. sclerotiorum* (**b&c**) hyphal deformation, swollen and dark staining indicating hyphae death. …….…….……	118

13. Suppression of sclerotia germination and mycelial growth after soaking into broth of the three *Streptomyces* spp. for 10, 20 & 30 days**.** …….. **121**