https://dx.doi.org/10.4314/ijs.v20i2.8

BIOCONTROL POTENTIAL OF *Bacillus thuringiensis* ISOLATED FROM SOIL SAMPLES AGAINST MOSQUITO LARVAE

Adeyemo I.A.[1]*, Abdul-Wahab S.O.[2] and Obadofin A.A.[3]

[1, 2] Department of Biological Sciences, Ondo State University of Science and Technology (OSUSTECH), Okitipupa, Nigeria.

[3]Department of Crop, Soil and Pest Management, Ondo State University of Science and Technology, (OSUSTECH), Okitipupa, Nigeria.

Corresponding Author: adeyemiyourfriend@gmail.com; Tel: (+234)8035671847

(Received: 23rd March, 2018; Accepted: 26th April, 2018)

ABSTRACT

A major challenge for achieving successful mosquito control is overcoming insecticide resistance. The potential of *Bacillus thuringiensis* isolated from different soil samples as a control strategy of mosquitoes and monitoring of larvae susceptibility was investigated in this research. Larvicidal activity of *Bacillus thuringiensis* on mosquito larvae was assessed by isolating them from different soil habitats at Okitipupa. The isolated organisms were confirmed as *Bacillus thuringiensis* based on biochemical characterization and microscopic observation. Two out of the six isolates of *Bacillus thuringiensis* obtained from the soil samples labeled 6ORD1 and 4OD3 were used for the purpose of this study. The larvicidal activities (which were measured by mortality rate and change in morphology of the larvae) were observed at 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} dilution factors and at intervals of 4, 12 and 24 hours on the mosquito larvae. The isolates of *Bacillus thuringiensis* showed a slight level of variation in their larvicidal activity. Both isolates 40D3 and 60D1 caused 100% mortality of the larvae at the highest concentration of 10^{-1} at 4 hours while 100% mortality was recorded in other dilution factors at 12 hours. In both control system (distilled water and rain water) 100% mortality was recorded at 24 hours. From this study, it is concluded that *Bacillus thuringiensis* is a very potent biolarvicide that brings about mortality of mosquito larvae at a short duration of time.

Keywords: Malaria, Mortality, Larvicidal, *B. thuringiensis*, Insecticides.

INTRODUCTION

Mosquitoes are vectors of various disease causing agents, responsible for transmission of pathogens causing more life threatening and debilitating human diseases than any other organism. Over one million people worldwide die from mosquito borne diseases which include malaria, filariasis, yellow fever, chikungunya and dengue fever yearly within disease-endemic countries (Boutayeb, 2006; Poopathi and Abidha, 2010; Nareshkumar *et al.*, 2012).

Chemical insecticides provide benefit in food production, human health and have proven very effective at increasing agriculture and forestry productivities. However, uncontrolled use of chemical insecticides has resulted in irreparable damage to environment (El-Kersh *et al.,* 2012). Radhika *et al.* (2011) reported that repetitive use of man-made insecticides for mosquito control disrupts natural ecosystems leading to reemergence of, and increase in mosquito populations. In their studies, Das *et al. (*2007) and Zhang *et al.* (2011) also pointed out that the continuous use of chemical-based insecticides has resulted in the development of resistance, detrimental effects on non-target organisms and human health problems. Consequently, they suggested the need for alternative control measures which leaves biological control as a viable alternative to chemical control. Microbial insecticides are especially valuable because their toxicity to non-target animals and humans is extremely low and a crucial part of integrated pest management (Aramideh *et al.,* 2010; El-kersh *et al.,* 2012). Compared to other commonly used insecticides, they are safe for both the pesticide user and consumers of treated crops.

Interestingly, *Bacillus thuringiensis* is an important insect pathogen which is highly toxic to mosquito larvae and related dipterans (Poopathi and Abidha, 2010; Zulfaidah, *et al.,* 2013). *Bacillus thuringiensis* is selectively active on pests and less likely to cause resistance hence it is considered beneficial to humans, animals and plants and also

as a suitable replacement to chemical pesticides in many countries.

Bacillus thuringiensis is a Gram-positive facultative anaerobe and spore forming saprophytic soil bacterium. The toxicity is attributed to an endotoxin, which is made of proteins that are produced and assembled when the bacteria sporulate (Haggag and Youssef, 2010; Sanahuja *et al.,* 2011).

Bacillus thuringiensis during the sporulation produces one or more proteinaceous parasporal crystals, recognized as delta-endotoxin. This crystal protein under alkaline condition of midgut of insects, gets solubilized, and then activated by intrinsic protease into an active toxin that selectively binds specific receptor in the cell membrane, leading to pore formation and consequent insect larvae death (Soberon *et al.,* 2000; Eswarapriya *et al.,*2010; El-kersh *et al.,* 2012).

Most *Bacillus thuringiensis* preparations available in the market contain spores with parasporal inclusion bodies composed of δ (delta) - endotoxins. In commercial production, the crystals and spores obtained from fermentation are concentrated and formulated for spray on application according to conventional agriculture practices. There are many strains of *Bacillus thuringiensis* having insecticidal activity against insect orders (eg Lepidoptera, Diptera, Homoptera, Mollaphage, and Coloptera). Only a few of them have been commercially developed.

The main objective of the study is the isolation of local strains of *B. thuringiensis* from soil habitats in Okitipupa and studying their killing effect on mosquito larvae. The specific objectives are to: breed mosquito larvae for the purpose of the experiment; isolate *Bacillus thuringiensis* from soil samples; observe the effect of the *Bacillus thuringiensis* on the larvae over a period of time and determine the effectiveness of a *Bacillus thuringiensis* against the larvae of mosquito as a biological control agent.

MATERIALS AND METHODS

Study Location

This study was carried out in Okitipupa Ondo State, Nigeria between July and November 2017. Soil samples were collected from four locations: Igodan Lisa, OSUSTECH Farm, OSUSTECH River and Okitipupa

Collection of Soil Samples

The soil samples were taken at a depth of 10 cm below the soil surface, after scrapping of the surface material with sterile spatula; the sample was placed in a sterile plastic bag and transferred to the laboratory for isolation of *Bacillus thuringiensis* from the sample. Eight soil samples were collected from four locations within Okitipupa town, two (2 samples) each from Igodan Lisa, OSUSTECH Farm, OSUSTECH River as well as Okitipupa township.

Isolation of B. thuringiensis from Soil Samples

Acetate selection method described by Travers *et al.* (1987) which inhibit non- spore forming bacteria was used. The medium contained: 10g tryptone, 0.5g yeast extract and 5g NaCl. prepared in an Erlenmeyer flask. Half a gram (0.5 g) of each soil sample was added to 10 ml of the broth medium. The broth was buffered with 0.25 M sodium acetate. The mixture was vortexed vigorously and incubated for 24 hours on a rotary shaker (Model KOMA) at a speed of 160 rpm at 37°±2 °C. At the end of this time, 0.5 ml of each soil suspension was drawn into test-tube and pasteurized in a water bath at 80 °C for 10 minutes to kill vegetative cells and non-spore forming bacteria. After cooling at room temperature, the mixture was then serially diluted with sterile distilled water of 4.5 ml in five folds. A volume of 0.1 ml of each dilution was streaked on nutrient agar medium. Plates were incubated at 37 °C for 24 hours.

Purification and Preservation of Typical B. thuringiensis Isolate

Colonies showing *B. thuringiensis* type morphology were rough, white and spread out over the plate. These colonies were selected and streaked again on nutrient agar and incubated for 48 hours at 37 °C to obtain pure culture. Isolates suspected to be *B. thuringiensis* were kept in nutrient broth medium at 4 °C for further characterization.

Identification of isolates

The isolates were identified using morphological

characterization and conventional biochemical procedures according to Claus and Berkeley (1986) and Barrow and Feltham (1993). Gram staining and spore staining procedures were performed. Biochemical tests conducted include catalase, indole, casein hydrolysis, and amylase activity.

Detection of Endospore, Crystal Proteins and Morphology

Bacillus species do not stain, and they may be seen as unstained bodies within bacterial cells stained with methylene blue. Smears of Bacillus isolates were prepared and they were fixed by heat. The bacterial smears were then flooded with methylene blue. Staining lasted for 5 minutes. Finally, destaining was performed by washing under slow running tap water. The stained bacterial colonies were observed under oil – immersion objective for endospore position, crystal production and morphology. The isolates having visible parasporal crystals next to the spore in the sporangium cells were identified as *Bt.* Isolates having ellipsoidal and subterminal spores in unswollen bacterial cells. The colonies showing morphology and crystal shape were scraped off from the plates and transferred into sterile vials containing 1 ml of nutrient broth. After vortex mixing, they were stored at 4 ^{o}C as stock.

Breeding of Mosquito Larvae

Water containers were left to stand in an open space at ambient temperature of about 30 ^{o}C for seven (7) days to facilitate laying of eggs by the mosquito. The water container was monitored daily to observe the emergence of the larvae. The larvae of the female anopheles mosquitoes were harvested using sieve and placed in a moistened cotton-wool to preserve them before use (Thomas *et al.,* 2014).

Bioassay and Activity of B. thuringiensis **against Mosquito Larvae**

In primary screening, two *Bacillus thuringiensis* isolates having different crystal shapes were selected and tested against larvae of mosquito. Five larvae were transferred into each test tube with labeled dilution factor of the *Bacillus thuringiensis* suspension. The *Bacillus thuringiensis* from broth slants were diluted to 10^{-1}, 10^{-2} and 10^{-3}, 10^{-4} and 10^{-5} with sterile distilled water by diluting 1 ml of the suspension in 9 ml of sterile distilled water. The test tubes were kept at 30 ^{o}C; the mortality rate was checked for each dilution factor. Larval mortality was recorded at 4, 12 and 24 hours. A control test was also carried out using distilled water and rain water.

Statistical Analysis

Data for mortality rate of the mosquito larvae were generated by the use of the two *B. thuringiensis* specie with two distinct morphological characteristics and quantitative measurement determined by counting. Duncan multiple range tests were employed to analyze the similarities and differences in the mean values of the quantitative characters.

RESULTS

Habitats and Locations of the Isolated B. thuringiensis

B. thuringiensis was isolated from the eight soil sample habitats with four (4) sandy soil samples from Okitipupa providing isolates 4OD1, 4OD3, 2ID3 and 2ID1 while the loamy soil samples from OSUSTECH school farms produced isolates 3OFD1 and 3OFD3. Clay soil samples from OSUSTECH River produced the last two isolates labeled 6ORD1 and 6ORD3. Table 1 shows the various habitats and locations from where isolations were made.

Table 1: Habitats and Locations of Bacillus thuringiensis **Isolates**

No	Habitat of Isolate	Location	Isolate code
1	Clay soil	OSUSTECH River	6ORD1
2	Loamy soil	OSUSTECH Farm	3OFD1
3	Loamy soil	OSUSTECH Farm	3OFD3
4	Sandy soil	Okitipupa	4OD1
5	Sandy soil	Okitipupa	4OD3
6	Clay soil	OSUSTECH River	6ORD3
7	Sandy soil	Igodan	2ID3
8	Sandy soil	Igodan	2ID1

Colonial and Cultural Characteristics of the Isolated B. thuringiensis

The cultural characteristics of the suspected *Bacillus thuringiensis* isolates were examined. Generally, colonies were cream in colour, tend to have large frosted glass appearance, initially, but become opaque and spread over the plates as shown in plate 1. Some colonies were mucoid in nature, others brittle. The eight isolates were Gram- positive and spore formers (Table 2).

Table 2: Morphological Characteristics of Bacillus thuringiensis **Isolates**

Characteristic	Isolate NO							
	6ORD1	3OFD1	3OFD3	4OD1	4OD3	6ORD3	2ID1	2ID3
Gram staining	+	+	+	+	+	+	+	+
Formation of spore	+	+	+	+	+	+	+	+
Position of spore	S	S	S	S	S	S	S	S
Shape of spore	O	O	O	O	O	O	O	O
Presence of crystal	+	+	+	+	+	+	+	+

O- OVOID S-SUBTERMINAL

Biochemical Characteristics of the Isolated B. thuringiensis

The isolates showed positive reactions with regard to catalase test. They showed negative results with regard to indole test while the other tests were positive. The summary of the test results is shown in table 3. After biochemical test and observation, phase-contrast microscopy were carried out, six of the isolates were characterized as *Bacillus thuringiensis* based on the crystal protein production. Cuboidal and bipyramidal crystals were observed in the isolates.

Activity of Bacillus thuringiensis **Strains against Mosquito Larvae**

Two of the isolated *Bacillus thuringiensis* (4OD3 and 6OD1) were investigated for their potential against the larvae. The dead larvae were examined under light microscope, it was observed that the midgut of the larvae were swollen and the general morphology of the dead larvae (as a result of feeding on the bacillus toxin) was different from the normal one not fed with the toxin of *Bacillus thuringiensis* (Plate 2). The mortality caused by the isolates amounted to 100% after the duration of time (24 hours).

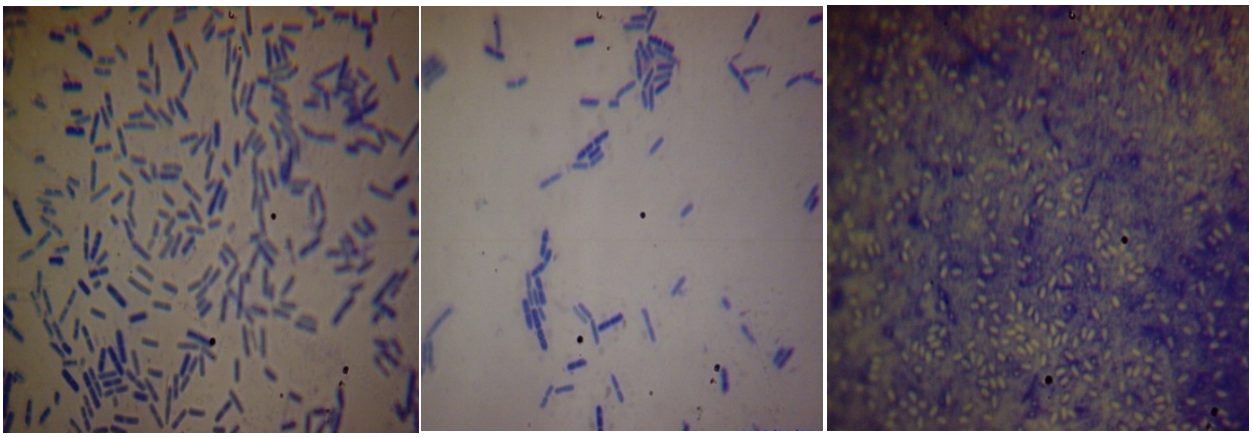
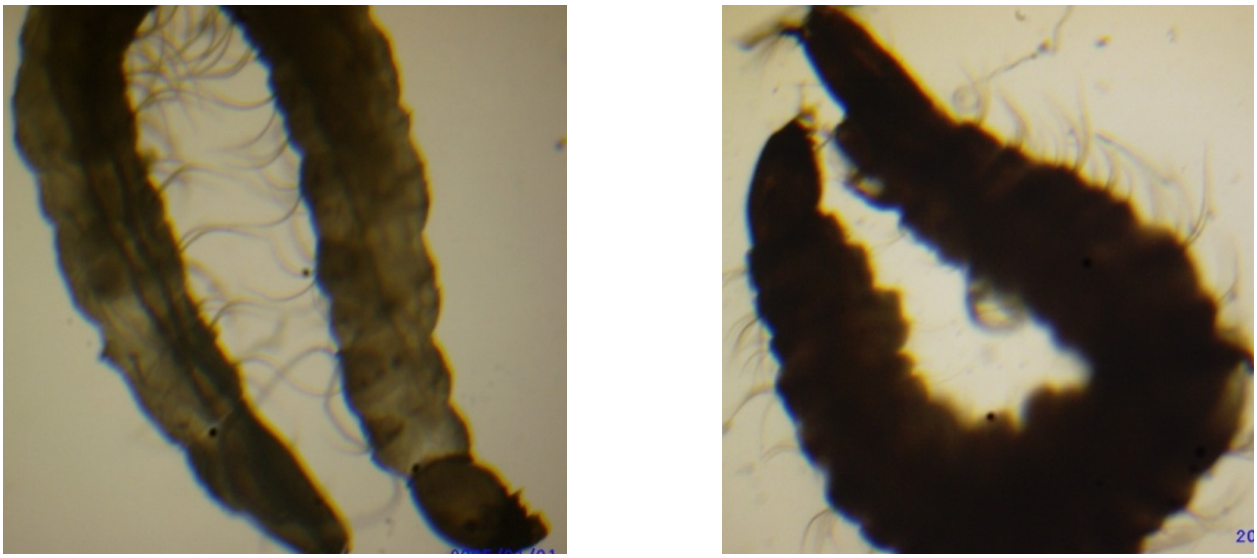


Plate 1: Phase Contrast Photograph of Bacillus thuringiensis **Isolates**



Before Ingestion of *B. thuringiensis* After Ingestion of *B. thuringiensis*

Plate 2: Phase Contrast Photograph showing Morphological features of larvae after ingestion of B. thuringiensis

Table 3: Biochemical Reactions of Bacillus thuringiensis **Isolates**

Biochemical Test	**Isolate No**							
	6ORD1	3OFD1	3OFD3	4OD1	4OD3	6ORD3	2ID3	2ID1
Hydrolysis of casein	+	+	+	+	-	+	+	+
Catalase test	+	+	+	+	+	+	+	+
Indole test	-	-	-	-	-	-	-	-
Amylase activity	+	+	+	+	+	+	-	+
Antibiotics resistance	+	+	+	+	+	+	+	+

Table 4: Bioactivity of Bacillus thuringiensis **Isolates 6OD3 and 4OD3 against Mosquito Larvae at 4 hours, 12 hours and 24 hours Intervals**

	4 hours				12 hours				24 hours			
	6OD3		**40D3**		**60D3**		**4OD3**		**6OD3**		**4OD3**	
Dilution Factor	No of live larvae	No of dead larvae	No of live larvae	No of dead larvae	No of live larvae	No of dead larvae	No of live larvae	No of dead larvae	No of live larvae	No of dead larvae	No of live larvae	No of dead larvae
10^{0}	0	5^{a}	0	5^{a}	0	5^{a}	0	5^{a}	0	5^{a}	0	5^{a}
10^{-1}	2^{c}	3^{c}	0	5^{a}	0	5^{a}	0	5^{a}	0	5^{a}	0	5^{a}
10^{-2}	1^{d}	4^{a}	2^{c}	3^{c}	0	5^{a}	0	5^{a}	0	5^{a}	0	5^{a}
10^{-3}	3^{c}	2^{c}	4^{a}	1^{d}	0	5^{a}	0	5^{a}	0	5^{a}	0	5^{a}
10^{-4}	5^{a}	0	4^{a}	1d	0	5^{a}	0	5^{a}	0	5^{a}	0	5^{a}
10^{-5}	5^{a}	0	5^{a}	0	0	5^{a}	0	5^{a}	0	5^{a}	0	5^{a}

DISCUSSION

Bacillus thuringiensis is a Gram-positive, spore-forming soil bacterium that produces insecticidal proteins during sporulation. *Bacillus thuringiensis* is the most widely used microbial control agent in the developed world. Biological pesticides based on *Bacillus thuringiensis* are becoming increasingly important in pest management programs, accounting for 80-90% of all biological pest control agents used worldwide.

A total of six out of eight samples of soil collected had *Bacillus thuringiensis*. The result of this study showed that *Bacillus thuringiensis* are abundant in soil samples. In the crystal protein morphology, the bipyramidal were the most frequent crystals in the isolates. The isolates obtained in this study were found to exhibit differences in form and size of crystal proteins. Most of the isolates have bipyramidal crystal proteins while others have cuboidal forms.

The unique character that distinguishes *Bacillus thuringiensis* from other *Bacillus* species is parasporal crystal formation. Kampfer (1991) reported that it is difficult to distinguish *Bacillus thuringiensis* from *Bacillus cereus* based on the colony morphology and almost all biochemical reactions. He also stated that analytic methods such as DNA homology, pyrolysis, gas chromatography and mass spectrometry failed to differentiate between these two species. Nevertheless, biochemical tests formed the basis of conventional bacterial classification.

In this study toxicity was demonstrated by death at a specified period of time and change in morphology of the larvae. Wei *et al.* (2003) demonstrated the toxicity by developmental, fecundity and gut morphology assays. One hundred percent mortality was recorded for both isolates (6OD1 and 4OD3) at 12 hours for all the dilution factors. The two isolates used for the purpose of this research work exhibits slight differences in their potentials against the mosquito larvae at the same period of time and same dilution factors. The control tubes which contain rain water and distilled water separately recorded 100% mortality at 24 hours. Table 4 shows the analysis of the two isolates for various dilutions at 4^{th}, 12^{th} and 24^{th} hour respectively. The activities of both isolates with the mosquito larvae for the various dilutions at the 12^{th} and 24^{th} hour were the same as all the mosquito larvae were dead at these hours of the experiment. However, at the 4^{th} hour of the experiment, there were significant differences in the values of the dead and live larvae at 10^{-2}, 10^{-3} and 10^{-4} dilutions respectively.

Change in morphology of larvae was viewed under the phase contract microscope and a change in the general morphology of the larvae was recorded which includes change in coloration, gut

shape and general body shape (Plates 1 and 2)

The role of *Bacillus thuringiensis* and its insecticidal crystal proteins in nature remains enigmatic. Glare and O'Callagan (2000) speculated that soil dwelling nematodes might have contributed to the evolution and/ or spreading of this bacterium and its crystal proteins. It seems plausible that a soil bacterium might take advantage of the fact that soil nematodes use bacteria as food source and evolve crystal proteins to help it propagate inside the host nematode once its spores and crystals are ingested.

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CONCLUSION

The results obtained in this study clearly demonstrated the efficiency of the *Bacillus thuringiensis* in controlling mosquito larvae. The use of *Bacillus thuringiensis* as a biocontrol agent against mosquito larva is preferred as it is environmentally friendly and does not deplete the ozone layer unlike the regular pesticides used in killing mosquitoes in most communities.

It is still necessary to search for more microbial toxins to control insects' orders which have the ability to develop resistance against selected insecticides. Screening of soil samples from different sources and habitats may be useful to obtain *Bacillus thuringiensis* strain with broader host ranges and novel crystal proteins.

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