



#### Suez Canal University Faculty of Science Ismailia

# Potency of actinomycete metabolites as biocontrol agents against cotton leafworm, *Spodoptera littoralis* (Boisduval)

A Thesis Submitted by Mohamed Khaled Ahmed Mohamed Diab B.Sc. (May, 2004)

> For The Degree of Master of Science (M.Sc.)

> > IN (Applied Microbiology)

#### TO

Botany Department Faculty of Science Suez Canal University

2019

#### **Abstract**

The present study aims to investigate the potency endophytic actinomycete of certain secondary metabolites in controlling Spodoptera littoralis. Under laboratory conditions, the efficiency of ethyl acetate extracts of seventy endophytic actinomycete strains against laboratory and field cultures of S. littoralis larvae (4<sup>th</sup> instar) were tested. The studied endophytic strains reflected the biochemical diversity of these strains in biocontrol aspect against S. littoralis larvae. The results indicated high potency for the crude metabolites of seven strains at a concentration of 100 mg/ml. The seven strains belonged to Streptomyces (2 strains), Nocardioides (2 strains), Kitasatospora (2 strains), Pseudonocardia (1 strain); and were originally recovered from the Asteraceae host plants Seriphidium herba-album and Artemisia judaica L. against S. littoralis. Bioactivity of the metabolic extracts ranged between direct toxicity on the 2<sup>nd</sup> day of feeding against the laboratory S. littoralis strain; to latent effects that appeared from the 6<sup>th</sup> day in the field *S. littoralis* strain. The molecular studies of the most potent endophytic strain have directly contributed to drawing the way to complete the evaluations of this strain. The assessment of volatile organic compounds of the most potent endophytic actinomycete strain, which belonged to the genus Kitasatospora, has shown the short-time effectiveness of controlling S. littoralis larvae. This promising result leads us in the future to the extensive

study of this technology and its application as an environmentally friendly. *Kitasatospora* ES2 crude metabolite resulted in significant histopathological impacts which explained a part of the biological activity. The biochemical assessments revealed significant deficiencies to  $\alpha$  esterase, protease, and lactate dehydrogenase enzymatic activities of *S. littoralis* differ from the commercial product, Radiant 12 % SC. The thin layer chromatographically analysis was a separator point for distinguishing between *Kitasatospora* ES2 crude metabolite and the commercial product, Radiant 12 % SC.

### **Contents**

### Subject

#### Page

Acknowledgement	i
Abstract	ii
Contents	iii
List of Figures	vii
List of Tables	viii
List of Abbreviations	ix
<b><u>1. Introduction</u></b>	1
1.1. Statement of problem	2
1.2. Aim of work	3
2. Literature Review	5
2.1. Actinomycetes	5
2.2. Endophytic actinomycetes	8
2.3. Importance of endophytic actinomycete metabolites	10
2.4. Importance of <i>Streptomyces</i> ' metabolites	12
2.5. Microbial consortia as a source of active metabolites	14
2.6. History of biocontrol and contribution of actinomycetes in that field	14

2.7. Spinosad, a commercial insecticide from actinomycetes, (CAS Number: 168316-95-8)	17
2.8. The Egyptian cotton leafworm, <i>Spodoptera littoralis</i> (Boisd.)	19
<b>2.9.</b> Microbial volatile compounds (mVOCs) and its role(s) in pests' control	22
2.10. Histological aspects in insecticides assays	25
2.11. Biochemical aspects in insecticides assays	27
2.11.1. Esterase (EST) enzymes	27
i. Specific esterase enzymes	27
ii. Non-Specific esterase enzymes	30
a. Alpha esterase [α-esterase (EC 3.1.1.1)]	30
b. Beta esterase [β-esterase (EC 3.1.1.2)]	31
2.11.2. Peptidases family enzymes; Protease (EC 3.4.21.112)	31
2.11.3. Oxidoreductases family enzymes; LDH- Lactate Dehydrogenase (EC 1.1.1.27)	32
3. Materials and Methods	33
3.1. Chemicals, reagents and tools	33
3.2. Cultures	33
<b>3.2.1.</b> Source of endophytic actinomycete strains	33

<b>3.2.2. Production of actinomycetes inocula</b> from the stock source	35
i. Culture refreshment on starch casein (SC) agar	35
ii. Maintainance in 20% glycerol	35
iii. Maintainance as lyophilized cultures	36
3.3. Fermentation and extraction	37
3.3.1. Secondary metabolites production	37
3.3.2. Extraction of organic metabolites	37
<b>3.4. Reared insects, laboratory and field</b> <i>Spodoptera littoralis</i> strains	47
<b>3.4.1. Rearing protocol of the laboratory</b> <i>S. littoralis</i> strain, and offspring	47
3.4.2. Rearing technique of the field S. <i>littoralis</i> strain	49
<b>3.5.</b> Commercial insecticide product, Radiant 12 % SC	5(
3.6. Screening of bioactivity	5
<b>3.6.1.</b> Bioactivity of the crude metabolites against the 4 <sup>th</sup> larval instar of the laboratory <i>S. littoralis</i> strain	51
<b>3.6.2.</b> Toxicity impacts of the crude metabolites on the field <i>S. littoralis</i> strain	52
<b>3.7.</b> Molecular confirmation of the most promising endophytic actinomycete strain	54

3.7.1. Taxonomic affiliation and phylogenetic	
analysis based on partial 16S rRNA gene	55
	33
i. PCR amplification	56
ii. PCR product purification and DNA sequencing	56
3.7.2. Computational analysis	57
<b>3.7.3.</b> Screening of the active biosynthetic PKS-I (EC 2.3.1.233) and NRPS (EC 6.3.2.1-49 group) genes of <i>Kitasatospora</i> ES2	58
<b>3.8. Detailed investigations of <i>Kitasatospora</i> ES2 secondary crude metabolites on <i>S. littoralis</i></b>	59
<b>3.8.1.</b> Toxic effects of <i>Kitasatospora</i> ES2 secondary crude metabolites	59
<b>3.8.2. Biological effects of <i>Kitasatospora</i> ES2 secondary crude metabolites</b>	60
<b>3.8.3.</b> Evaluation of 1 <sup>st</sup> , 2 <sup>nd</sup> and 3 <sup>rd</sup> extract of <i>Kitasatospora</i> ES2 extracts	60
<b>3.8.4.</b> Effect of the crude volatile organic compounds (VOCs) of <i>Kitasatospora</i> ES2 on <i>S. littoralis</i>	61
3.8.5. Histopathological investigations of <i>Kitasatospora</i> ES2	63
<b>3.8.6.</b> Biochemical effects of <i>Kitasatospora</i> ES2 (Biochemical assay) on <i>S. littoralis</i>	64
3.8.6.1. Samples preparation	64
3.8.6.2. Total soluble protein	
assessment	66

3.8.6.3. Ace	tylcholines	sterase	(EC
3.1.1.7) determination .			•••••
3.8.6.4. Nor determination	ı–specific	este	erases
3.8.6.5. Determ activity, protease enzyn	ination one (EC 3.4	f prote .21.112)	olytic
3.8.6.6. Lactate EC 1.1.1.27) determina	Dehydrog tion	genase (]	LDH,
3.9. Thin layer chromat bioactive fraction(s) crude metabolite	ography ( of <i>Kitasa</i>	(TLC) fo tospora	or the ES2
3.10. Statistical analysis	;		••••
1 Doculto			
<b>4.</b> <u><b>NESUITS</b></u>	•••••		••••
4.1. Screening of bioact	ivity		• • • • • •
4.1.1. Bioactivity o extracts against the 4 laboratory <i>S. littoralis</i> s	f the cru <sup>th</sup> larval i train	de meta instar o	abolic f the
4.1.2. Toxicity in metabolites on the field	npacts of <i>S. littorali</i>	f the o is strain	crude
4.2. Molecular analys taxonomic affiliatior FS2	is for co 1 of	nfirming <i>Kitasato</i>	g the spora
4.2.1. Phylogenetic	analysis e	stablish	ed on
partial 165 rKNA gene	sequence		
4.2.2. Phylogene Kitasatospora ES2 (EM	etic an CC2291) s	nalysis train	of

4.2.3. Screening for active biosynthetic	
PKS-I (EC 2.3.1.233) and NRPS (EC 6.3.2.1-49 group) genes for Kitasatospora ES2EMCC2291	86
4.3. Detailed investigations of <i>Kitasatospora</i> ES2 (EMCC2291) secondary crude metabolites on <i>S. littoralis</i>	86
4.3.1. Toxic effects of <i>Kitasatospora</i> ES2 (EMCC2291) secondary crude metabolites	86
4.3.2. Biological effects of <i>Kitasatospora</i> ES2 (EMCC2291) secondary crude	
metabolites	88
<b>4.3.3.</b> Evaluation of 1 <sup>st</sup> , 2 <sup>nd</sup> and 3 <sup>rd</sup> extract of <i>Kitasatospora</i> ES2 (EMCC2291)	90
4.3.4. Effect of the volatile organic compounds (VOCs) of <i>Kitasatospora</i> ES2 (EMCC2291)	92
4.3.5. Histopathological investigations of <i>Kitasatospora</i> ES2 (EMCC2291)	93
i. Deformities in the cuticle layer and muscles of the treated <i>S. littoralis</i>	93
ii. Deformities in the midgut tissues of the treated <i>S. littoralis</i>	96
4.3.6 Biochemical effects of <i>Kitasatospora</i> ES2 (EMCC2291) (Biochemical assay)	100
4.3.6.1. Acetylcholinesterase (EC 3.1.1.7) determination	100
4.3.6.2. Non–specific esterases determination	100

4.3.6.3. Determination of proteolytic activity, protease enzyme (EC 3.4.21.112)	100
4.3.6.4. Lactate Dehydrogenase (LDH, EC 1.1.1.27) determination	101
4.4. Thin layer chromatography (TLC) for the bioactive fraction(s) of <i>Kitasatospora</i> ES2 (EMCC2291) crude metabolite	108
5. Discussion	110
Summary	118
Conclusion and recommendations	121
References	123
Appendices	167
Arabic Summary	

#### **List of Figures**

#### Title Page **Figure** (1): Crop damage caused by *S. littoralis* in 3 Egypt. **Figure** (2): Phylogenetic tree of Actinobacteria 7 based on 1,500 nucleotides of 16S rRNA (Gao and Gupta, 2012). Figure (3): Growth of actinomycetes on solid agar 8 media (Qinyuan et al., 2016). 9 **Figure** (**4**): Plant-endophyte symbiosis and ecological perspectives (Wani et al., 2015). Figure (5): 9 Graphical representation of natural product(s) discovery approach from endophytes (Alvin et al., 2014). Streptomyces spp. role in production of **Figure** (**6**): 13 metabolites and enzymes for supporting crop protection (Rey and Dumas. 2017). **Figure** (7): Molecular structure of the commercial 18 product Spinosad (Cas no.: 168316-95-8). Spinosyn A, R=H & Spinosyn D, R=CH<sub>3</sub> (Crouse, et al., 2007). **Figure** (**8**): Graphical representation focusing on 25 the role of volatile compounds released bv bacteria and their biocontrol potential impacts and applications (Audrain et al., 2015). **Figure** (**9**): Structure of acetylcholinesterase 29

(AChE, EC 3.1.1.7) (Heide, 2012).

\_\_\_\_

Figure (10):	Mode of action of acetylcholinesterase 30 (AChE, EC 3.1.1.7) in neurotransmission (Čolović <i>et al.</i> , 2013).	)
Figure (11):	Relation between the studied 34 endophytic actinomycete strains.	ł
Figure (12):	Preparation of actinomycetes crude 39 metabolites.	)
Figure (13):	Schematic flowchart showing the steps 40 of experimental design.	)
Figure (14):	Cotton leafworm, <i>S. littoralis</i> , life <b>49</b> cycle.	)
Figure (15):	Flowchart illustrating the treatment of laboratory and field <i>S. littoralis</i> with crude actinomycete metabolites under constant laboratory conditions.	3
Figure (16):	Experimental design to evaluate the 62 crude volatile organic compounds effect of <i>Kitasatospora</i> ES2.	2
Figure (17):	Histological wax blocks and slides for $64$ the $4^{\text{th}}$ treated larval instar of <i>S</i> . <i>littoralis</i> .	1
Figure (18):	TLC chromatography for preliminary 72 fractionation of <i>Kitasatospora</i> ES2 crude metabolite.	2
Figure (19):	Lethality and developmental defects of the <i>Kitasatospora</i> ES2 crude metabolite on 4 <sup>th</sup> instar laboratory <i>S. littoralis</i> larvae.	5
Figure (20):	Toxicity and latent effects of <b>78</b> actinomycetes' metabolic extracts on the field <i>S. littoralis</i> strain.	3

- Figure (21): Nucleotide sequence of the partial 82 sequencing 16S rRNA gene of *Kitasatospora* ES2 (EMCC2291) strain.
- Figure (22): G + C content plotting of 82 *Kitasatospora* ES2 (EMCC2291) sequence.
- Figure (23): 16S rRNA partial sequencing of 83 *Kitasatospora* ES2 (EMCC2291).
- Figure (24): Microphotograph of *Kitasatospora* ES. 84
- Figure (25): Neighbor-joining phylogenetic position 85 of *Kitasatospora* ES2 and related taxa based on 16S rRNA partial gene sequences.
- Figure (26): Toxic effects of *Kitasatospora* ES2 87 (EMCC2291).
- Figure (27): Biological effects of *Kitasatospora* 89 ES2 (EMCC2291).
- **Figure (28):** Biological effects of 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> **90** extract of *Kitasatospora* ES2 (EMCC2291).
- **Figure (29):** Comparison of 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> extract **91** effects of *Kitasatospora* ES2 (EMCC2291).
- Figure (30): Effect of volatile fraction(s) of 92 *Kitasatospora* ES2 (EMCC2291).
- Figure (31): Light micrographs of longitudinal 94 sections of histopathological deformities on the *S. littoralis* larval cuticle layer after 3 days posttreatment, Showing muscles deformities, (100× H&E).

- Figure (32): Light micrographs of longitudinal 95 sections of histopathological deformities on the *S. littoralis* larval cuticle layer after 3 days posttreatment, (400× H&E).
- Figure (33): Light micrographs of transverse 98 sections of histopathological deformities in *S. littoralis* larval midgut tissue after 3 days posttreatment, (100× H&E).
- Figure (34): Light micrographs of transverse 99 sections of histopathological deformities in *S. littoralis* larval midgut tissue after 3 days post treatment, (400× H&E).
- Figure (35): Showing Acetylcholinesterase (EC 102 3.1.1.7) determination.
- Figure (36): Showing  $\alpha$ -esterase (EC 3.1.1.1) 103 determination.
- Figure (37): Showing  $\beta$ -esterase (EC 3.1.1.2) 104 determination.
- Figure (38): Showing Protease (EC 3.4.21.112) 105 determination.
- Figure (39): Showing Lactate Dehydrogenase 106 (LDH, EC 1.1.1.27) determination.
- Figure (40): Biochemical assessment of the 108 enzymatic activities of the laboratory *S. littoralis* treated with *Kitasatospora* ES2 extract.
- Figure (41): Preliminary separation and detection 109 for the fraction(s) of *Kitasatospora* ES2 (EMCC2291) crude metabolite.

#### **List of Tables**

#### Title Page

- Table (1):Selected actinomycete strains and<br/>their respective host plants (El-<br/>Shatoury et al. 2006).41
- Table (2):The 70 studied actinobacterial<br/>strains, their host plants,<br/>physiological activities, and<br/>bioactivities (El-Shatoury et al.<br/>2006).43
- Table (3):Bioactivity of the crude metabolic<br/>extracts against the 4<sup>th</sup> laboratory<br/>*S. littoralis* larvae at concentration<br/>100 mg/mL.77
- Table (4):Bioactivity of the crude metabolic<br/>extracts against the 4<sup>th</sup> field S.<br/>*littoralis* larvae at concentration<br/>100 mg/mL.79
- Table (5):Sequence similarity showing the<br/>query and identity of<br/>*Kitasatospora* ES2 and its closest<br/>type strains based on the partial<br/>16S rRNA gene sequence.81
- Table (6):Biological effects of *Kitasatospora*89ES2 (EMCC2291) crude extract.
- Table (7):Biological effects of 1st, 2nd and 3rd91extract of Kitasatospora ES2<br/>(EMCC2291).ES2

- Table (8):Biochemical<br/>analysisanalysis<br/>forfor102Acetylcholinesterase(AchE, EC3.1.1.7)enzyme activity.
- Table (9):Biochemical analysis for α- 103<br/>esterase (EC 3.1.1.1) enzyme<br/>activity.
- Table (10):Biochemical analysis for  $\beta$  104esterase (EC 3.1.1.2)enzymeactivity.
- Table (11):Biochemical analysis for protease105(EC 3.4.21.112) enzyme activity.
- Table (12):Biochemical analysis for Lactate106Dehydrogenase(LDH, EC1.1.1.27) enzyme activity.
- **Table (13):** Statistical and biochemical analysis**107**for some enzymatic activitieswhich might be responsible for thephenotype potency.





## فعالية نواتج أيض الأكتينوميسيتات في المكافحة الحيوية لدودة ورق القطن

رسالة مُقدمة من محمد خالد أحمد محمد دياب بكالوريوس العلوم (مايو ٢٠٠٤)

للحصول على درجة الماجستير في العلوم في علم النبات (الميكروبيولوجي التطبيقي)

إلى

7.19