



Zagazig University
Faculty of Science
Botany and Microbiology Department

**STUDY THE PESTICIDAL ACTIVITY OF THE
BIOFILM PRODUCER GRAM POSITIVE *BACILLI*
AGAINST COWPEA WEEVIL.**

A Thesis

Submitted for the degree

Of

Doctor of philosophy in Science

(Microbiology)

By

Shahera Moabed Mohamed El-Sayed

M.Sc. of Science (Microbiology) Zagazig University (2016)

Botany and Microbiology Department
Faculty of Science
Zagazig University

2021

ABSTRACT

The present study aimed to determine and developed a new bioinsecticidal compounds which can be used as alternative to synthetic insecticides. It was considered more safety to human and the environment, in addition to it was more efficient and less toxic. So a laboratory study was investigated the effect of different *Bacillus* biofilms and their extracellular matrix (ECM) on the adult's mortality and reproduction of the cowpea weevil, *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae). In the investigated study among thirty Gram positive bacilli bacterial isolates, only four isolates recorded the highest abilities to biofilm formation also showed the heights efficacy against the cowpea weevil, at different concentrations 5, 10 and 20%. Results determined that the percentage of mortality of *C. maculatus* adults ranged between 13.36 and 63.43%, and it significantly increased by increasing the concentration of ECMs. Whereas the latent larval mortality did not exceed 31.42%, which was recorded at the highest concentration (20%) with ECM of the isolate no. 13, moreover, all the tested concentrations recorded a significant reduction in egg hatchability and ,female fecundity but the highest activity recorded by ECM of isolate no. 13 compared to other tested isolates. So, the most potent biofilm-producing isolate no. 13 was identified by using 16S rDNA gene sequence as *Bacillus flexus* S13, and submitted in GenBank under accession number MK292147. Also, this study throw light on the chemical composition of the extracellular matrix (ECM) of *Bacillus flexus* S13; biofilm' and its potential activity as insect biocontrol agents, in addition to investigated the insecticidal efficacy of ECM of *Bacillus flexus*; S13 against adults of *Callosobruchus maculatus*. The toxicity results revealed LC₅₀ values as (22.6455 g/100ml and 10.8524 g/100ml) after 24 and 48 hr. respectively. Also, the Biochemical assays revealed a significant

CONTENTS

Subject	Page
INTRODUCTION	1
Aim of the work	4
REVIEW of LITERATURE	5
I- Cowpea weevil, <i>Callosobruchus maculatus</i> . (Coleoptera: Bruchidae)	5
II- Microorganisms as biocontrol of insect	6
1- Important of <i>Bacillus</i> as biological control agent	8
2- Characteristic of <i>Bacillus flexus</i>	11
3- Rhizosphere: A unique source of microorganisms	11
III- Bacterial biofilms	12
IV -Factors affecting on biofilm formation	16
1- Effect of temperature	16
2- Effect of incubation period	18
3- Effect of growth media	18
4- Effect of carbon Carbon sources	19
V - Biochemichal studyies of biofilm on cowpea weevil	20
VI - Toxicological impacts	22

MATERIALS AND METHODS	24
I- Collection of soil and insect samples	24
II- Isolation technique	25
III- Determination of Biofilm formation	26
IV- Production of biofilm and ECM extraction	29
V- Bioassay of biofilm (ECM) activity against cowpea weevil	31
VI- Identification of the selected bacterial isolates	31
VII- Factors affecting biofilm formation by <i>Bacillus flexus</i> S13	38
VIII- Toxicological and biological impacts of <i>Bacillus flexus</i> S13; ECM on cowpea weevil.	39
RESULTS	43
1- Isolation of bacterial isolates from different soil samples.	43
2 - Screening of biofilm formation by <i>Bacillus</i> isolates	44
3- Entomopathogenicity of biofilm produced by the tested isolates.	46
4- Identification of the most effective bacterial isolates	52
5- Environmental factors affecting biofilm formation of <i>Bacillus flexus</i> S13	56
a- Effect of incubation temperatures	56
b- Effect of incubation period	58
c- The effect of different carbon source	60
d- The effect of growth media	61

6- Chemical characterization of ECM extract of <i>B. flexus</i> S13	63
7- Toxicological and biological impacts	68
8-Biochemical response of Cowpea weevil to <i>B. flexus</i> S13 ECM	70
DISCUSSION	72
SUMMARY	84
CONCLUSION	86
REFERENCES	87
ARABIC SUMMARY	

List of Table

No.	Title	Page
1	Phosphate Buffer	28
2	Isolation, purification isolates from different soil	43
3	Qualitative and quantitative determination of the biofilm formation by <i>bacillus</i> isolates and screening for insecticidal activities of cowpea weevil	45
4	Effect of different concentration of ECM from the four selected isolates on Mortality percentage of adults of cowpea weevil	48
5	Susceptibility of cowpea weevil adults towards different <i>bacillus</i> isolates ECM after 24hr exposure.	51
6	Susceptibility of cowpea weevil adults towards different <i>Bacillus</i> isolates ECM after 48hr. exposure	52
7	Morphological characteristics, biochemical confirmatory tests for the most potent bacterial isolate	53
8	Effect of incubation temperatures on growth and biofilm formation by <i>B. flexus</i> S13	57
9	Effect of incubation period on growth and biofilm formation by <i>B. flexus</i> S13.	59
10	Effect of carbon source on growth and biofilm formation by <i>B. flexus</i> S13	60
11	Effect of growth media on growth and biofilm formation by <i>B. flexus</i> S13.	62

No.	Title	Page
12	Cumarine compounds identified by GC/MS.in ECM of <i>B. flexus</i> S13.	63
13	Flavonoid compounds identified by GC/MS.in ECM of <i>B. flexus</i> S13.	64
14	Terpenoid compounds identified by GC/MS.in ECM of <i>B. flexus</i> S13.	66
15	Susceptibility of cowpea weevil adults towards <i>Bacillus flexus</i> S13 ECM after 24 and 48hr. exposure	68
16	Effect of LC ₅₀ of ECM produced by <i>B. flexus</i> S13 on cowpea weevil	69
17	Effect of LC ₅₀ of ECM produced by <i>B. flexus</i> S13 on total protein, total lipid and chitin contents of adults expressed as mg/g. body weight (M ± SE).	70

List of Figures

No	Title	Page
1	Stages of biofilm formation	15
2	Qualitative assaying of the biofilm formation by isolate No.13 using Congo red agar method (CRA).	36
3	Effect of different concentration of ECM from the four selected isolates on Mortality percentage of adults represents as (M±SE) and statistical treatments were performed using ANOVA for differences compare means in each concentration separately using Tukey's HSD test. ANOVA parameters of 5, 10, 20% conc. respectively were (LSD =1.628, 1.711, 1.509; P<0.05).	49
4	Effect of different concentration of ECM from the four selected isolates on number of eggs per female represent means (M±SE) and statistical treatments were performed using ANOVA for differences compare means in each concentration separately using Tukey's HSD test. ANOVA parameters of 5, 10, 20% conc. respectively were (LSD =1.628, 1.711, 1.509; P<0.05) .	49
5	Effect of different concentration of ECM from the four selected isolates on Hatchability percentage % (M±SE) and statistical treatments were performed using ANOVA for differences compare means in each concentration separately using Tukey's HSD test. ANOVA parameters of 5, 10, 20% conc. respectively were (LSD =1.628, 1.711, 1.509; P<0.05) .	50

No	Title	Page
6	Effect of different concentration of ECM from the four selected isolates on Mortality percentage of larval represent means (M±SE) and statistical treatments were performed using ANOVA for differences compare means in each concentration separately using Tukey's HSD test. ANOVA parameters of 5, 10, 20% conc. respectively were (LSD =1.628, 1.711, 1.509; P<0.05) .	50
7	Alignment sequence of <i>B. flexus</i> S13	54
8	Phylogenetic trees analysis of <i>Bacillus flexus</i>	55
9	Agarose gel electrophoresis of 16SrDNA gene. Lanes: 1.DNA Ladder; <i>Bacillus flexus</i> S13	56
10	The effect of different temperature on growth and biofilm formation by <i>B. flexus</i> S13.	58
11	The effect of different incubation period on growth and biofilm formation by <i>B. flexus</i> S13	59
12	The effect of different carbon source on growth and biofilm formation by <i>B. flexus</i> S13	61
13	The effect of different growth media on growth and biofilm formation by <i>B. flexus</i> S13	62
14	GC/MS chromatogram of methanol extract of ECM produced by <i>B. flexus</i> S13, and the major bioactive compounds	67

No	Title	Page
15	Effect of LC ₅₀ of ECM produced by <i>B. flexus</i> S13 on total protein ,total lipid and chitin contents of adults expressed as mg/g. body weight (M ± SE), and statistical treatments were performed using ANOVA for differences comparing means with Tukey's HSD test. ANOVA parameters were LSD = 1.628, 1.711, 1.509; P < 0.05	71