Egyptian Journal of Biological Pest Control

RESEARCH Open Access

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

Fifi M. Reda[1*], Wesam A. Hassanein[1], Shahera Moabed[2] and Samah N. El-Shafiey[2]

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

* Correspondence: afmo67@yahoo.com; Fifi.reda133@yahoo.com
[1]Dept. Botany and Microbiology, Faculty of Science, Zagazig University, Sharkia, Zagazig 44519, Egypt
Full list of author information is available at the end of the article

(FDA) and the United States Environmental Protection Agency (EPA) for controlling plant diseases caused by phytopathogenic microorganisms (Borriss, 2011 and Calvo et al., 2017).

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 (Lasa 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 100 μl of the 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 KH_2PO_4, 10 mM Na_2HPO_4, 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.

Table 1 Qualitative and quantitative determination of the biofilm formation by *Bacillus* isolates

Sample no.	No. of isolates	CRA	OD 570 nm
1	1	-	0.050
	2	-	0.031
	3	+	0.143
	4	-	0.062
2	5	-	0.027
	6	-	0.045
	7	-	0.043
	8	+	0.165
3	9	-	0.084
	10	-	0.092
	11	++	0.298
	12	-	0.056
4	13	++	0.433
	14	-	0.041
	15	++	0.312
	16	++	0.344
	17	-	0.061
	18	-	0.055
	19	-	0.021
5	20	-	0.031
	21	-	0.041
	22	+	0.138
	23	-	0.077
	24	-	0.068
6	25	+	0.240
	26	-	0.066
	27	-	0.054
	28	-	0.087
	29	+	0.211
	30	-	0.059

OD < 0.120 = No biofilm; OD 0.120:0.240 = Moderate; OD > 0.240 = Strong
CRA Congo red agar

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

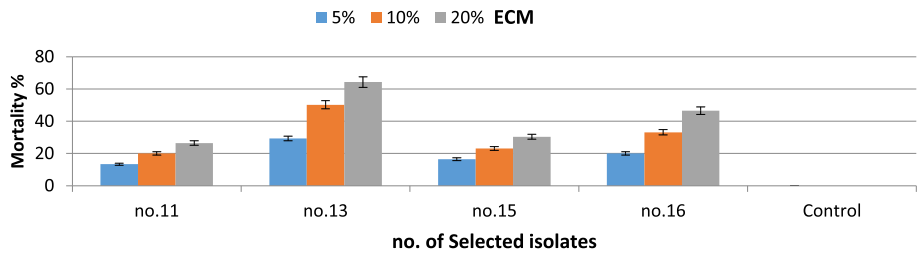

Fig. 1 Effect of different concentrations of ECM from the 4 selected isolates on mortality percentage of adults are represented as M ± SE, and statistical treatments were performed using ANOVA for differences comparing means in each concentration separately using Tukey's HSD test. ANOVA parameters of 5, 10, and 20% concentrations, respectively were LSD = 1.628, 1.711, 1.509; $P < 0.05$

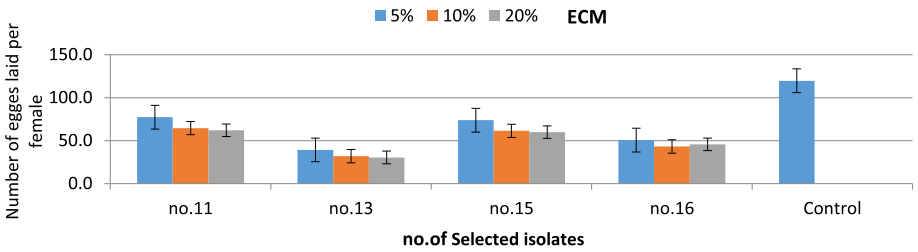

Fig. 2 Effect of different concentrations of ECM from the 4 selected isolates on number of eggs per female are represented as means (M ± SE), and statistical treatments were performed using ANOVA for differences comparing means in each concentration separately using Tukey's HSD test. ANOVA parameters of 5, 10, and 20% concentrations, respectively were LSD = 1.628, 1.711, 1.509; $P < 0.05$

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

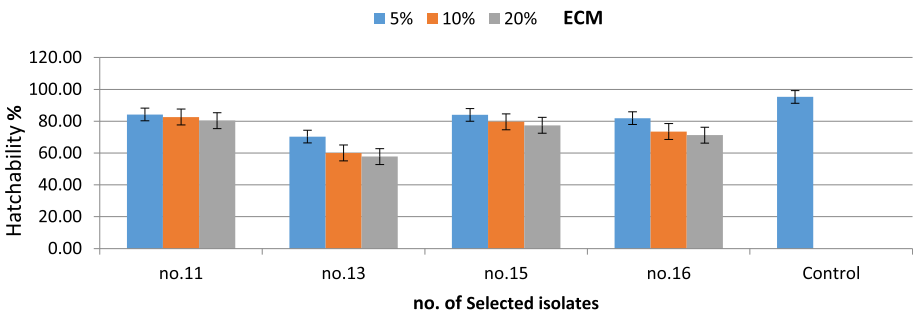

Fig. 3 Effect of different concentrations of ECM from the 4 selected isolates on hatchability percentage (%; M ± SE), and statistical treatments were performed using ANOVA for differences comparing means in each concentration separately using Tukey's HSD test. ANOVA parameters of 5, 10, and 20% concentrations, respectively were LSD = 1.628, 1.711, 1.509; $P < 0.05$

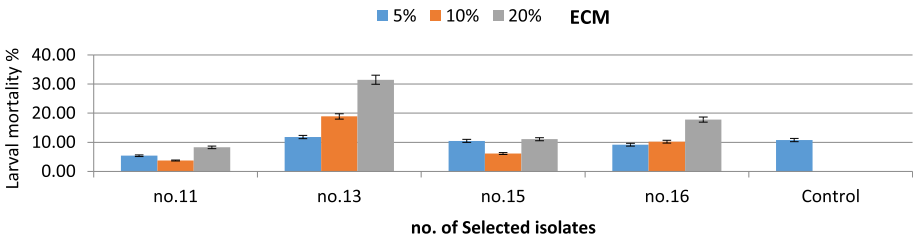

Fig. 4 Effect of different concentrations of ECM from the 4 selected isolates on mortality percentage of adults represented as means (M ± SE), and statistical treatments were performed using ANOVA for differences comparing means in each concentration separately using Tukey's HSD test. ANOVA parameters of 5, 10, and 20% concentrations respectively were LSD = 1.628, 1.711, 1.509; $P < 0.05$

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

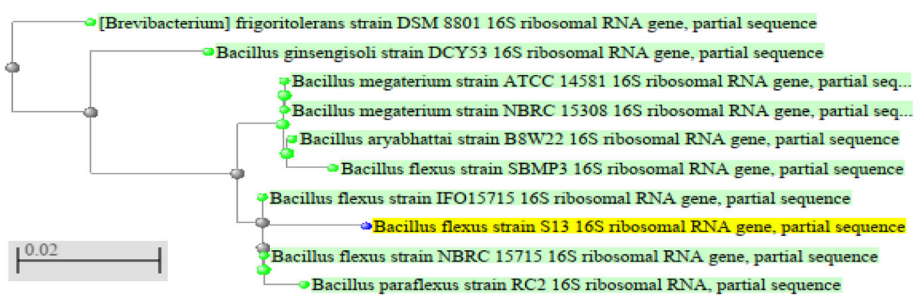

Fig. 5 Phylogenetic tree analysis of *Bacillus flexus* S13

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

BHI: 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

Acknowledgements

Thanks are due to Prof. Dr. Ali Elsheikh of Plant Protection Research Institute for his effort and help to carry out this experimental work.

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.

Funding

This work was not supported by any funding body but personally financed.

Availability of data and materials

All data are available in the manuscript, and the materials used in this work are of high transparency and grade.

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals.

Consent for publication

The manuscript has not been published in complete or in part elsewhere.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Dept. Botany and Microbiology, Faculty of Science, Zagazig University, Sharkia, Zagazig 44519, Egypt. [2]Plant Protection Research Institute, Agricultural Research Center, Dokki, Giza 44516, Egypt.

Received: 7 January 2020 Accepted: 18 February 2020
Published online: 27 February 2020

References

Aider FA, Kellouche A, Fellag H, Debras JF (2016) Evaluation of the bio-insecticidal effects of the main fatty acids of olive oil on *Callosobruchus maculatus* F. (Coleoptera: Bruchidae) in cowpea (*Vigna unguiculata* (L.)). J of Plant Diseases and Protection 123(5):235–245

Ajayi OE, Oladipupo SO, Jegede OJ (2018) Comparative and synergistic influence of extracts of two tropical plants on the activity of the cowpea weevil, *Callosobruchus chinensis*. Medicinal Plant Res. 8(8):60–73. https://doi.org/10.5376/mpr.2018.08.0008

Altschul SF, Gish W, Miller W, Myers EW, Lipmanl DJ (1990) Basic local alignment search tool. J Mol. Biol. 215:403–410

Borriss R (2011) Use of plant-associated *Bacillus* strains as biofertilizers and biocontrol agents in agriculture. In: Bacteria in Agrobiology: Plant Growth Responses. Springer, Berlin Heidelberg, Berlin, Heidelberg, pp 41–76. https://doi.org/10.1007/978-3-642-20332-93

Bose S, Khodke M, Basak S, Mallick SK (2009) Detection of biofilm producing staphylococci: need of the hour. J Clinical and Diagonstic Res. 3(6):1915–1912

Calvo H, Marco P, Blanco D, Oria R, Venturini ME (2017) Potential of a new strain of *Bacillus amyloliquefaciens* BUZ-14 as a biocontrol agent of postharvest fruit diseases. Food Microbiol. 63:101–110. https://doi.org/10.1016/j.fm.2016.11.004

Castelijn GA, van der Veen S, Zwietering MH, Moezelaar R, Abee T (2012) Diversity in biofilm formation and production of curlifimbriae and cellulose of *Salmonella typhimurium* strains of different origin in high and low nutrient medium. Biofouling. 28:51–63. https://doi.org/10.1080/08927014.2011.648927

Chiba A, Sugimoto S, Sato F, Hori S, Mizunoe Y (2014) A refined technique for extraction of extracellular matrices from bacterial biofilms and its applicability. Microbial Biotechnol 8:392–403

De Sá L, Reis VK, Machado O, Tavares F, Sales K, Xavier-Filho J, Oliveira AA (2018) Toxic effect of *Canavalia ensiformis* seed coat on larval development of *Callosobruchus maculatus*. J of pest sci. 91:313–326

Flemming HC, Wingender J (2010) The biofilm matrix. Nat. Rev. Microbiol. 8: 623–633

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (1994) Bergey's manual of determinative bacteriology, 9th edn. The Williams & Wilkins Co. USA, Baltimore

Hyronimus B, Le Marrec C, Urdaci MC (1998) Coagulin, a bacteriocin-like-inhibitory substance produced by *Bacillus coagulans* I. Journal of applied microbiology 85(1):42–50

Johnson LE, Curl EA, Bond JH, Fribourg HA (1959) Methods for studying soil microflora-plant diseases relationship. Burgess Publ. Co. Minn, USA

Kellouche A, Soltani N (2004) Biological activity of the powders of 5 plants and essential oil against *Callosobruchus maculatus* (F.). Internat J of Tropical Insect Sci 24(1):184–191

Lasa I, Penadés JR (2006) Bap: a family of surface proteins involved in biofilm formation. Res. Microbiol. 157:99–107

Lienard V, Seck D, Lognay G, Gaspar C, Severin M (1993) Biological activity of *Cassia occidentalis* L. against (F.) (Coleoptera: Bruchidae). J Stored Prod. Res 29:311–318

López D, Vlamakis H, Kolter R (2010) Biofilms. Cold spring harbor perspectives in biology 2(7):a000398

Maphosa Y, Jideani VA (2017) The role of legumes in human nutrition. M. Chavarri. Funct. Improve Health through Adequate Food, 103–109.

Mbatchou VC, Dickson RA, Amponsah IK, Mensah AY, Habtemariam S (2018) Protection effect of the anthraquinones, cassiatorin and aurantio-obtusin

from seeds of *Senna tora* against cowpea weevil attack. Asian Pac J Trop Biomed. 8:98–105
Parrbruno MJ, Tranmonique S, Simmonds D, Peter F (1996) Credland: oviposition behavior of the cowpea seed beetle, *Callosobruchus maculatus*. Physiol. Eniomol 21(107–1):17
Radha R, Murugan K (**2011**) Bio efficacy of plant derivatives on the repellency, damage assessment and progeny production of the cowpea weevils, *Callosobrucrhus maculatus* (F.) (Coleoptera: Bruchidae). Internet J Food Saf 13: 115–123
Reda FM (2019) Antibacterial and anti-adhesive efficiency of *Pediococcus acidilactici* against foodborne biofilm producer *Bacillus cereus* attached on different food processing surfaces. Food Sci Biotechnol 28:841–850
Singh SR, van Emden HF (1979) Insect pests of grain legumes. Annu. Rev. Entomol. 24:255–278
Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30:2725–2729
Thompson JD, Higgins DG, Gibson TJ (1994) Clustral W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22(22):4673–4680
Tiroesele B, Thomas K, Seketeme S (2015) Control of cowpea weevil, *Callosobruchus Maculatus* (F.) (Coleoptera: Bruchidae), using natural plant products. Insects 6:77–84
Vlamakis H, Chai Y, Beauregard P, Losick R, Kolter R (2013) Sticking together: building a biofilm the *Bacillus subtilis* way. Nat. Rev. Microbiol 11(3):157–168
Weng J, Wang Y, Li J, Shen Q, Zhang R (2013) Enhanced root colonization and biocontrol activity of *Bacillus amyloliquefaciens* SQR9 by abrB gene disruption. Applied microbiology and biotechnology 97(19):8823–8830
Wollum AG (1982**)** Culture methods for soil microorganisms. In method of soil analysis, part 2. Chemical and microbiological properties Agronomy monograph no. 9 (2nd Edition). ASA-SSSA, 677 S. Segoe RD, Madison, WI 53711, USA.

Publisher's Note