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Pathogenicity of Sudan Isolates of *Bacillus* spp to the Greater Wax Moth *Galleria mellonella* L.

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Authors' contributions

This research work was carried out in collaboration between all authors. Author NEG proposed the study, performed the statistical analysis and wrote the paper. Author HAD supervised in the field of microbiology and author HAE supervised in the field of entomology and biological control. They closely followed the study from the proposal of the study till the writing of the work. Author HAH went through the written work and revised the manuscript. All authors read and approved the final manuscript.

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Short Research Article

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ABSTRACT

Aims: To evaluate the pathogenicity of *Bacillus* spp isolated from Sudan against the greater wax wax moth *Galleria mellonella* L. measured as mortality percentages and reduction in the amount of food consumption.
Study Design: This study is a laboratory experiment in a completely randomized design.
Place and Duration of Study: Department of Biopesticides and Biofertilizers, Environment, Natural Resources Research Institute, during 2010.
Methodology: In this study 500 ppm of liquid suspension of the dried spores-crystal mixture of 39 Sudanese *Bacillus* spp were prepared. This suspension was mixed with the food provided to the

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greater wax moth larvae. Larvae in the control were fed on food mixed with sterile distilled water only. Dead larvae were daily counted for ten days and the amount of food consumed was calculated by the end of the experiment.

Results: Up to 81% mortality was recorded by isolate Wh-5 (Bt- JX674041) compared to 12.5% in the larvae fed on untreated food. However, the lowest mortality was recorded by isolate Om-5 (Bt-JX660701), which is 21.8%. Larvae exposed to this isolate consumed greater amount of food than that consumed by larvae fed on untreated food but are not significantly different. Significant differences were observed between different isolates in the mortality percentages and in the amount of food consumed by the different isolates. The least amount of food consumed was that of larvae served food treated with isolate Po-2 (*Bacillus* sp-KF 305081) which was one-third of that consumed by larvae fed on untreated food. While consumption of larvae exposed to some isolates was greater than that consumed by larvae in the control.

Conclusion: This study showed the potentiality of the Sudanese *Bacillus* strains in controlling the greater wax moth. Detailed studies for determination of the lethal doses and specification for optimum production condition is important step for formulation, registration and commercialization.

Keywords: Wh-5 (Bt- JX674041); dried spores-crystal mixture; greater wax moth; food consumption; mortality %.

1. INTRODUCTION

More than 10 million species of insects are known; about 15 thousand species are considered pests and about 300 species require some form of control [1]. The greater wax moth (*Galleria mellonella* L.) is one of the most destructive insect pests that threaten apiculture. Newly hatched larvae seek out honey, nectar and pollen, and then chew their way down to the midrib of the comb. This tunneling destroys the wax cells of the comb. It causes complete destruction of the bee colonies and affects the produce and its salability. In a survey study 100% infestation of bee combs with wax moth was reported in Gezira and Khartoum States, with the mean infestation percentage in different parts of Sudan of 86% [2]. The control of this pest is a universal problem, particularly in warm climates, and the best way to control this pest is to keep a strong, healthy colony [3]. In the past this pest was effectively controlled through fumigation by chemical pesticides, such as methyl bromide, ethylene dibromide (EDB) and paradichlorobenzene (PDB). Of these only one, paradichlorobenzene, appears to have a long-term future as a registered pesticide against the wax moth. Unfortunately, it does not kill all stages of wax moth and does not clean up a severe infestation; it is only a preventative method [4]. In addition, these chemicals are very poisonous to bees and humans and leave residues in the honey. Hot and cold temperatures treatments, and fumigation with carbon dioxide [5] are used in small scale [3].

Bacillus thuringiensis (Bt) is a soil bacterium that produces spores and insecticidal crystal proteins having specific toxicity to insect larvae [6,7]. This bacterium is used in controlling insect pests, and characterized with its specificity, high toxicity to insect, environmental safety and lack of toxicity to vertebrates [8]. Spraying the combs with a watery suspension of *Bacillus thuringiensis* is practiced and persists for several weeks [4]. Previously, Bt and its relatives were characterized from soil collected different locations from Sudan and from dead insects debri in stored products [9]. Pathogenicity of these isolated strains was assessed against the red flour beetle and the house mosquito. Finding a potent strain among the local isolate will solve the problem of this destructive insect in an ecologically sound method. Accordingly, this study was proposed to evaluate the pathogenicity of the indigenous strains against the greater wax moth and their effect on the amount of food consumed.

2. MATERIALS AND METHODS

2.1 Sources of the Bacillus

Thirty nine bacteria**l** isolates were obtained from soil, air and infested stored grains from Sudan following [10] method. Biochemical characterization of these bacteria identified 29 of them as Bt. However, sequencing of the 16S rRNA for 26 of them resulted in various species and genera, which are *Bacillus thuringiensis*, *Paenibacillus popilliae*, *Lysinibacillus sphaericus*, *Bacillus* sp, and

P. sp. [9]. All the thirty nine species were included in this study.

2.2 Revival of the Culture

Nutrient agar medium was prepared in petri dishes plated, and then all isolated bacterial cultures were streaked on this medium. The plates were then incubated at 37º C for 24 hours. Slants were prepared and kept in the freezer.

2.3 Production of Spore Crystal Powder

Five loopful of the 24 hours old culture on Nutrient agar were used to inoculate T3 broth medium [10]) and incubated in an incubator-shaker at 37ºC, 60 rpm for nine days until the spores and crystalline bodies were formed. The suspension was then centrifuged for 10 minutes at a speed of 3000 rpm twice. The precipitates of the spores and crystalline bodies were removed from the vegetative cells [11]. Precipitate was freeze dried and kept in the freezer for further studies.

2.4 Insect Rearing

Bee colony infested with the greater wax moth larvae was brought from Shambat (Faculty of Agriculture Apiary) to the laboratory and kept in a honey bee rearing box with dimensions of 51x32.5x26 cm. Emerging greater wax moth adults were caught and put in a glass jar (15.5x6.5 cm) and fed a sugary solution provided in a small vial covered with cotton. Hatched larvae were fed on natural products of the honey bee comb after autoclaving.

2.5 Bioassays

A concentration of 500 ppm was prepared from the freeze-dried spores of the 39 species in sterilized distilled water. This suspension was mixed with autoclaved 2 gms, of natural food of the greater wax moth (honey bee comb products) this is the method of Dulmage and Co-operators [12]. This food was fed to eight second and third instar larvae of the greater wax moth kept in a glass jar. The glass jar was tightly covered with a piece of cloth and fixed with an elastic band. The food in the control was mixed with the same volume of water. These jars were randomly arranged in the laboratory. Each treatment was replicated four times; the number of dead larvae was recorded daily for ten days.

2.6 Effect on Food Consumption

The amount of food provided to the larvae was weighed before served to the larvae and again at the end of the experiment. The amount of food (g) consumed was calculated for each isolate and for the control.

2.7 Statistical Analysis

Data were subjected to square root transformation. Then, the significance of the differences between the different isolates was evaluated by one way analyses of variance, in a completely randomized design. Duncan Multiple Range Test was used for the comparison of means.

3. RESULTS AND DISCUSSION

3.1 Pathogenicity of the Isolated Strains

Dead larvae were observed within the first 24 hours of exposure to 75% of the isolates, although no mortality was recorded on larvae exposed to other strains such as isolate St-13 up to the fifth day (Table 1). Generally, before death, infected larvae were slow in movement and they were seen out of their tunnels. Brown colour was observed in small areas of the abdomen, and then spread to cover the whole body, finally turned black. The larvae became very soft; lost their body integrity, and smell fusty. Most of the dead larvae were found inside their woven threads or their moulting skins.

About 49% of the isolates were recorded as non-pathogenic to the greater wax moth larvae, as less than 50% mortality was calculated for up to ten days after treatment. The result of fourteen bacterial strains was shown in Fig. 1. During the first five days only three isolatesWh-5 (Bt, JX674041), Kh-3 (*Paenibacillus sp,* JX674042) and St-23(Bt, JX674040), were found to cause mortality percentages more than 50% (Fig. 1). Mortality percentages achieved by these three strains continued to be the highest; where 81% mortality was achieved by Wh-5. The lowest mortality percentage, 21.8%, was recorded on larvae treated with isolates Ab-1 and Po-4 while only 12.5% mortality was recorded on the control larvae (Fig. 1). Only two isolates, 10% of the tested strains, from the twenty toxic isolates achieved mortality above 70% ten days post exposure. These were Wh-5, 81%, Kh-3, 75%, (Fig. 1). Significant differences ($P \leq 0.05$) in

mortality were daily recorded between the different isolates as indicated by analysis of variance (ANOVA). These differences were existed between the means of the mortality of larvae exposed to the different isolates (Table 1). From this table it is clear that isolate Wh-5 achieved the greatest mean mortality which is significantly different from all other isolates, while isolate Om-5 (Bt, JX660701) is the least pathogenic one. Although, Strain St-2 (Bt, KC201677) resulted in less than 10% mortality in the first five days, the mortality approaching 70% in ten days. On the other hand, strain St-23 (Bt, JX674040) achieved more than 60% mortality in five days and less than 70% in ten days. However, 20% of the larvae exposed to strain Po-41 were dead in five days and no additional dead larvae were seen up to day ten.

Table 1. Cumulative daily means† mortality of larvae of *Galleria mellonella* treated with the *Bacillus* spp strains

Isolate	Days after treatment									
	1	2	3	4	5	6	7	8	9	10 ± S.E
Po-41	0.00	0.00	0.85	1.10	1.29	1.29	1.29	1.29	1.29	1.29±0.17 fghi
Sa-49	0.00	0.00	0.50	1.49	1.65	1.78	1.78	1.78	1.78	1.78±0.16 bcdefg
Ab-1	0.00	0.00	0.00	0.50	1.06	1.14	1.14	1.14	1.14	1.14±0.38 ghi
Om-5	0.00	0.00	0.25	0.50	0.60	0.68	0.68	0.68	0.68	0.68±0.42 i
Gf-18	0.75	0.85	1.04	1.46	1.56	1.80	1.80	1.93	1.93	1.93±0.12 abcdef
Ab-12	0.60	0.85	1.62	1.81	1.87	1.92	1.97	2.03	2.03	2.03±0.34 abcd
Wh-5	1.53	1.78	2.03	2.03	2.16	2.22	2.39	2.55	2.55	2.55±0.05 a
Sa-2	0.43	1.41	1.90	2.03	2.10	2.19	2.19	2.19	2.19	2.19±0.25 abcde
Kb-26	0.00	0.85	1.35	1.41	1.61	1.72	1.82	1.82	1.82	1.82±0.25 bcdefg
Dn-1	0.75	1.04	1.64	1.90	2.02	2.14	2.14	2.14	2.17	2.27±0.19 abcde
Kb-29	1.06	1.18	1.62	1.62	1.74	1.81	1.91	1.91	1.91	1.91±0.17 abcdef
St-23	0.60	1.80	2.03	2.15	2.21	2.27	2.27	2.33	2.33	2.33±0.13 abc
Ab-31	1.31	1.65	1.77	1.78	1.83	1.90	2.10	2.15	2.16	2.16±0.17 abcde
Ab-33	0.50	0.85	1.49	1.49	1.49	1.49	1.72	1.72	1.72	1.72±0.11 cdefg
Ab-4	0.75	1.54	1.62	1.68	1.81	1.92	1.92	2.08	2.08	2.08±0.22 abcde
Po-42	0.00	0.68	0.93	1.65	1.72	1.79	1.79	1.79	1.85	1.85±0.14abcdefg
Fh-6	0.25	0.85	1.10	1.10	1.29	1.39	1.79	1.79	1.79	1.79±0.13 bcdefg
Sa-8	1.04	1.39	1.39	1.68	1.68	2.05	2.12	2.12	2.12	2.12±0.06 abcde
Po-1	1.06	1.06	1.41	1.49	1.57	1.92	2.17	2.23	2.29	2.29±0.05 abcd
Sd-2	0.60	1.21	1.47	1.55	1.78	1.79	1.79	1.79	1.99	1.99±0.10 abcdef
Kh-3	1.16	1.22	1.66	1.95	2.28	2.33	2.39	2.45	2.45	2.45±0.08 ab
Po-7	0.71	1.14	1.65	1.85	2.05	2.05	2.21	2.21	2.21	2.21±0.20 abcde
Dn-4	0.00	0.00	1.21	1.39	1.53	1.54	1.64	1.71	1.77	1.77±0.20 bcdefg
Po5	0.00	0.00	0.50	1.31	1.39	1.39	1.39	1.54	1.70	1.70±0.19 cdefg
Om-6	0.00	0.25	0.50	1.21	1.28	1.46	1.54	1.54	1.64	1.64±0.14 cdefgh
Po-2	0.50	0.85	0.85	1.10	1.39	1.39	1.39	1.87	1.93	1.93±0.06 abcdef
Sh-3	0.25	0.25	0.60	0.85	1.39	1.72	1.93	2.06	2.24	2.24±0.00 abcde
Wh-4	0.85	0.85	0.85	0.85	1.60	1.72	1.96	2.10	2.12	2.12±0.06 abcde
Ab-3	0.60	0.71	0.79	0.78	1.39	1.54	1.62	1.68	1.85	1.85±0.14 abcdefg
Om-4	0.50	0.60	0.60	1.06	1.31	1.31	1.64	1.79	1.93	1.93±0.06 abcdef
St-2	0.25	0.25	0.25	0.25	0.60	1.60	2.12	2.18	2.23	2.33±0.13 abc
Kb-30	0.35	0.60	0.60	0.60	1.35	1.43	1.43	1.49	1.60	1.68±0.25 cdefgh
Wh-1	0.50	0.50	0.75	0.75	1.21	1.50	1.77	1.96	2.04	2.10±0.15 abcde
Sh-13	0.25	0.25	0.50	0.50	0.96	1.54	1.85	2.12	2.12	2.12±0.06 abcde
St-13	0.00	0.00	0.00	0.00	0.75	1.00	1.11	1.47	1.51	1.56±0.43 efgh
Sh-14	0.25	1.21	1.46	1.75	1.83	1.83	2.05	2.17	2.23	2.34±0.06 abc
St-14	0.00	0.71	1.06	1.41	1.41	1.49	1.57	1.64	2.17	2.34±0.11 abc
St-6	0.71	0.87	1.57	1.64	1.82	1.82	1.90	1.98	1.98	1.98±0.16 abcdef
Gz-6	0.00	0.79	1.18	1.52	1.52	1.60	1.60	1.60	1.60	1.60±0.55 defgh
Control	0.00	0.25	0.75	1.00	1.00	1.00	1.00	1.00	1.00	1.00±0.00 hi
Mean	0.45	0.76	1.08	1.31	1.53	1.66	1.78	1.86	1.90	1.92
SE (±)	0.26	0.30	0.26	0.24	0.22	0.24	0.22	0.21	0.21	0.20

†Means of four replicates, square-root transformed; those followed by the same letters are not significantly different at P ≤ 0.05 by Duncan's Multiple Range

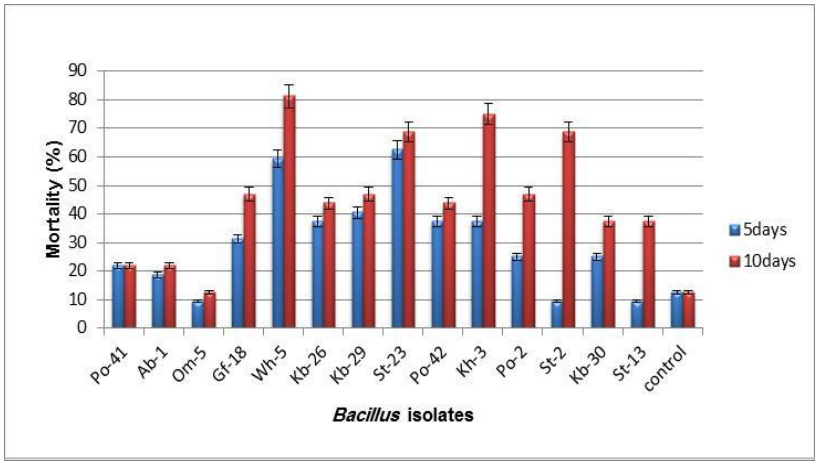

Fig. 1. Cumulative mortality (%) of *Galleria mellonella* larvae exposed to Sudan *Bacillus thuringiensis* strains and relatives 5 and 10 days post exposure

3.2 Effect on Food Consumption

Significant differences ($P \leq 0.05$) were observed in the amount of food consumed by larvae exposed to the different isolates (Table 2). Larvae treated with isolates Po-41, Ab-1, Kb-30 and Om-5 showed an increased amount of food consumption compared to the control, while isolate Gf-18 consumed the same percentage of food as the control (Fig. 2). The greatest amount of food (93% of the food provided) was consumed by larvae exposed to isolates Po-41and Ab-1, followed by larvae treated with Kb-30 (86.25%). On the other hand, larvae in the control treatment consumed 85% of the amount of the food provided to them. However, larvae exposed to food treated with isolate Po-2 consumed the least amount of food (11.25%) as shown in Fig. 2. Comparison of means (Table 2) showed no significant differences ($P \leq 0.05$) in the amount of food consumption between the larvae in the control treatment and those fed on food mixed with isolates; Om-5, Sa-49, Sh-13 and Gf-18, while the rest were significantly different from the control (Table 1). Larvae exposed to isolates Ab-1 and Po-41consumed significantly greater ($P \leq 0.05$) amount of food than that consumed by larvae in the control.

4. DISCUSSION

Chemical pesticides are efficiently used against insect pests [13]. However, their draw backs and hazardous effects are well known [14,15]. These draw backs forced scientists to search for safer alternatives; of which biological control methods or biopesticides are the preferred ones [16].

In this study, Sudanese Bt and relatives showed different activity against the grater wax moth. In general, mortality percentages of the larvae fed on food mixed with Bt increased with time. This is logic as time is needed for the activation of the protoxin inside the mid-gut after ingestion by susceptible larvae [17]. In addition, time is also needed for multiplication of ingested spores. Death of larvae exposed to the toxin is a result of successive events following release of the toxin starting from binding to the specific receptors to the interruption of ion exchange. In addition, the dose taken by the treated larvae was increased by continued eating from the food mixed with the bacteria. The infected larvae become more exposed to removal from the hives by the honey bee workers, as they were weakened and observed out of their silken tunnels. In addition, their capabilities to destroy the hive and damage its components were also decreased. This is expected because of their slow movement and reduced food intake resulted from infection. In this study up to 81% mortality of the larvae was achieved by isolate Wh-5. This percentage is comparable to that obtained by *Bt aizawi* where 80 and 90% mortality of the greater wax moth larvae was reported after one and two weeks, repectively, [18].

The affected insect activities such as feeding and movement before insect death may compensate for the delayed death. This inactivity could be attributed to the effect of Bt, in general, on the muscles and tracheoles. [19] Observed complete relaxation of the muscles of the red flour beetle fed upon Bt treated food. The reduction in food consumption aggravated the toxicity of the Bt, as starvation could be additional cause of death.

Table 2. Comparison of mean amounts of food consumed (g) by larvae of G. *mellonella* exposed to the *B. thuringiensis* isolates

Isolate code	Mean food consumed±S.E		Isolate code	Mean food consumed±S.E	
Po-41	1.368±0.04	a	St-14	0.997±0.03	Cdefgh
Ab-1	1.362±.06	a	Om-6	0.996±0.19	Cdefgh
Kb-30	1.310±0.06	ab	Dn-4	0.960±0.15	Defghi
Om-5	1.308±.0.06	abc	Kb-26	0.952±0.04	Defghi
Control	1.299±0.13	abc	Wh-5	0.858±0.16	Efghik
Gf-18	1.298±0.08	abc	Ab-31	0.840±0.03	Fghik
Sa-49	1.284±0.12	abc	Om-4	0.835±0.08	Fghik
Sh-13	1.282±0.02	abc	Sa-2	0.829±0.10	Fghik
Ab-33	1.242±0.03	abcd	St-23	0.812±0.10	Ghik
Gz-6	1.228±0.03	abcd	Po-7	0.788±0.18	Ghik
St-6	1.207±0.14	abcd	Ab-12	0.785±0.05	Ghik
Wh-1	1.170±0.05	abcd	Kb-29	0.780±0.00	Ghik
St-13	1.160±0.01	abcde	Wh-4	0.721±0.2013	Hikl
St-2	1.124±0.06	abcdef	Sd-3	0.667±0.00	Ikl
Sh-14	1.067±0.08	abcdefg	Dn-1	0.637±0.09	Kl
Po-5	1.009±0.05	bcdefgh	Ab-3	0.591±0.15	Kl

Figures are square root-transformed, those followed by the same letters are not significantly (P ≤ 0.05) different

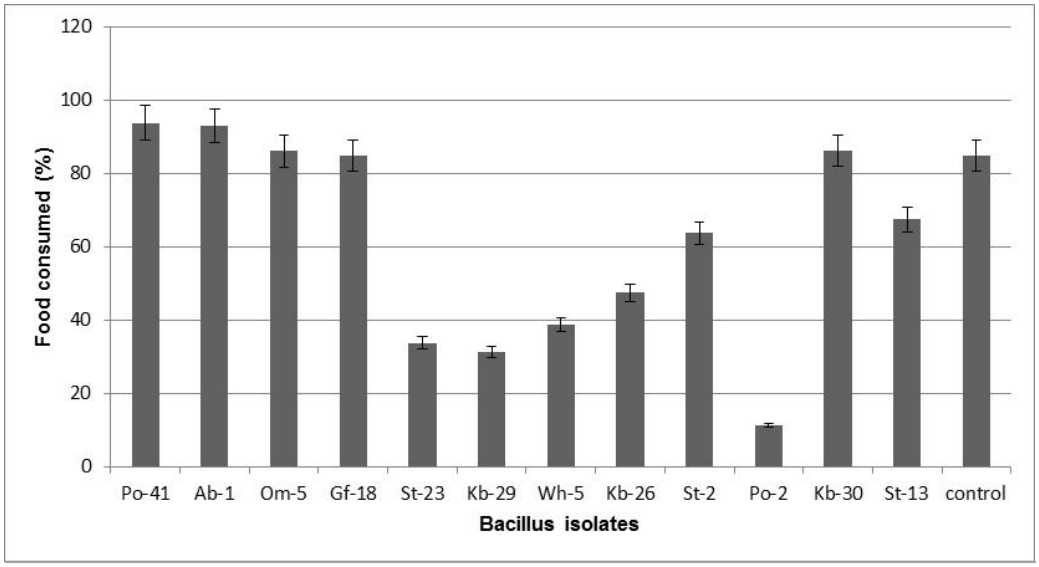

Fig. 2. Percentages of food consumed by larvae of *Galleria mellonella* exposed to the *Bacillus thuringiensis* strains and relatives

Isolates caused maximum reduction in food consumption could be combined with that caused the highest mortalities to increase the efficiency of the isolates. In this study, up to 86.9% reduction in the amount of food consumption was achieved by isolate Po-2 that resulted in only 46.75% mortality. This isolate could be integrated with Wh-5 which gave the highest mortality to achieve better control. Detailed studies to evaluate these strains are needed such as determination of the lethal doses and the optimum production conditions for the most efficient strains, specially Wh-5 and Kh-3. These should be followed with determination of the possibility of production of new modified efficient biocide through gene transfer between two or more strains example Wh-5 and Po-2.

Mapping the distribution of *cry* genes in the whole country should be done, as diversity of *cry* genes could be associated with the geographical areas [20,21]. Continued isolation and toxicity

tests may results in more potent strains. This study is considered as a starting point in this subject in Sudan and should be continued to end in a registered biocide. This includes detailed studies on toxicity of the different strains and the possibility of aggravating the pathogenicity through integrating two or more strains.

5. CONCLUSION

This study showed the potentiality of Sudanese Bt strains in combating the greater wax moth which is a devastating insect pest of the honey bee comb. This finding paves the way for further studies leading to the formulation and commercialization of effective bio-pesticide against the wax moth from the local strains.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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