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EVALUATION OF *BACILLUS THURINGIENSIS* H-14 ISOLATES FROM NIGERIAN SOILS FOR USE IN MOSQUITO CONTROL

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Abstract—Eleven mosquitocidal *Bacillus thuringiensis* isolates from Nigerian soils were screened for the degree of toxicity against *Aedes aegypti* larvae. The six most larvicidal isolates, code-named OBGI, OBG8, BUS4, BAR3, GSC3 and GNA13, were identified as subsp. *israelensis (B.t.i).* Spores of the six isolates of a *B. t. i.* from a commercial powder (R-153-78, Roger Bellon Laboratory, Belgium) and of IPS 82 (Institut Pasteur (Standards)) were subjected to ultraviolet irradiation (280–350 nm) for up to 75 min. After irradiation, the toxicity of OBG8, OBGI, BUS4, and BAR3 was higher than that of R-153-78 but lower than that of IPS 82.

Key Words: *Bacillus thuringiensis* H-14 isolates, Nigerian soils, ultraviolet irradiation effects, mosquito control

Résumé—Onze isolats (aux propriétés anti-moustiques) de *Bacillus thuringiensis* provenant des sols nigérians ont été passés au crible concernant leur degré de toxicité a l'égard des larves d'*Aedes aegypti.* Les 6 isolats ayant les plus fortes activités larvicides et désignés sous les codes OBG1, OBG8, BUS4, BAR3, GSC3 et GNA13, ont été identifiés en tant que sous-espèces *israelensis* (*B. t. i.*). Les spores des 6 isolats d'une *B. t. i.* provenant d'une poudre commerçiale (R-153-78; Laboratoire Roger Bellon, Belgique) et celle du IPS 82 (Institut Pasteur (Standards)), ont été soumis à l'irradiation aux ultra-violets (280–350 nm) pendant 75 min. Aprés irradiation, la toxicité de OBG8, OBG1, BUS4 et BAR3 a été supérieure à celle de R-153-78, mais inférieure à celle de IPS 82.

Mots Clés: isolats de *Bacillus thuringiensis* H-14, sols Nigérians, effets d'irradiation aux ultra-violets, lutte anti-moustique

INTRODUCTION

The discovery of *Bacillus thuringiensis* subsp. *israelensis (B. t. i.)* (Goldberg and Margalit, 1977) and *B. spaericus* (Singer, 1975) has revolutionised the methods for controlling mosquitoes and black flies, two major disease vectors. The discovery has shifted research, production and application from synthetic chemical insecticides to biological agents to control both vectors. The product *B. t. i.* is a potent bioinsecticide and is used to achieve control of mosquitoes and black flies in the USA, Canada, West Africa, Europe and Asia (Walsh, 1986; Guillet et al., 1990; Molloy, 1990; Mulla, 1990).

Since the original isolation of *B. t. i.* in Israel (Goldberg and Margalit, 1977), many strains have been isolated in other parts of the world (e.g., Balaraman et al., 1981; Zhang et al., 1984; Abdel-Hameed et al., 1990). Scientists have advocated a continued search for new *B. thuringiensis* strains with improved toxicity, and new microbial pathogens (Davidson and Sweeny, 1983; de Barjac, 1990; Sutherland, 1990). Intensification of screening programmes, especially where the vectors are endemic in the tropics, could yield strains that are more potent than the existing ones.

In this study, we evaluated strains of *B. thuringiensis* isolated in Nigeria, an area where

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mosquitoes and black flies are endemic, for use as mosquito control agents. The susceptibility of each isolate to inactivation by ultraviolet irradiation was also assessed.

MATERIALS AND METHODS

Source of B. thuringiensis *strains*

Eleven mosquitocidal strains of *B. thuringiensis (B.t.)* were selected from 85 crystal-forming *Bacillus* spp. that were isolated from soils in different parts of Enugu and Kogi States of Nigeria between February 1991 and March 1992. A standard IPS 82 preparation was kindly supplied by Dr. H. de Barjac (Institut Pasteur, Paris), while a strain of *B t. i.* from a commercial powder (R-153-78) of Roger Bellon Laboratories, Belgium), commonly used in this laboratory, was obtained from Mr. Robert Manasherob.

Screening the 11 B. t. *isolates for toxicity*

A loopful from the slant of each isolate was used to inoculate a test tube containing sterile 5 ml LB broth containing in deionised water (g/l): tryptone (Difco), 10; yeast extract (Difco), 5; NaCl, 10; pH 6.8. The tubes were incubated overnight (for 16 h) on a gyratory water bath shaker (New Brunswick model G76) at 200 rpm and 32°C. After checking for purity, 0.2–0.5 ml of the culture was diluted in a 100-ml Erlenmeyer flask containing 20 ml of LB broth to a turbidity of 5 Klett units (KU) at 600 nm in a Klett-Summerson photoelectric colorimeter (Model 800 3). A 2-ml volume of the 5 KU culture was used to inoculate duplicate fresh Erlenmeyer flasks of LB broth and incubated as previously described. Turbidity was measured at 30-min intervals until the culture reached 40 KU.

A volume of 1.5 ml of the 40 KU culture of each *B. t.* isolate was used to inoculate a 125-ml Erlenmeyer flask containing 20 ml of LB broth and incubated for 48 h on the shaker as previously described. At 24 and 48 h, the culture was examined microscopically for degree of sporulation; it was then diluted and assayed against early fourth instar larvae of *Aedes aegypti*. Isolates of *B. t.* which produced 80% mortality and above at 0.5×10^{-5} dilution in the 24-h bioassay were selected for further evaluation. A strain of *B. t. i.* (R-153-78) was included in all the experiments for comparison.

Preparation of primary powders of selected B. t. *isolates and* B. t. i. *(standard)*

The medium used for the production of primary powders contained, in deionised water (g/l): tryptone (Difco), 5; yeast extract (Difco), 3; glucose, 3; NaCl, 5; $MnCl_2.4H_2O$, 0.02 and a pH of 6.8 adjusted with 1N NaOH. This medium allowed sporulation better than LB broth and had a reproducible pH of the final whole culture (FWC). A volume of 100 ml of the medium was dispensed into each 500-ml Erlenmeyer flask and sterilised at 121°C for 15 min. Three percent of 40 KU culture (v/v) of each *B. t.* isolate and of *B. t. i.* was used to inoculate each production flask with the medium. The flasks were shaken at 30°C until sporulation and lysis (above 95%) were evident microscopically (usually after shaking for 37–48 h). The spore-delta-endotoxin complexes were recovered from the FWC as bacterial powders by the acetone lactose coprecipitation method (Dulmage et al., 1970). A cell-free supernatant of the FWC was tested for larvicidity against *A. aegypti* larvae.

Bioassays

A stock suspension of the primary powder (*B. t.* isolates, *B. t. i.* and IPS 82) was prepared (de Barjac, 1983) by homogenising 50 mg of the powder with 10 ml deionised water in a 20-ml penicillin flask containing 6-mm glass beads. A volume of 0.1 ml of the suspension was mixed with 9.9 ml deionised water and 80, 60, 40, 20, 16 and 10 µl of the suspension added to triplicate assay cups each containing 100 ml deionised water and 20 early fourth-instar *A. aegypti* larvae to obtain a final concentration of 0.04, 0.03, 0.02, 0.01, 0.008 and 0.005 mg/l. A final concentration of 0.2 to 0.1 mg/l was used for assays of *B. t.* isolates and *B. t. i.* Each assay was scored after 24 h and repeated three times on different days.

Calculation of the results

The average percentage mortality was analysed by the probit transformation (Fisher and Yates, 1964). The level of regression was calculated by the method of Busvine (1971). Mortality among control larvae was rare and needed no correction with Abbott's formula (1925); rather, the assays were rejected when control mortality exceeded 3.3%. The potency of each primary powder was expressed in International Units (ITU) per mg by comparing with the standard IPS 82, which was assigned 15,000 ITU/mg.

Effect of ultraviolet irradiation (UV) on the spores and larvicidal activities

A 100-ml homogenised suspension containing 10 mg of a bacterial powder was prepared in deionised water in a 400-ml beaker and continuously stirred (magnetic stirrer) at the same rate in a chamber (100

41 cm), 10 cm under a 60-cm UV (280–350 np. Samples (0.1 ml) were withdrawn at 15- tervals for up to 75 min and appropriate ns were plated in triplicate by spreading evenly gar plates. The plates were incubated at 32°C h and colonies counted (New Brunswick Counter) on plates with 20–300 colonies. samples (0 min) were pasteurised at 70°C nin before plating. Each experiment was d three times.

suspension-volume yielding 92–94% y before UV irradiation was determined in ioassays (as above) for each powder in nary experiments. The volume served in the ys after UV irradiation. Three assay cups t up for each powder for each irradiation , each with the same sample volume of the suspension, 50 ml deionised water and 10 larvae, and incubated as described above. An assay was rejected when mortality in the control was higher than 3.3%. The assays were repeated three times on different days.

Identification of B. t. *isolates*

Six isolates code-named OBG1, OBG8, BUS4, BAR3, GSC3 and GNA13, which showed good larvicidal activities, were sent to Dr. H. de Barjac's laboratory, Institut Pasteur (Paris) for identification.

RESULTS

The six most larvicidal *B. t.* isolates were identified by Dr. de Barjac as strains of subsp.

Sources and features of *Bacillus thuringiensis* isolates: Toxicity against early fourth-instar *Aedes aegypti* d sporulation efficiency after 48 h growth in LB broth

Sources of soil samples	Mortality % at dilution of 1×10^{-5}	0.5×10^{-5}	1×10^{-6}	% sporulation
Grassland in mechanic village, Nsukka, Enugu State	95	10	0	98
	100	100	45	98
Groundnut farm (1) at Akpanya Kogi State	100	95	10	96
Groundnut farm (2) at Akpanya Kogi State	100	65	0	95
Grassland in a valley at Obimo, Enugu State	85	15	0	99.8
Bamboo grove at Obaka Unadu, Enugu State	100	100	15	97
Bamboo grove at Ohomu Unadu, Enugu State	100	45	0	98.8
Bamboo trees along Aku Road Nsukka, Enugu State	100	100	5	97
A large bamboo grove along Obechara, Road Nsukka, Enugu State	100	100	40	96
	100	5	0	97
	100	100	30	98
53-78) standard	100	100	30	98

Larvicidal activities: LC_{50}, LC_{90} and ITU values of primary powders of *Bacillus thuringiensis* H-14 isolated erian soils (Table 1) and of the standard *B. t. i.* and IPS 82 against early fourth-instar larvae of *Aedes aegypti*

Spore counts/ mg powder $\times 10^7$	LC_{50} value $\times 10^{-2}$ (mg/ 100 ml)	95% confidence interval $\times 10^{-2}$ (mg/ 100 ml)	LC_{90} value $\times 10^{-2}$ (mg/ 100 ml)	95% confidence interval $\times 10^{-2}$ (mg/ 100 ml)	Slope of regression	χ^{2*} df = 4	ITU+/mg vs *A. aegypti* 4th-instar larvae
22.0	0.1	0.09 – 0.12	0.18	0.16 – 0.21	5.3	0.14	15,000
1.9	0.5	0.43 – 0.51	0.69	0.63 – 0.75	7.5	3.16	3,500
1.5	0.8	0.71 – 0.87	1.33	1.20 – 1.48	5.6	6.85	2,100
1.5	1.0	0.92 – 1.16	1.68	1.50 – 1.89	6.1	2.85	1,600
1.2	1.0	0.92 – 1.14	1.62	1.46 – 1.81	6.3	0.50	1,600
1.2	0.7	0.67 – 0.81	1.10	1.00 – 1.21	7.4	0.33	2,200
1.3	1.8	1.22 – 1.53	2.41	2.51 – 3.28	6.3	1.53	1,200
2.2	0.9	0.84 – 0.99	1.37	1.27 – 1.49	7.3	2.17	1,800

value for the Chi-square with 4 degrees of freedom = 7.8.
lues were calculated by comparison with the standard IPS 82 powder assigned 15,000 ITU/mg.

israelensis. The exact source of each isolate and the results of the screening bioassay for the degree of their larvicidal activity against early fourth-instar *A. aegypti* larvae are shown in Table 1. Table 2 summarises the LC_{50} and LC_{90} values of the primary powders, and of *B. t. i.* (R-153-78) grown under the same conditions, and their ITU/mg against such larvae, calculated by comparison with that of the standard IPS 82 preparation (assigned 15,000 ITU). Toxicity of the isolates was relatively low, but those of OBG1 (3,500), OBG8 (2,100) and GSC3 (2,200) were higher than that of *B. t. i.* (1,800) grown under the same conditions. The LC_{50} and LC_{90} values also indicate that OBG1 and GSC3 are more larvicidal than the *B. t. i.* The cell-free supernatant of all the Nigerian isolates had no activity against *A. aegypti* larvae.

Effects of ultraviolet irradiation (UV)

The number of surviving spores of the isolates *B. t. i.* and IPS 82 are shown in Fig. 1. After 15 min of irradiation, reduction in spore counts ranged from 49% in BUS4 to 66% in OBG1; the concentration of UV resistant colony-forming units (cfu/ml) of *B. t. i.* and IPS 82 were also reduced by 54 and 58%, respectively (Data not shown). Spore counts in all cultures were reduced by more than 90% after 30 min of irradiation. The effects of UV irradiation on the larvicidity of all the cultures is shown in Fig. 2. The concentration of bacterial powder ($x10^{-2}$ mg/50 ml) that produced 92–94% mortality (Fig. 2) before UV irradiation (0 min) were: IPS 82, 0.10; OBG1, 0.40; OBG8, 0.64; BUS4, 0.84; BAR3, 0.85; GSC3, 0.60; GNA13, 1.70 and *B. t. i.*, 0.70. Figure 2 shows that after 75 min of UV irradiation, IPS 82 had the highest larvicidal activity against *A. aegypti* larvae followed by OBG8, OBG1, BUS4, BAR3, *B. t. i.*, GSC3 and GNA13, respectively.

DISCUSSION

All the highly active mosquitocidal *B. t.* strains isolated from Nigerian soils were assigned to subsp. *israelensis*. Similar surveys for mosquitocidal *B. t.* strains in India (Balaraman et al., 1981), China (Zhang et al., 1984), Israel (Brownbridge and Margalit, 1986) and Egypt (Abdel Hameed et al., 1990) resulted in isolation of strains of the *B. t. i.* This seems to confirm the views of Priest (1992) that mosquitocidal *B. t.* strains are commonly associated with serotype 14 and that the variety may be closely associated with dipteran breeding grounds.

A spore count of 2.2×10^8/mg obtained for IPS 82 is 10-fold the counts of our laboratory-prepared *B. t. i.* (R-153-78) primary powder and 11-fold the OBG1 counts which had the highest spore counts of all the Nigerian isolates (Table 2). It is therefore, unreasonable to compare the larvicidal activities of the Nigerian isolates with those of IPS 82. Rather they are comparable with *B. t. i.* (R-153-78) produced under the same conditions. The LC_{50} and LC_{90} values (Table 2) indicate that OBG1 is more active than *B. t. i.* while GSC3 and OBG8 are as active as *B. t. i.* The LC_{90} values (mg/litre) of the isolates ranged from 0.07 (OBG1) to 0.24 (GNA13). These figures compared well with the 0.4 to 0.5 mg/litre reported for most *B. t. i.* primary products, and with 0.17, 0.70 and 0.48 mg/litre obtained for Bactimos, Sandos and IPS 78, respectively (Mulla, 1990).

A reduction in spore counts of 49.0–66.0% and above 90% occurred in all the cultures after 15, and 30 min of UV irradiation, respectively. Such rapid spore inactivation of *B. t.* strains has been previously reported (Cantwell and Franklin, 1966; Griego and Spence, 1978). The decrease in larvicidities of the isolates and standards after irradiation was not as rapid and severe as observed in spore viability (Fig. 2). However, after 75 min of irradiation, the toxicity against *A. aegypti* larvae was decreased in all the cultures by up to 70%; IPS 82 was still more larvicidal than the rest of the cultures, followed by OBG8, OBG1, BUS4, BAR3, *B. t. i.*, GSC3 and GNA13 (Fig. 2). The toxicity of the cultures was thus not equally susceptible to inactivation by UV irradiation. Raun et al. (1966) attributed the low persistence of *B. t.* preparations in the field partly to inactivation by UV wavelengths in the sunlight. They also reported that larvicidity of *B. t.* serotype 1 against *Ostrinia nubilalis* and *Spodoptera frugiperda* was completely eliminated after 72 h of exposure to 15W germicidal UV lamp. On the contrary, Cantwell (1967) reported that 120 min UV irridiation did not affect the larvicidity of *B. t.* subsp. *sotto* against larvae of *Bombyx mori*.

The findings of this work indicate that two Nigerian *B. t.* H-14 isolates, IBG1 and OBG8, are potentially useful in mosquito control. The spore counts in both isolates can be increased by formulating adequate media for growth as well as manipulating the fermentation conditions. These will further increase their larvical activity and their capability to resist inactivation by ultraviolet irradiation.

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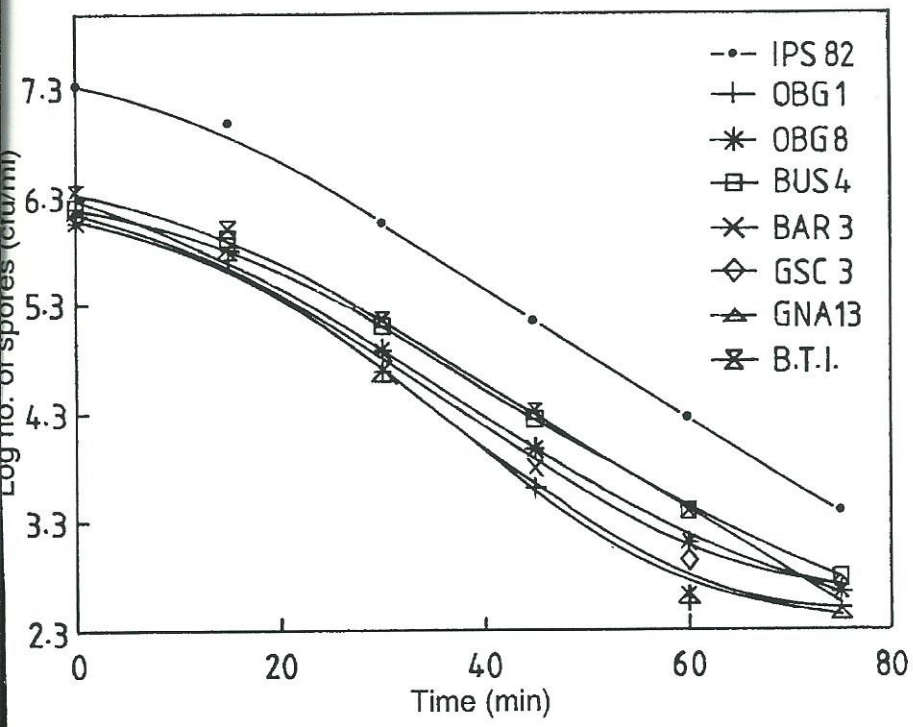
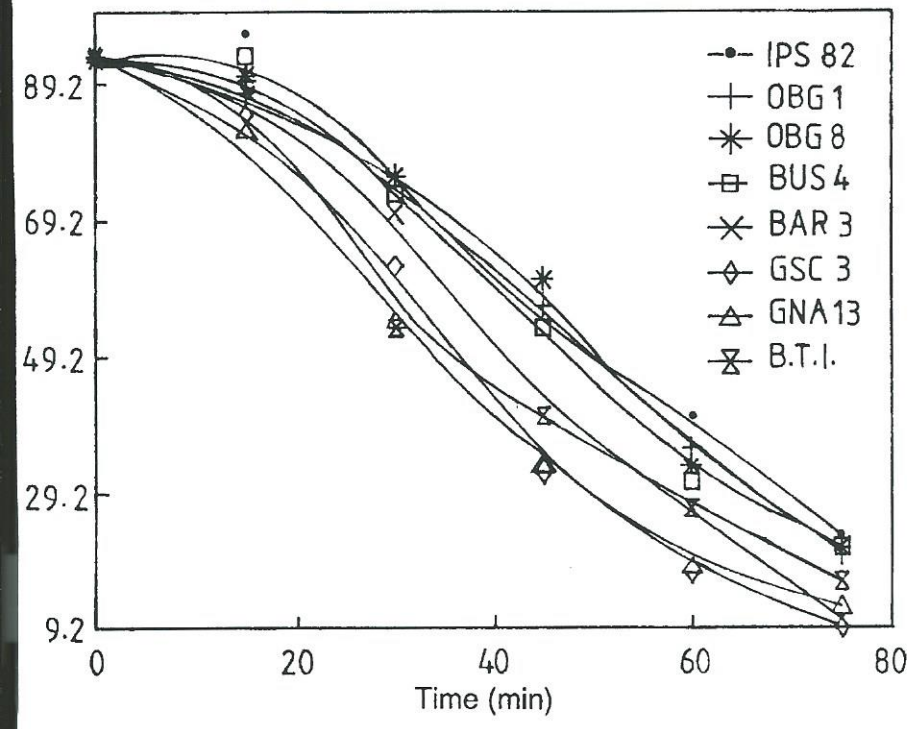


ects of UV irradiation on spore viability of *Bacillus thuringiensis* H-14 isolated from Nigerian soils and the standards *B. t. i.* and IPS 82



cts of UV irradiation on larvicidal activities of primary powders of *Bacillus thuringiensis* H-14 m Nigerian soils (Table 1) and the standards *B. t. i.* and IPS 82 against early fourth-instar *Aedes* ae

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