Potential exploitation of Bacillus flexus biofilm against the cowpea weevil, *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae)

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**Abstract**

The present study aimed to investigate new insecticidal compounds used as environmentally safe alternative to synthetic insecticides. A laboratory study was conducted to investigate the effects of *Bacillus* biofilms and their extracellular matrix (ECM) on the adult’s mortality and reproduction of the cowpea weevil, *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae). Among 30 bacterial isolates, only 4 exhibited high abilities to form biofilm and showed potentials against the pest at different concentrations 5, 10 and 20%. Results showed that the mortality percentage of *C. maculatus* adults ranged between 13.36 and 63.43% overall and it increased significantly with raising the concentration of ECMS. While the latent larval mortality did not exceed 31.42%, which was recorded with ECM of isolate no. 13 at the highest concentration (20%). Further, all tested concentrations reduced female fecundity and egg hatchability. The most potent biofilm-producing isolate having the highest adverse activity of ECM extract was identified as *Bacillus flexus* S13, using 16S rDNA gene sequence and submitted in GenBank under accession number MK292147. These results demonstrated that *B. flexus* S13 biofilm is a good strategy for the development of new biocontrol agents against *C. maculatus*.

**Keywords:** Biocontrol, Biofilm, Cowpea weevil, *Callosobruchus maculatus*, Bacillus flexus

**Background**

Legumes are a sustainable and inexpensive meat alternative because of its high protein content, thus are considered the second most important food source after cereals. Cowpea, *Vigna unguiculata* (L) (Walpers, Fabaceae), is an important edible legume crop in many parts of the world, especially in tropical and subtropical regions (Maphosa and Jideani, 2017). Production of cowpea is affected by the cowpea weevil, *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae) which causes economic losses. It is considered a cosmopolitan pest ranked as the principal post-harvest pest of cowpea in the tropics, causing substantial quantitative and qualitative losses manifested by seed perforation, deformation and reductions in weight so the market value and germination ability of seeds decrease (Singh et al., 1979).

Misuse of insecticides harmed the non-target pests and may accelerate resistance development, so various strategies have been developed to control the target pest in a safe manner; microorganisms are indeed an inexhaustible source of wide range of novel useful bioactive molecules to be used as biological control agents on different economic pest.

In this status, fetching economic, ecologically safe, and sustainable strategies became a priority. Some species of the genus *Bacillus* fulfill all the above-mentioned requirements; therefore, exploitation and trading have been recommended by the U.S. Food and Drug Administration.

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Bacillus genus biofilms could represent as a good strategy for the development of new antimicrobial agents for fighting plant diseases, whether by increasing its resistance to environmental stresses and allowing colonization in the plant rhizosphere or more steadily enhancing plant growth and health. Mostly, it all returns to the extracellular matrix (ECM) composition (López et al., 2010 and Vlamakis et al., 2013). The extracellular matrix of biofilms consist of polysaccharides, extracellular DNA, and proteins (Lasag and Penadés 2006); in spite of their composition, they differ largely from species to species (Flemming and Wingender 2010).

Despite the utilization of biofilms as inoculants, extracellular matrix benefits and characterization are less studied. Therefore, this study aimed to investigate the potential role of biofilm produced by Bacillus spp. to control the cowpea weevil. Up till now, there are no previous biocontrol studies on cowpea weevil by Bacillus biofilms.

Materials and methods
Isolation of Bacillus spp. from soil
Six soil samples were collected from different localities in Sharkia province, Egypt according to the methods described by Johnson et al. (1959). The protocol for isolation of Bacillus spp. from soil, described by Wollum (1982), was used with some modifications. One gram of each soil sample was mixed with 10 ml sterile distilled water. Thereafter, supernatants were diluted by 10-fold serial dilution as 10^2 up to 10^-7 dilutions in saline solution. One milliliter of soil dilution was spread on a nutrient agar plate (4 plates for each dilution) and incubated at 35 °C for 24 h. The different-looking bacterial colonies developed on each plate were selected to sub-culturing and purifying, using nutrient agar medium. These colonies were characterized according to the key of Bergey’s Manual of Systematic Bacteriology (Holt et al., 1994) and depending on Gram’s stain reaction, morphological and biochemical tests. The confirmed Bacillus isolates were tested for their ability to produce biofilm.

Detection of biofilm-forming isolates
Congo red agar (CRA) method described by Bose et al. (2009) was used to detect biofilm production in CRA medium, which was prepared with brain heart infusion: sucrose: agar and Congo red stain (37:50:10 and 8 g/l, respectively). Congo red stain was separately prepared as concentrated aqueous solution and autoclaved at 121 °C for 15 min, then added to the autoclaved brain heart infusion agar with sucrose at 55 °C, CRA plates were inoculated with test organisms and incubated aerobically at 37 °C for 24 h. Black colonies with a dry crystalline consistency indicated biofilm production. The experiment was repeated 3 times.

Quantity estimation of biofilm formation
Thirty Bacillus cultures were isolated and screened for their biofilm formations. The overnight cultures were grown in 96-well micro titer plate, filled with brain heart infusion broth medium (BHI), then the plates were incubated for 48 h at 30 °C.

The biofilm formation by each Bacillus culture was measured and quantified, using Crystal Violet (CV) assay as described by Castelijn et al. (2012). After incubation, the wells were gently washed 3 times with phosphate-buffered saline (PBS) pH 7.1 (1 mM KH2PO4, 10 mM Na2HPO4, 3 mM KCl, 140 mM NaCl). The attached bacteria were fixed with 0.1% (W/V) crystal violet for 30 min. The wells were washed 3 times with PBS. Thereafter, 0.2 ml of ethanol (70% (V/V)) were added to each well and incubated for 30 min to dissolve bounded biofilm. The optical density (OD) of solubilized bounded biofilm in each well was measured at 595 nm, using an ELISA Microplate Reader. The negative control was determined in uninoculated BHI broth under the same conditions.

Biofilm production and ECM extraction
Single colony of tested isolates were inoculated into 3 ml of BHI medium and incubated overnight at 37 °C with shaking. The overnight cultures were 1000-fold diluted in 10 ml of the Brain heart infusion medium supplemented with 1% glucose and incubated at 37 °C for 24 h under static conditions. After the incubation, the cultures were centrifuged at 6000 rpm for 10 min at 25 °C, and the pellets were collected for extraction of biofilms. Pellets were weighted and suspended with 1.5 M NaCl solution at various concentrations 5, 10, and 20% (w/v). The suspensions were centrifuged at 1000 rpm for 10 min at 25 °C, and the supernatants were transferred to a new test tube as ECM which would be subjected to bioassay (Chiba et al., 2014).

Insect culture and experimental conditions
Insect cultural rearing and experiments were carried out under controlled environmental conditions in the Department of Pest Physiology, Plant Protection Research Institute, Sharkia branch, Egypt. C. maculatus adults, collected from naturally infested cowpea seeds, were reared on infested cowpea seeds. The cultures were maintained in a controlled chamber under a 12/12 h light: dark photoperiod at 28 ± 2 °C and 70 ± 5% relative humidity (RH). Newly emerged adult weevils were used for bioassay.
Bioassays of biofilm against cowpea weevil, C. maculatus

Totally, 90 seeds of cowpea were taken in a conical flask and separately mixed with each concentration of ECM previously prepared, and seeds treated with NaCl 1.5 M solution alone was used as a control. The treated seeds were air-dried and they were separated into 3 plots, each having 30 seeds, each placed in a separate plastic container; 5 pairs of newly emerged adults were introduced into the containers. All were maintained for 15 days under experimental conditions. Adult mortality, number of eggs laid, eggs hatching, larval development, and adult emergence were recorded in both treated and control seeds.

Molecular identification of the potent bacterial strains

Genomic DNA from the most potent bacterial strain was extracted as reported by Hyronimus et al. (1998). Bacterial DNA was subjected to the polymerase chain reaction (PCR), using universal primers; F (5'-AGAGTT TGATCCTGGCTCAG-3') and R (5'-GGTTACCTTG TTACGACTT-3') and recommended thermal cycling conditions (activation 95 °C for 10 min and 35 cycles of 95 °C for 30 s, 65 °C for 1 min, 72 °C for 1 min and 30 s, extension 72 °C for 10 min) to amplifying 16S rDNA gene fragments.

The PCR products were cleaned with the QIAquick gel extraction kit (Qiagen Inc., Valencia, CA) then, purified PCR product was sequenced by Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA). Using a ready-reaction BigDye Terminator v3.1 Cycle Sequencing Kit (Perkin-Elmer/Applied Biosystems, Foster City, CA), with Cat. No. 4336817. A BLAST (Basic Local Alignment Search Tool) analysis was initially performed to establish sequence identity to GenBank accessions (Altschul et al., 1990).

Phylogenetic analysis

A comparative analysis of sequences was performed, using the Clustal W multiple sequence alignment program, version 1.83 of MegAlign module of DNASTAR Lasergene software Pairwise, which was designed by
Thompson et al. (1994), and phylogenetic analyses were done, using maximum likelihood, neighbor joining and maximum parsimony in MEGA6 (Tamura et al., 2013).

Results and discussion
Isolation and screening of biofilm formation by Bacillus isolates
Thirty Bacillus cultures were isolated and screened for their ability to form biofilms, using CRA plates and crystal violet method. Four isolates (nos. 11, 13, 15, and 16) were selected as the highest biofilm-producing bacteria and were subjected for further studies (Table 1). Reda (2019) reported that Bacillus cereus isolated from the milky machine surface was a good biofilm producer. Also, Weng et al. (2013) reported that the colonization and biocontrol efficacy of Bacillus spp. could be significantly improved by improving its ability to form biofilms.

Lethal effects of ECM on cowpea weevil, C. maculatus
Adult weevils contact seed in various ways; these were used to examine the host quality, using different organs (antenna, legs, thorax, mouthparts, and ovipositor; Martin et al., 1996). The effects of different concentrations of ECM (5, 10, and 20%) produced by the 4 selected isolates were determined against cow pea weevil adults. The initial mortality after 24 h of adult–seed contact exhibited significant differences among the 3 tested concentrations, within the 4 tested ECM (Fig. 1). Data in Fig. 1 showed that the isolate no. 13 recorded the highest mortality percentages (29.3, 50.2, and 63.43%) at 5, 10, and 20% of ECM, respectively, followed by isolate no.16. Meanwhile, the lowest mortality percentages were obtained by both isolate nos. 11 and 15. Also, the results revealed that the mortality percentage of cowpea weevil was gradually increased by raising concentrations of ECM in all tested isolates. These results agree with those of Tiroesele et al. (2015) and Mbatchou et al. (2018) who revealed that the natural compounds and plant extracts caused high adult mortality of the cowpea weevils that contacted treated seeds. On the contrary, natural seed coats (seed capsulation) and other manufactured seed treatments recorded lower mortality rates in adult weevils on the treated seeds (De Sá et al., 2018).

Effect of ECM on egg laying
The reduction in egg laying was calculated as mean number of eggs laid per female to express fertility of adults and to avoid the decrease due to mortality. Notable reduction was observed with all treatments compared to control (119.6 eggs/female; Fig. 2). ECM from isolate no.13 showed the lowest number of eggs (30.36 eggs/female) at...
20% ECM concentration, followed by 31.866 and 39.133 eggs/female at 5 and 10%, respectively. While, the ECM from isolate nos. 11 and 15 recorded low reduction and high egg number per female, independent to the tested concentration. In general, the results exhibited that the reduction in egg numbers by ECM of all tested isolates showed highly significant results than the control. On the other hand, egg numbers showed a significant reduction in fecundity, when ECM concentration increased, regardless to ECM source. This reduction in egg laying on treated cowpea seeds may be due to the coating film of the treated seeds, and its physical mechanism that reduced the adhesion of the eggs to the seed surface. This seed coating films may not constitute a suitable surface for laying eggs as reported by Kellouche and Soltani (2004) and Aider et al. (2016). Regarding fecundity, the reduction was not only related to the shortened period of laying eggs or survival of the females, but it could also be a result of disturbances in the vitellogenesis process (Lienard et al., 1993).

**Effect on egg hatching**

Data in Fig. 3 showed that hatchability percentages of all treatments were higher than 70%, except in case of isolate no. 13, which recorded 57.7% at 20% concentration. Furthermore, the hatchability percentages of isolate nos. 11, 15, and 16 were 80.3, 77.3, and 71.2%, respectively at the highest concentration (20%). In summary, ECM extract from isolate no.13 was the most effective in reducing egg hatching than other treatments and control. Meanwhile, mild-significant effects were noticed between other treatments compared to control seeds. The results explained that the reduction in egg hatchability may be due to the interruption occurring in embryo development by fetal constituents of ECM treatments; besides, physical occlusion of their micropyle causes egg death. Generally, egg hatching refracts fertility of adult males but undoubtedly can be caused by the embryonic development disturbance (Kellouche and Soltani, 2004). Aider et al. (2016) reported that the treatments of seeds with olive oil, 2 fatty acids (oleic acid, and linoleic acid separately), and their mixtures caused a significant reduction in longevity, fecundity, number of hatched eggs, and emergence of *C. maculatus*. Also, Ajayi et al. (2018) estimated that the ethanolic extracts of *Zingiber officinale* rhizome and *Moringa oleifera* seeds recorded the highest inhibitory effects on egg laying as well as egg hatching.

**Effect on larval mortality**

The results in Fig. 4 indicated that all the tested treatments had a non-significant toxic effect on the *C. maculatus* larvae at low concentrations (5 and 10%) of ECM than the control group, which recorded normal mortality rate in larvae as 10.7%. A mild significance appeared in the highest concentration (20%), where ESM of isolate no.13 recorded 31.4% larval mortality. Similar results were presented by Aider et al. (2016) and De Sá et al. (2018) when essential oil treatments and natural seed coats were used. They recorded a low mortality rate in
latent developed larvae of cowpea weevil. Radha and Murugan (2011) which confirmed that the biocontrol agents from natural sources affect the physiology of insects or modify their behavior that can lead to insect’s death, development impedance, cause of fecundity loss or egg production viability and, therefore, reduction in the number of offspring. Those compounds not only act by ingestion, but can also act via cuticle contact or fumigant action.

Molecular characterization of the selected bacterial strains
The above concluded data of biofilm production and adverse effects of ECM against cowpea weevil showed that the isolate no.13 exhibited the highest biofilm production as well as adverse activities. So, it was selected and preliminarily identified as Bacillus sp. depending on morphological and biochemical characteristics described in Bergey’s Manual of Systematic Bacteriology (Holt et al., 1994). PCR amplification of 16S rRNA gene confirmed the identification of selected strain as B. flexus S13, the partial nucleotide sequence of the amplified gene was submitted in GenBank (http://www.ncbi.nlm.nih.gov/GenBank/update.htm) under accession number MK292147 (Fig. 5).

Conclusion
Obtained results demonstrated that the biofilms produced by Bacillus spp. and their ECM extracts showed significant adverse effects against cowpea weevil. These effects may be due to ECM extracts containing compounds which serve as alternatives to insecticides. Therefore, ECM extract can be widely used as biocontrol material as it is low cost and long acting.

Abbreviations
BHII: Brain heart infusion broth medium; C. maculatus: Callosobruchus maculatus; CRA: Congo red agar; CV: Crystal violet; EPA: Environmental Protection Agency; ECM: Extracellular matrix; FDA: Food and Drug Administration; no.: Number; RH: Relative humidity

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Authors’ contributions
The conception and design of the study were done by all authors. Authors FMR and WAH, isolated and purified bacteria, screened bacterial isolates for their abilities to produce biofilm. Authors SM and SNES studied the screening of bacterial isolates for their mortality effect against Callosobruchus maculatus. The identification of the most potent bacteria was done by FMR and WAH. All authors read and approved the final manuscript.

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The authors declare that they have no competing interests.

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