

Pathogenicity of different isolates of *Bacillus thuringiensis* to the larvae of the red flour beetle *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae)**¹Naiema E. Gorashi, ²Hamadttu, A. Elshafie, ²Hamid A. Hamid and ²Dirar H. Dirar**¹*Environment and Natural Resources Research Institute, National Centre for Research, Khartoum, Sudan.*²*Faculty of Agriculture, University of Khartoum, Shambat, Sudan.***ABSTRACT**

In this study, several samples were obtained from soils collected from different locations in Sudan and from stored products dust and dead insects, with the objective of isolating entomopathogenic *Bacillus* strains. Thirty nine strains were isolated and identified morphologically and biochemically and their pathogenicity to the red flour beetle *Tribolium castaneum* was evaluated. The larvae of red flour beetle were introduced to wheat bran treated with 500 ppm concentration from each isolate. The isolates differed significantly in their pathogenicity, where mortality percentages ranged from 12.5% to 85% compared to 7.5% in the untreated control larvae. Approximately 59% of the 39 Bt isolates were found pathogenic (mortality $\geq 50\%$) to the red flour beetle. The maximum reduction in the amount of food consumed by the treated larvae was 50%, compared to that consumed by the larvae in the untreated control. The least amount of food consumed (14%) was observed when larvae were fed upon food treated with isolate Sh-13. The untreated control larvae consumed 26% of the amount of food provided. Regression analysis revealed differences in the lethal times between different isolates. The LT_{50} values varied from 96.7 hours to 188 hours.

Key words:* *Tribolium castaneum*; pathogenicity; *Bacillus thuringiensis*; mortality; isolates; microbial control.*Introduction**

Tribolium castaneum (Herbst) is a cosmopolitan pest that attacks mainly; the germ of stored grains and other farinaceous products resulting in discolored flours with unpleasant odours.

It has been found in association with a wide range of commodities including, grain, flour, peas, beans, cacao, nuts, dried fruits and spices, but milled grain products such as flour appear to be their preferred food (Good, 1936). It can be found all over the world, mainly because of the international trade of agricultural products (Sokoloff, 1974). This insect is highly tolerant to climatic changes and is one of the most harmful pests occurring in processed grains (wheat flour, corn flour) particularly when in bulk (Rees, 1996).

The red flour beetle *Tribolium castaneum* has been reported to cause both quantitative and qualitative damage to wheat and other grains (Ahmedani *et al.*, 2008), these losses can be summarized in weight loss, loss of nutritional value, increased levels of rejects in the grain mass and loss of industrial (baking) characteristics. Among qualitative losses, reduction in germination percentage is the most important, as it has direct impact on lowering wheat yield (Gallo *et al.*, 1978). Different biological control methods were practiced against this pest including the use of traditional plant-based pesticides for grain protection (Othira *et al.*, 2009), and the microbial control with the entomopathogenic fungus *Beauveria bassiana* for the control of the adult insects (Padin *et al.*, 1997) in addition to the use of *Bacillus thuringiensis* (Ahmedani *et al.*, 2008).

Fumigants such as phosphine and methyl bromide are used as quick and effective tools for insect control in food commodities (Othira *et al.*, 2009). Despite their significance in assuring quality, several fumigants have been withdrawn or discontinued on ground of environmental safety, cost, carcinogenicity, ozone depletion, insect resistance and other factors (Chaudry, 2000). Frequent use and exposure to increasing doses of insecticides have caused *Tribolium castaneum* strains to develop resistance to some pesticides such as pyrethroids, malathion, dichlorvos, chlorpyrifos-methyl and phosphine (Zettler, 1991).

Bacillus thuringiensis seems to be indigenous to many environments (Bernhard *et al.*, 1997). Strains have been isolated world-wide from many habitats, including soils (Carrozi *et al.*, 1991), water and plants. Parasporal crystalline inclusions of *Bacillus thuringiensis* subspecies are among the most promising bacterial biopesticides available for use today (Schnepf *et al.*, 1998). As a whole, these proteins (*B. thuringiensis* toxins) demonstrate great specificity toward certain orders of insects and to date, have shown no known side effects for non target animals (Keeton *et al.*, 1998). recent reference.

This study was proposed with the objectives of isolation and characterization of *Bacillus thuringiensis* strains from Sudan environment and to evaluate their pathogenicity to the red flour beetle *Tribolium castaneum*.

Materials and Methods

Corresponding Author: Naiema E. Gorashi, Environment and Natural Resources Research Institute, National Centre for Research, Khartoum, Sudan.
E-mail: naiemaeltayeb@yahoo.com, Tel:00249-922007559

Sample collection:

Samples were collected from different habitats in Sudan, including; agricultural lands and infested stored products. Collected samples were brought to the laboratory and stored. Samples were drawn from the soil by scraping off the upper surface of the soil. Then about 5 g samples were taken with a sterile spatula from a depth of 2-5 cm. Collected samples were brought to the laboratory in plastic bags and kept at about 4°C in the refrigerator until used for bacterial isolation. Five grams from stored product dirt containing insect debris were obtained from warehouse, store or kitchen? in plastic bags and brought to the laboratory for bacterial isolation.

Isolation:

The method described by Traverse *et al.*, (1987) was followed for the isolation of *Bacillus thuringiensis*. Random colonies from the isolated bacteria were sub-cultured on Nutrient Agar Petri-dishes for purification and then slanted and refrigerated at 4°C for further investigations.

Morphological Identification:

A Smear of 24-48 hours old culture was spread on a clean microscope slide, and left to dry and heat fixed, then stained with gram stain as described by Tortora *et al.* (1998) and then examined under oil immersion (X100). Cell shape, presence or absence of spores and their positions were recorded for each isolate.

Detection of crystal proteins:

According to the method described by Tortora *et al.* (1998), Bt suspension was streaked on Nutrient Agar medium. The plates were then incubated at 37°C for 24 hours. Five loopful of each of the 24 hrs inocula on Nutrient Agar were transferred to flasks of Nutrient broth and shaken for 9 days using electric shaker at speed of 60 rpm until the spores and crystalline bodies were formed. The suspension was then centrifuged for 10 minutes at a speed of 3000 rpm. The precipitates of the spores and crystalline bodies were removed from the vegetative cells. The precipitate was suspended in sterile distilled water; a small loop of this suspension was spread over clean microscopic slide, stained and examined under high power to detect the parasporal crystals.

Biochemical Characterization:

The isolated bacilli that showed gram positive reaction and spore formation were subjected to biochemical tests like catalase production, motility (Collee *et al.*, 1996) ability to hydrolyze starch, gelatin, casein and Indole production was also tested.

Production of gas and acid from glucose, tolerance to sodium chloride, Urease production and others as mentioned in Claus and Berkeley (1986) were also checked.

Bioassay:

Bacterial isolates were grown on T3 broth (Traverse *et al.*, 1987) medium for at least 9 days, then centrifuged and the pellets were freeze dried and kept in the refrigerator. A concentration of 500 ppm from each dried bacterial isolate was prepared then mixed with food (wheat bran), as mentioned by Dulmage and Co-operators (1981), in petri-dishes, in addition to the untreated control. Ten larvae of the middle instars were added to each petri-dish and observed daily and the number of dead larvae was recorded.

Effect of inoculation with Bt isolates on food consumption:

The amount of food provided to the treated larvae was weighed before and after consumption. The amount of food (g) consumed was calculated for each isolate and for the untreated control.

Statistical analysis:

Data were subject to square root transformation and one way analyses of variance, in a completely randomized design, was carried out to evaluate the significance of differences between the different isolates. Each treatment was replicated in four Petri-dishes. Duncan Multiple Range Test was used for the comparison of means.

Probit analysis for mortality percentages equal to or greater than 50% was used to calculate the lethal times using Minitab software version 13.20.

Results:

Approximately 58.9% of the isolated strains were found to be pathogenic to the red flour beetle *Tribolium castaneum*, at a concentration of 500 ppm, resulting in mortality percentages equal to or greater than 50% ten days after exposure. The treated larvae were observed to have slow movement, avoided feeding as they were seen away from the food material, and finally died. Discolored small areas were observed in the ventral side, which increased through time.

In most of the treatments, mortality of the treated larvae started on the second day post-exposure, and the mortality percentages increased with time (Fig.1). Table (1) shows that about 33% of the isolates did not succeed in causing mortality in the treated larvae in two days, and by the third day, this value decreased to 18%. After four days all isolates except isolate Kb-26 were able to cause mortality to the exposed larvae. However, maximum increase in the mortality percentages was obtained between the fourth and the seventh day after treatment (Fig.1). By day ten, 85% mortality was obtained when the larvae were exposed to isolate Wh-5 and isolate St-6, while those treated with isolate Ab-1 showed 12.5% compared to 7.5% in the control (Fig.1, d). It was observed that although isolate kb-26 was slow at the beginning, 57% mortality was achieved after 10 days of exposure.

Analysis of variance for the square root-transformed daily mortality data showed significant difference ($P \geq 0.05$) between the different treatments or isolates and the untreated control.

Significant differences were detected between the different isolates (Table 1). Isolates Ab-1, Ab-3 and Om-6 were recorded the lowest mean mortality values which were not significant different from the control. However, larvae exposed to isolates Wh-5, St-6 and Sh-13 showed the highest mortality mean values and were statistically at par with each other.

Larvae of the red flour beetle responded differently to the different Bt isolates as seen in Fig. 2. This figure shows the response of the larvae to isolates Wh-5, Gz-6, Sd-2 and Ab-12. Isolate Wh-5 showed the least lethal times values, while isolate Ab-12 showed the least toxicity effect, with Lt_{50} of 96.77 hours for the first one and 169.33 hours for the second one (Table 2). The additional times required to kill 90% of the larvae exposed to the different isolates were similar, which was 2.26 times of that required to kill 50% (Table 2). Different potencies were recorded for the different isolates. Isolate Ab-12 showed the least potency while isolate Wh-5 was the most potent (Table 2).

Effect of inoculation with Bt isolates on food consumption:

The amounts of food consumed by the red flour beetle larvae exposed to the different Bt isolates were calculated at the end of the experiment. Food consumed by larvae exposed to some isolates was presented in Fig. (3). The food (mixed or not mixed with the Bt isolates) consumption percentages is less than 40% of the food provided to the larvae, except that mixed with isolates St-2 and Wh-4 (78%). The least amount of food consumed (14%) was observed when larvae were fed upon food treated with isolate Sh-13. The untreated control larvae consumed 26% of the amount of food provided. This indicated that larvae exposed to food treated with isolate Sh-13 consumed half of the amount consumed by the larvae fed on untreated food. On the other hand, larvae exposed to food treated with isolates St-2 and Wh-4 were active feeders as the percentage of food consumed by them was three times that consumed by the untreated control larvae. Where larvae exposed to food treated with isolates Po-7 and Ab-4 consumed the same amount as that consumed by the larvae in the untreated control (Fig. 2). Analysis of variance indicated the existence of highly significant difference ($P \geq 0.01$) in the amount of food consumed by larvae exposed to the different isolates and to the untreated control.

Data for the amount of food consumed by larvae exposed to each isolate revealed clear and significant differences ($P \geq 0.05$) to exist between the different isolates and the control (Table 3).

Discussion:

Conventional chemical control is the most frequently used technique to protect agricultural products against insect attacks (White and Leesch, 1996). Chemical control is responsible for the increase in the development of resistance among several insect species as well as for food contamination by chemical residues (de Olivera *et al.*, 2006).

The value of *Bacillus thuringiensis* in controlling insects that destroy crops and transmit human diseases is well established. It could be integrated with entomopathogenic nematodes or with pesticides to control insect pests inhabiting the soil and foliage in the green house (Kaya *et al.*, 1995).

Accordingly, this study was proposed to isolate strains of *Bacillus thuringiensis* from Sudan local habitat and to test their pathogenicity. In this study 39 isolates closely resembling *Bacillus thuringiensis* were subjected to further characterizations. Previous workers were also able to isolate this entomopathogenic bacterium from different habitats in Sudan including soil, insects and phylloplane (Omer, 1990; Hamza, 2002; Ahmed, 2004;

Elyass 2004, and Ahmed, 2005). *Bacillus thuringiensis* was also isolated from different habitats by worldwide investigators (Travers *et al.*, 1987; Martin and Travers, 1989 and Binh *et al.*, 2005).

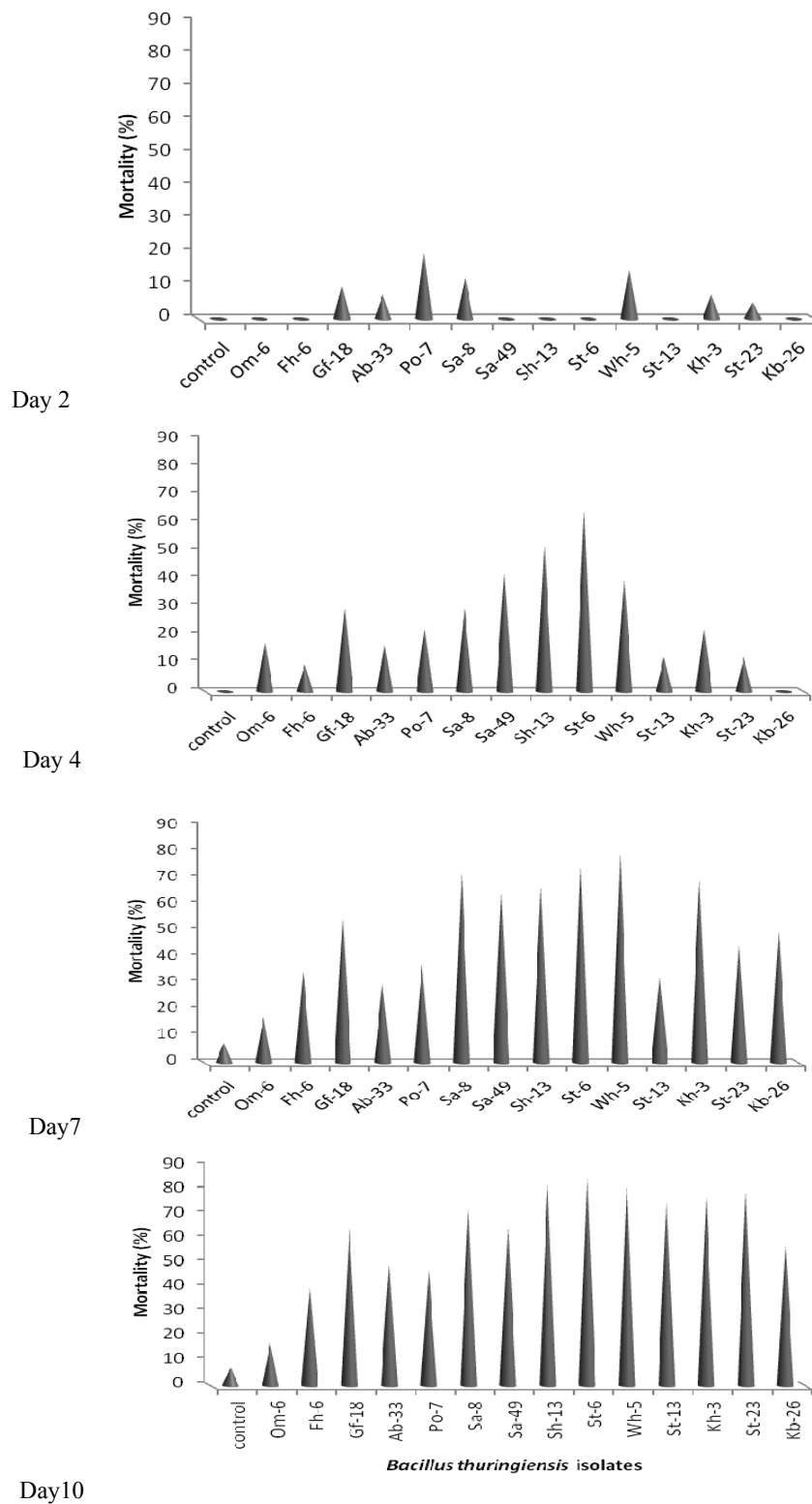


Fig. 1: Cumulative mortality (%) of *T. castaneum* larvae exposed to the isolates of *Bacillus thuringiensis*

Table 1: Cumulative daily means[†] of dead larvae of *Tribolium castaneum* treated with the *Bacillus thuringiensis* isolates.

	Day 2	Day 4	Day 7	Day 9	Day10
Control	0.0	0.75	0.75	0.75	0.75 l
Om-5	0.0	1.103	0.957	1.732	1.992 fgh
Om-6	0.0	0.25	0.603	0.853	1.103 l
Ab-3	0.50	0.603	1.353	1.286	1.103 l
Om-4	0.60	1.036	1.207	1.493	2.153 cdefgh
Po-2	0.25	1.103	1.310	2.825	2.819 ab
Wh-4	0.0	1.457	1.496	1.845	1.845 ghi
St-2	0.0	1.299	1.496	1.732	1.854 ghi
Wh-1	0.50	0.786	1.207	1.319	1.319 lkl
Sa-2	0.25	1.207	1.573	2.137	2.137 defgh
Sh-14	0.50	0.957	1.414	1.845	1.845 ghi
Fh-6	0.0	1.219	1.795	1.831	1.978 fgh
Ab-4	0.25	1.366	1.469	1.640	1.719 hik
Gf-18	0.683	1.742	2.342	2.391	2.532 abcdef
Ab-33	0.683	1.219	1.732	2.230	2.230 bcdefgh
Po-7	1.219	1.742	1.898	2.110	2.153 cdefgh
Po-1	1.103	2.045	2.396	2.413	2.486 abcdef
Sa-8	0.954	2.051	2.688	2.691	2.638 abcde
Po-42	0.786	1.707	2.342	2.440	2.582 abcdef
Ab-31	1.457	1.858	1.866	1.965	1.992 fgh
Sd-3	0.50	2.007	2.391	2.440	2.445 abcdefg
Ab-12	0.603	1.523	1.788	2.636	2.685 abcde
Sa-49	0.0	1.683	2.547	2.547	2.547 abcdef
Sh-13	0.0	2.051	2.593	2.782	2.871 a
Dn-4	0.353	1.559	2.171	2.386	2.498 abcdef
Kb-29	0.603	0.603	2.011	2.575	2.593 abcdef
Sh-2	0.933	1.040	2.289	2.437	2.737 abcd
St-6	0.0	1.559	2.825	2.871	2.914 a
Wh-5	1.207	2.118	2.852	2.914	2.914 a
St-13	0.0	0.957	1.786	2.638	2.737 abcd
Kh-31	0.603	1.390	2.642	2.782	2.782 abc
Gz-6	0.25	1.493	2.642	2.737	2.737 abcd
St-23	0.353	0.786	2.177	2.733	2.825 ab
Kb-30	0.25	0.853	2.289	2.381	2.825 ab
Kb-26	0.0	0.0	2.220	2.360	2.391 abcdefg
Po-5	0.0	0.25	2.177	2.539	2.642 abcde
Ab-1	0.0	0.25	0.75	1.103	1.103 l
Po-41	0.0	0.25	1.140	1.207	1.207 kl
Dn-1	0.25	0.5	1.469	2.090	2.090 efgh
St-14	0.25	0.353	1.140	1.845	1.845 ghi
Mean ±S.E	0.397 ±0.297	1.168 ±0.306	1.854 ±0.177	2.138 ±0.16	2.215 ±0.18

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

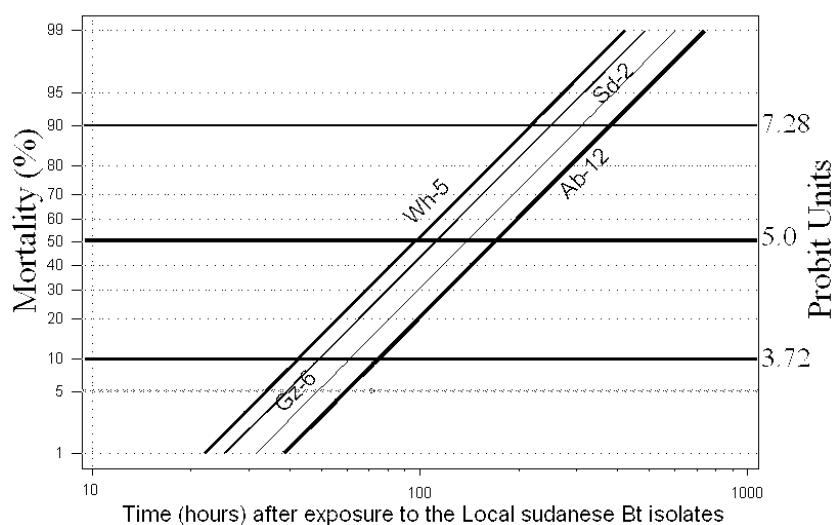


Fig. 2: Time-response of *Tribolium castaneum* exposed to the *B. thuringiensis* isolates Wh-5, Sd-2, Gz-6, and Ab-12 at 500 ppm.

The affected insect activities such as feeding and movement before insect death is an advantage for microbial control, as it takes long time to kill the treated insects. It was observed that treated larvae were inactive, sometimes unable to get out of their moulting skins. This could be attributed to the effect of *Bacillus thuringiensis*, in general, on the muscles and tracheoles as reported by Abdel Razak *et al.*, (2002) who investigated the changes on tissues of larvae of *Tribolium castaneum* and *Plodia interpunctella* that appear after ingestion of Bt subspecies *indiana* and subsp. *morrisoni*. These authors observed complete relaxation of the muscles after feeding on 5000 µg of Bt subsp. *morrisoni*/ g of grains.

In general, the mortality percentages of the treated larvae, increased with time, which could be attributed to the fact that the bacteria needed time to be activated inside the midgut after being ingested. In addition, daily dose ingested by the treated larvae was increased by eating from the treated food. Time was also needed for the subsequent events in the midgut starting from the ingestion, activation of the toxin, disruption of ion stability, and finally death of the treated insects (Lonc *et al.*, 2003).

In this study approximately 58.9% of the isolated *Bacillus thuringiensis* strains were lethal to the red flour beetle, where mortality percentages between 12.5% and 85% were obtained by the various isolates compared to 7.5% in the control larvae after ten days. This high mortality, 85%, is comparable to that reported by Ahmedani *et al.*, (2008) who exposed adults of *Tribolium castaneum* to Ecotech Pro7.5 (transconjugant Bt *kurstaki* x Bt *aizawi*), and resulted in 79.6% cumulative mortality after 7 days. Finding an isolate with high potentiality gives hope in controlling this devastating pest, which can live for up to three years (Mason, 2003). This insect acquired resistance to most insecticides, while the most effective ones, such as methyl bromide were banned (Ahmedani *et al.*, 2008).

Probit analysis showed clear differences, even between isolates showing no differences in mortality percentages such as isolates Wh-5 and St-6. These isolates resulted in the same cumulative mortality percentages after 10 days (85%), while in this analysis approximately nine hours were needed by isolate St-6 over that needed by isolate Wh-5 to kill 50% (Lt_{50}) of the treated larvae. The same could be applied for isolates Sa-8 and St-13 that achieved the same mortality (82.5%) 10 days after exposure but the difference in the Lt_{50} was 63 hours. The range of the calculated Lt_{50} values were 96.7 hours for isolate Wh-5 to 188 hours for isolates Kb-26 and Ab-33. Elyass (2004), examined Sudanese isolates against *Henosepilachna elaterii* (Coleoptera) and reported Lt_{50} to range from 48.6 to 72 hours for the higher concentrations examined. While the reference strain tested with these local isolates (*Bacillus thuringiensis* var. *tenebrionidae*) failed to achieve 50% mortality even after 72 hours.

Table 2: Time-response analysis for *Tribolium castaneum* exposed to some Bt isolates, Wh-5, St-6, Sd-2, Gz-6, Gf-18 and Ab-12 at 500 ppm.

Isolate	Lethal times (hours)	Fiducial limits	Lt_{90}/Lt_{50}	Chi-square	df	slope	Relative poter
Wh-5	Lt_{10} : 42.8858	35.4303-50.2278	2.25643	57	29	0.2758	0.5715
	Lt_{50} : 96.7688	85.6692-108.5687					
	Lt_{90} : 218.3520	191.9177-353.2935					
St-6	Lt_{10} : 46.8437	38.9720-54.5885	2.256429	57	29	0.2758	0.6242
	Lt_{50} : 105.6994	93.8371-118.4920					
	Lt_{90} : 238.5032	208.9351-278.1389					
Sd-2	Lt_{10} : 61.2427	52.2156-70.1059	2.25643	57	29	0.2758	0.8161
	Lt_{50} : 138.1899	123.7289-154.6298					
	Lt_{90} : 311.8158	270.1509-370.1413					
Gz-6	Lt_{10} : 49.4985	41.4440-57.3891	2.256429	57	29	0.2758	0.6595
	Lt_{50} : 111.6899	99.5842-124.8273					
	Lt_{90} : 252.0203	220.7559-294.3054					
Gf-18	Lt_{10} : 67.8437	58.3715-77.1692	2.256429	57	29	0.2758	0.9040
	Lt_{50} : 153.0844	137.2318-171.5535					
	Lt_{90} : 345.4241	297.5613-413.5115					
Ab-12	Lt_{10} : 75.0454	64.8905-85.1701	2.256429	57	29	0.2758	1.0
	Lt_{50} : 169.3347	151.1623-402.8473					
	Lt_{90} : 382.0918	320.1741-402.8473					

Some of the isolates examined could be regarded as highly pathogenic compared to the others, as they resulted in significant reduction of the amount of food consumed combined with higher mortality percentages. An example of this is the isolate designated Sh-13. The red flour beetle larvae exposed to this isolate consumed the least amount of food. Also it is one of the isolates which caused high cumulative mortality (82.5%) within 10 days. However, isolates such as St-2 and Wh-4 were regarded as non-pathogenic to the red flour beetle larvae as the mortality percentages achieved after 10 days were less than 50%. In addition, larvae exposed to these isolates were active feeders and they consumed significantly greater amounts of food, compared with the larvae in the control. These isolates might affect the odor and/or the taste of the food making it more attractive or digestible to the larvae. Detailed studies are needed to understand the actual cause of this behavior. Ibargutxi *et al.* (2006) proved the existence of non-toxic cry proteins, such as cry 2A that caused significant inhibition of

growth. This will result in decreased number of generations in a given time and so the damage to the produce would be minimized.

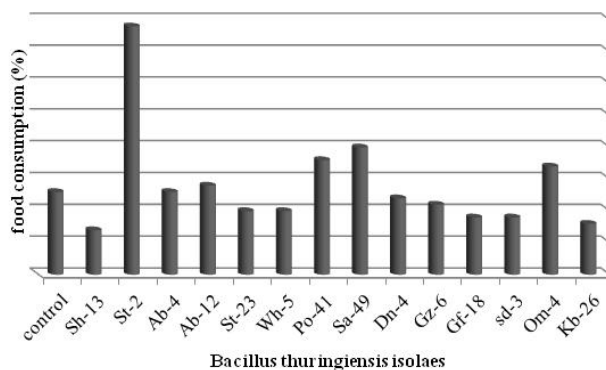


Fig. 3: Percentages of food consumed by the larvae of *Tribolium castaneum* exposed to the *B. thuringiensis* isolates.

Table 3: Comparison of mean [†]amounts of food consumed (g) by larvae of *T. castaneum* exposed to the *B. thuringiensis* isolates.

Isolate code	Mean food consumed (g)
St-2	0.9871 a
Wh-4	0.9871 a
Sa-49	0.7021 b
Po-41	0.6697 bc
Om-4	0.6511 bcd
St-14	0.6112 bcde
Dn-1	0.5900 bcdef
Kh-3	0.5900 bcdef
Ab-12	0.5900 bcdef
Ab-31	0.5875 bcdef
Sa-2	0.5861 bcdefg
Po-7	0.5689 bcdefg
Control	0.5689 bcdefg
Ab-4	0.5689 bcdefg
Ab-33	0.5437 cdefgh
Dn-4	0.5437 cdefgh
Om-6	0.5225 cdefgh
Po-42	0.5225 cdefgh
Fh-6	0.5225 cdefgh
St-6	0.5225 cdefgh
Gz-6	0.5186 cdefgh
Wh-1	0.5110 defgh
Sh-14	0.5110 defgh
Po-1	0.5045 defgh
Po-5	0.4974 defgh
Sa-8	0.4974 defgh
Wh-5	0.4974 defgh
St-23	0.4974 defgh
Ab-3	0.4930 efgh
Kb-29	0.4859 efgh
Ab-1	0.4723 efgh
Gf-18	0.4723 efgh
Po-2	0.4647 efgh
St-13	0.4647 efgh
Sd-3	0.4647 efgh
Om-5	0.4607 efgh
Kb-30	0.4607 efgh
Kb-26	0.4395 fgh
Sd-2	0.4319 gh
Sh-13	0.4144 h

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

Reduction in food consumption was observed in Bt isolates such as isolates Ab-1 and Ab-3 that were not able to kill 50% of the treated larvae, even after 10 days. These isolates could be considered as feeding deterrents, and so the possibility of their integration with the highly pathogenic ones should be examined.

Conclusions:

Few perspectives seem to emerge from this study; firstly, Sudan is rich in *Bacillus thuringiensis* and this study shows that, approximately 58.9% of the isolated strains were pathogenic to the red flour beetle *Tribolium castaneum*, at a concentration of 500 ppm 10 days after treatment. Significant differences in the amount of food consumed by larvae exposed to the different locally isolated strains were recorded.

Further molecular identification is urgently needed in addition to detailed pathogenicity studies to determine the lethal doses towards different larval instars. Those isolates with lower mortality percentage rates against the red flour beetle could be pathogenic towards other insect orders, so they should be examined against them. Further investigations are needed concerning pathogenicity and formulations to end up with a registered microbial insecticide.

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