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ASSESSMENT OF THE LARVICIDAL POTENTIAL OF *BACILLUS THURINGIENSIS* ISOLATES FROM SOIL AGAINST *CULEX QUINQUIFASCIATUS*

***[1]Bello, A., [2]Hussaini, I. M. and [3]Gide, S.**

[1]Department of Integrated Science, School of Science, Federal College of Education, Zaria Nigeria
[2] Department of Microbiology, Faculty of Life Sciences, Ahmadu Bello University, Zaria, Nigeria
[3] Desert Research Monitoring and Control Centre, Yobe State University, Damaturu, Nigeria

*Corresponding authors' email: adxzabello@gmail.com Phone: +2348065636427

ABSTRACT

Environmental hazard and detrimental health effects associated with usage of chemical insecticides has made the search for a safer alternative means of controlling disease vectors and pest necessary. In this study, the larvicidal potential of *Bacillus thuringiensis* isolates against *Culex quinquefasciatus* was evaluated. *B. thuringiensis* were isolated and characterized from different organic rich soils collected from various locations in Zaria, Kaduna State, Nigeria. The larvicidal potential of the isolates against *Cx. quinquefasciatus* larvae was conducted by exposing the larvae to spore crystal mixture at 50, 75 and 100 ppm concentrations. Ten larvae of *Cx. quinquefasciatus* were exposed to each concentration and the test was conducted in triplicates. Probit analysis was used to determine the LC_{50} (concentration that kills 50% of the exposed larvae) for each of concentrations assessed. For 100 ppm concentration, the mean mortality of the larvae was found to be between 33.33% and 96.67%. As for 75 ppm concentration, mean mortality range of 73.33% and 23.33% was observed while mean mortality range of 53.33% and 10.0% was observed for 50 ppm concentration. The LC_{50} was estimated to be between 148.73 ppm and 39.81 ppm. The results of this research show that *B. thuringiensis* isolated from organic rich soil samples in Zaria has promising larvicidal potential in the control of *Cx. quinquifasciatus* larvae; hence, reducing number of adult mosquitoes that serve as vector of diseases.

Keywords: *Bacillus thuringiensis, Culex quinquifasciatus*, Larvicidal, soil, activity

INTRODUCTION

Bacillus thuringiensis is a spore forming Gram-positive facultative anaerobic rod shaped saprophytic bacterium commonly found in the soil and other environment (Sanahuja *et al*., 2011; Adeyemo *et al*., 2018). They are ubiquitously found in the environment occurring naturally in soils (Crickmore *et al.*, 2005), aquatic ecosystem (Ichimatsu *et al.*, 2000), plant parts (Maduell *et al.*, 2002), dead insects (Cavados *et al.*, 2001) as well as animal excreta (Lee *et al.*, 2003).

The ubiquitous nature of *B. thuringiensis* necessitated the screening of isolates for biocontrol potentials and has led to the identification and characterization of more than 300 crystal proteins. *Bacillus thuringiensis* strains have been reported to exhibit insecticidal activity as well as activity against protozoa, mites and nematodes (Crickmore *et al.*, 2005).

Bacillus thuringiensis have been reported to be an important insect pathogen with high toxicity against larvae of mosquito as well as other related dipterans (Poopathi and Abidha, 2010; Zulfaidah *et al*., 2013). Their toxicity is attributed to the production of parasporal crystals protein called delta-endotoxin produced and assembled during sporulation (Haggag and Youssef, 2010; Sanahuja *et al.*, 2011). When ingested, the crystal protein which acts as a protoxins gets solubilized under the alkaline condition of the insects' midgut (pH>10), it then gets transformed to an active toxin through action of the intrinsic protease. The active toxin subsequently binds to a specific receptor on cell membrane selectively, thereby resulting in formation of pore and subsequent death of the insect larvae (El-kersh *et al.*, 2012; Adeyemo *et al*., 2018).

Mosquitoes serve as vectors of various aetiological agents of diseases most of which life threatening. Mosquito transmits diseases such as malaria, filariasis, yellow fever, chikungunya and dengue fever that are responsible for the death of over one million people globally, yearly (Nareshkumar *et al.*, 2012; Adeyemo *et al*., 2018).

Culex quinquefasciatus also "Southern House Mosquito" belongs to the *Culex pipiens* species complex. Principally, it is the vector of bancroftian filariasis. It has also been identified as a potential vector transmitting *Dirofilaria immitis*, West Nile Virus, St. Louis encephalitis virus, Rift Valley fever virus and *Plasmodium relictum.* It select and breed in polluted surface water as well as those rich organically, domestic container, shallow ponds and streams (Bhattacharya and Basu, 2016). During blood meals, a female *Culex quinquefasciatus* might transmit lymphatic filariasis and various arboviruses to humans.

The worldwide socioeconomic and health burden of vector transmitted diseases is on the increase partly as a result of failure of vector control measures. Hence, it is necessary to identify novel biocontrol measures effective against insecticide resistant vectors (Adam *et al.*, 2020; Achs and Malaney, 2002).

Frequent usage of chemical base insecticides is associated with environment damages (El-Kersh *et al*., 2012), disruption of the natural ecosystems resulting in the increase as well as resistant mosquito reemergence (Radhika *et al*., 2011; Zhang *et al.*, 2011) and detrimental effects on beneficial organisms (Das et al., 2007; Zhang *et al.*, 2011).

These drawbacks have necessitated the search for ecofriendly alternative control means such as use of biological control. Use of microbial derived insecticides is valuable since they have low or no toxicity on humans and animals (Aramideh *et al*., 2010; El-kersh *et al*., 2012).

The development of new control strategies, especially naturally occurring larvicidal and mosquitocidal agents is vital in combating the emergence of resistant vectors and the undesirable effects on beneficial organisms in the environment (Cetin and Yanikoglu, 2006). Therefore, the aim of this study is to isolate strains of *Bacillus thuringiensis* from

different soil types and to test for their larvicidal potential against *Culex quinquefasciatus* larvae. The study revealed the occurrence and distribution of *B. thuringiensis* different soil samples in Zaria with larvicidal activity against larvae of *Cx. quinquefasciatus* in organic rich soils within Zaria.

MATERIALS AND METHODS

Isolation and characterization

Organic rich soils collected from different sites within Zaria, Kaduna State, Nigeria were used for *B. thuringiensis* isolation. The soil samples were collected between September and November, 2019. Prior to collection of samples, the soil surface of each of the sampling sites was scrapped with a sterile spatula and then 10 g of soil samples were collected from 5 cm depth. The samples were placed in a sterile plastic bag and transported to the laboratory. Thirty (30) samples consisting of 10 from each of the three different soil types (Agricultural soil, Waste dump site and Cow range land) were collected; these soil types were selected due to the high abundance and accumulation of organic matter as well as the presence of insects. To 10 mL of LB (Luria Bertani) medium (10 g of Tryptone, 5 g of yeast extract and 5 g of NaCl per 1 L) supplemented with 0.25 M of CH3COONa (sodium acetate), 1 g of soil was added. The inoculated media were incubated for 4 hours at 30 °C in a shaker incubator at 250 rpm. Then 2 mL of each sample was place in a water bath set 80 °C to heat shocked for 20 minutes. Afterward, each of the treated samples were serially diluted using sterile distilled water, inoculated onto T3 agar (3 g of Tryptone, 1.5 g of Yeast extract, 2 g of Tryptose, 0.005 g of $MnCl_2$, 0.05M of Sodium phosphate and 15 g of Agar per 1 L) and then incubated for 48 hrs at 26 °C. Distinct colonies that appear as off white dry colonies with entire margin were sub-cultured onto LB agar plates (3 g of Tryptone, 1.5 g of Yeast extract, 2 g of Tryptose, 0.005 g of $MnCl_2$, 6.9 g of NaH_2PO_4, 8.9 g of Na_2HPO_4 and 15 g of Agar per 1 L) (Travers *et al.,* 1987).

The presumptive *B. thuringiensis* isolates were characterized by Gram staining, endospore staining, test for motility, ability to grow above 45 °C, catalase test and Voges Proskauer test. Identity of the isolates was confirmed using Microgen™ Bacillus-ID.

Evaluation of the larvicidal potential of *B. thuringiensis* isolates against larvae of *Culex quinquifasciatus*

Spore crystal mixture preparation

The mixtures were obtained by inoculating single colonies from overnight culture of *B. thuringiensis* on LB plates into 10 mL of T3 sporulation medium (composed of tryptone (3 g), tryptose (2 g), yeast extract (1.5 g), sodium phosphate (0.05 g) and $MnCl_2$ (0.005 g) per 1 L at pH 6.8) and incubating for 60 hours at 30ºC in a shaker incubator. After the incubation, the medium was centrifuged for 10 mins at 7000 g and washed using sterile distilled water twice to obtain the pellet of spore and crystal mixtures which were kept at 4ºC for storage (Gorashi *et al*., 2012).

Raising and collection of mosquito larvae

Blood fed adult females *Culex quinquefasciatus* were trapped with test tubes and introduced into cages designed for entomological studies. Bowls containing distilled water were placed in the cages to facilitate oviposition. Enclosed larvae from the eggs were nurtured on a diet of biscuit and barkers yeast (Ratio of 3:1) till when they moult into the 3rd instar larvae which were used for bioassay (Adebote *et al*., 2011).

Larval selection and counting

With the aid of a modified pasteur pipette, early 3rd instar (L3) larvae of *Cx. quinquefasciatus* were selected and placed into test tubes. For each test tube, 10 early L3 larvae were selected and transferred and distilled water was used to make the volume to 10 mL.

Bioassay

The larvicidal activity was evaluated by exposing the larvae to spores and crystal mixture to concentrations of 100 ppm, 75 ppm and 50 ppm. For each concentration the assay was conducted in triplicate and the control used was a test tube containing distilled water with 10 early L3 larvae. After 24 hrs, the larvae were observed for mortality and recorded (Naiema *et al.,* 2012).

Potency of the spore and crystal mixture against the larvae was assessed using probit analysis. Empirical Probit of kill for the tested concentrations was calculated using probit table and the corresponding mortality of each concentration. Regression equation derived from the graph of empirical probit of kill against the log of concentration was used for the calculation of median lethal concentration (LC_{50}).

Data analysis

Mean mortality of the concentrations of spore and crystal mixture for each isolate were compared using Analysis of Variance (ANOVA).

RESULTS

Twelve isolates of *B. thuringiensis*; consisting of 5 (41.67%) isolates from agricultural soil, 2 (16.67%) isolates from refuse dump site and 5 (41.67%) isolates from cow range land were isolated from the soil samples collected (Figure 1).

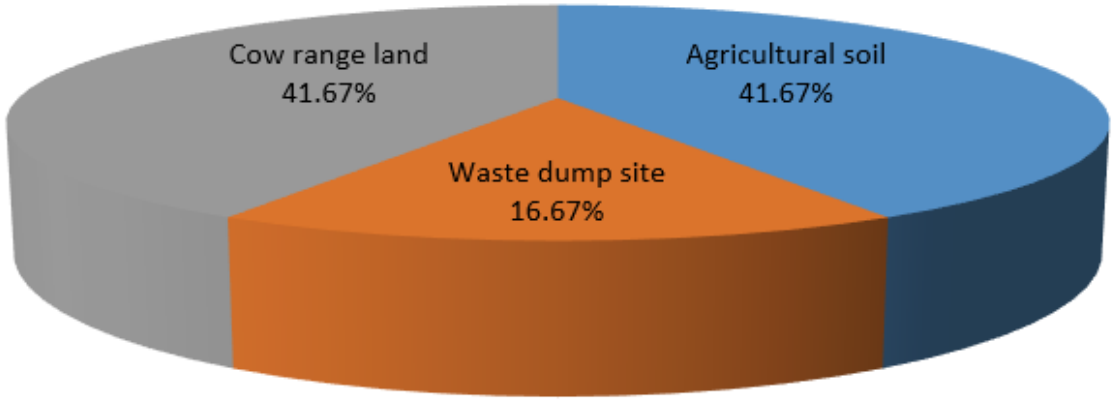

Figure 1: Distribution of *Bacillus thuringiensis* in soil from various sources.

Larvicidal activity was observed for the *B. thuringiensis* isolates at all the concentrations tested (100, 75 and 50 ppm) after exposure for 24 hrs. Highest mean mortality of *Cx. quinquefasciatus* larvae was observed at 100 ppm followed by 75 ppm while least mean mortality was observed at 50 ppm for all the isolates. Isolate L3 showed the highest larvicidal activity (96.67% mean mortality) at 100 ppm concentration of spore crystal mixture while isolate D2 showed the lowest larvicidal activity (33.33% mean mortality) against *Cx. quinquefasciatus* larvae at 100 ppm concentration of spore crystal mixture. Statistically significant differences were observed in mean mortality of *Cx. quinquefasciatus* larvae at different concentrations (Table 1).

Table 1: Mean mortality of *Culex quinquefascitus* larvae exposured to various spore-crystal mixtures at concentrations.

Isolates	Conc. (ppm)	No. of larvae		Mean mortality(95% CI ±SE)	ANOVA	
		Exposed*	Dead		F-Value	P-Value
A1	0(Control)	30	0	0.00^{d} (0.00-0.00±0.00)	124.422	$4.73x10^{-7}$
	50	30	7	2.33^{c} (0.89-3.77±0.34)		
	75	30	16	5.33^{b} (3.88-6.77±0.34)		
	100	30	22	7.33^{a} (5.88-8.77±0.34)		
C2	0(Control)	30	0	0.00^{c} (0.00-0.00±0.00)	24.4278	$2.216x10^{-4}$
	50	30	7	2.33^{b} (-0.53-5.18±0.66)		
	75	30	14	4.67^{a} (3.22-6.11±0.34)		
	100	30	15	5.00^{a} (2.51-7.48±0.00)		
D1	0(Control)	30	0	0.00^{c} (0.00-0.00±0.00)	32.523	$7.865x10^{-5}$
	50	30	8	2.67^{b} (1.22-4.11±0.34)		
	75	30	12	4.00^{ab} (1.51-6.48±0.58)		
	100	30	21	7.00^{a} (7.00-7.00±0.00)		
E2	0(Control)	30	0	0.00^{d} (0.00-0.00±0.00)	111.789	$7.189x10^{-7}$
	50	30	7	2.33^{c} (0.88-3.77±0.34)		
	75	30	11	3.67^{b} (2.22-5.11±0.34)		
	100	30	12	4.00^{a} (1.51-6.48±0.34)		
D2	0 (Control)	30	0	0.00^{c} (0.00-0.00±0.00)	16.801	$8.174x10^{-4}$
	50	30	3	1.00^{b} (1.00-1.00±0.00)		
	75	30	8	2.67^{a} (1.22-4.11±0.34)		
	100	30	10	3.33^{a} (0.47-6.18±0.66)		
N2	0(Control)	30	0	0.00^{c} (0.00-0.00±0.00)	110.683	$7.467x10^{-7}$
	50	30	14	4.67^{b} (3.22-6.11±0.34)		
	75	30	22	7.33^{a} (5.88-8.77±0.34)		
	100	30	27	9.00^{a} (6.51-11.48±0.58)		
N3	0(Control)	30	0	0.00^{d} (0.00-0.00±0.00)	207.152	$6.388x10^{-8}$
	50	30	7	2.33^{c} (0.88-3.77±0.34)		
	75	30	16	5.33^{b} (3.88-6.77±0.34)		

	100	30	18	6.00[a] (1.70-10.29±1.00)		
L1	0(Control)	30	0	0.00[b] (0.00-0.00±0.00)	25.7684	$1.830x10^{-4}$
	50	30	11	3.67[a] (2.22-5.11±0.34)		
	75	30	17	5.67[a] (2.81-8.52±0.66)		
	100	30	21	7.00[a] (2.70-11.29±1.00)		
L2	0(Control)	30	0	0.00[c] (0.00-0.00±0.00)	27.3988	$1.467x10^{-4}$
	50	30	8	2.67[b] (1.22-4.11±0.34)		
	75	30	11	3.67[b] (2.22-5.11±0.34)		
	100	30	12	4.00[a] (1.51-6.48±0.58)		
L3	0(Control)	30	1	0.00[b] (0.00-0.00±0.00)	76.3103	$3.155x10^{-6}$
	50	30	16	5.33[a] (2.47-8.18±0.66)		
	75	30	21	7.00[a] (7.00-7.00±0.00)		
	100	30	29	9.67[a] (8.22-11.11±0.34)		
L6	0(Control)	30	0	0.00[c] (0.00-0.00±0.00)	15.4026	$1.096x10^{-3}$
	50	30	3	1.00[bc] (-1.48-3.48±0.58)		
	75	30	7	2.33[ab] (0.88-3.77±0.34)		
	100	30	12	4.00[a] (1.51-6.48±0.58)		
L7	0(Control)	30	0	0.00[b] (0.00-0.00±0.00)	82.1062	$2.379x10^{-6}$
	50	30	15	5.00[a] (5.00-5.00±0.00)		
	75	30	20	6.67[a] (5.22-8.11±0.34)		
	100	30	21	7.00[a] (7.00-7.00±0.00)		

Means with same superscript for each isolate are not significantly different (P>0.05).
* 10 larvae in triplicate = 30 larvae.

Table 2 shows LC_{50} of *Bacillus thuringiensis* isolates against *Cx. quinquefasciatus* larvae. The LC_{50} value of the isolates was found to range from 39.81 ppm to 148.73 ppm (Table 2).

Table 2: LC_{50} (ppm) of *Bacillus thuringiensis* isolates against larvae of *Cx. quinquefasciatus*

Isolates	Conc. (ppm)	Log of conc	Mort. (%)	EPK	RE	R^2	LC_{50} ppm
A1	100	2.00	73	5.61	Y= 4.3673x-3.1107	0.9991	71.96
	75	1.87	53	5.08			
	50	1.67	23	4.26			
	0	-	0	-			
C2	100	2.00	50	5.00	Y= 2.4663x-0.1558	0.8937	92.06
	75	1.87	47	4.92			
	50	1.67	23	4.26			
	0	-	0	-			
D1	100	2.00	70	5.25	Y= 2.7263x-0.256	0.9656	84.68
	75	1.87	40	4.75			
	50	1.67	27	4.39			
	0	-	0	-			
E2	100	2.00	40	4.75	Y= 4.2263x-2.986	0.9036	77.53
	75	1.87	37	4.67			
	50	1.67	23	4.26			
	0	-	0	-			
D2	100	2.00	33	4.56	Y= 2.7724x-0.9148	0.9443	135.95
	75	1.87	27	4.39			
	50	1.67	10	3.72			
	0	-	0	-			
N2	100	2.00	90	6.28	Y= 4.3528x-2.4639	0.9929	51.84
	75	1.87	73	5.61			
	50	1.67	47	4.92			
	0	-	0	-			
N3	100	2.00	60	5.25	Y= 8.2105x-9.81	0.9095	63.63
	75	1.87	53	5.08			
	50	1.67	23	4.26			
	0	-	0	-			
L1	100	2.00	70	5.52	Y= 0.4175x+4.093	0.0561	148.73
	75	1.87	57	5.18			
	50	1.67	37	4.67			
	0	-	0	-			

L2	100	2.00	40	4.75	Y= 3.5158x-1.656	0.8650	78.18
	75	1.87	37	4.67			
	50	1.67	27	4.39			
	0	-	0	-			
L3	100	2.00	97	6.88	Y= 1.2407x+3.0472	0.8589	55.79
	75	1.87	70	5.52			
	50	1.67	53	5.08			
	0	-	0	-			
L6	100	2.00	40	4.75	Y= 3.3026x-1.8775	0.9958	120.89
	75	1.87	23	4.26			
	50	1.67	10	3.72			
	0	-	0	-			
L7	100	2.00	70	5.52	Y= 0.9078x+3.5475	0.4101	39.81
	75	1.87	67	5.44			
	50	1.67	50	5.00			
	0	-	0	-			

Conc. = concentration, Log = logarithm, Mort. = mortality, ACM = Abbott's corrected mortality, EPK = Empirical probit of kill, RE = Regression equation R^2 = Coefficient of determination LC_{50} = Median Lethal concentration.

In terms of the degree of the larvicidal activity of the isolates, no insecticidal activity between the range 0.00%-25.00% against *Cx. quinquefasciatus* was seen in all the isolates. Three isolates from agricultural soil and two from cow rangeland had insecticidal activity between the range of 25.01%-50.00% against *Cx. quinquefasciatus* so also two isolates from these two sites and one from waste dump soil had activity between the range 50.01%-75.00% against *Cx. quinquefasciatus*. One isolate each from refuse dump soil and cow rangeland had activity between the range 75.01%-100.00% against *Cx. quinquefasciatus* all at 100 ppm concentration (Figure 2).

Larvicidal activities of most of the isolates (83%) against *Cx. quinquefasciatus* larvae were between 25.01 - 75.00%. Some of the isolates (17%) had larvicidal activity ranging between 75.00%-100.0%. Figure 4 represents the percentage distribution of the larvicidal activity of 100 ppm spore and crystal mixture against *Cx. quinquefasciatus* larvae.

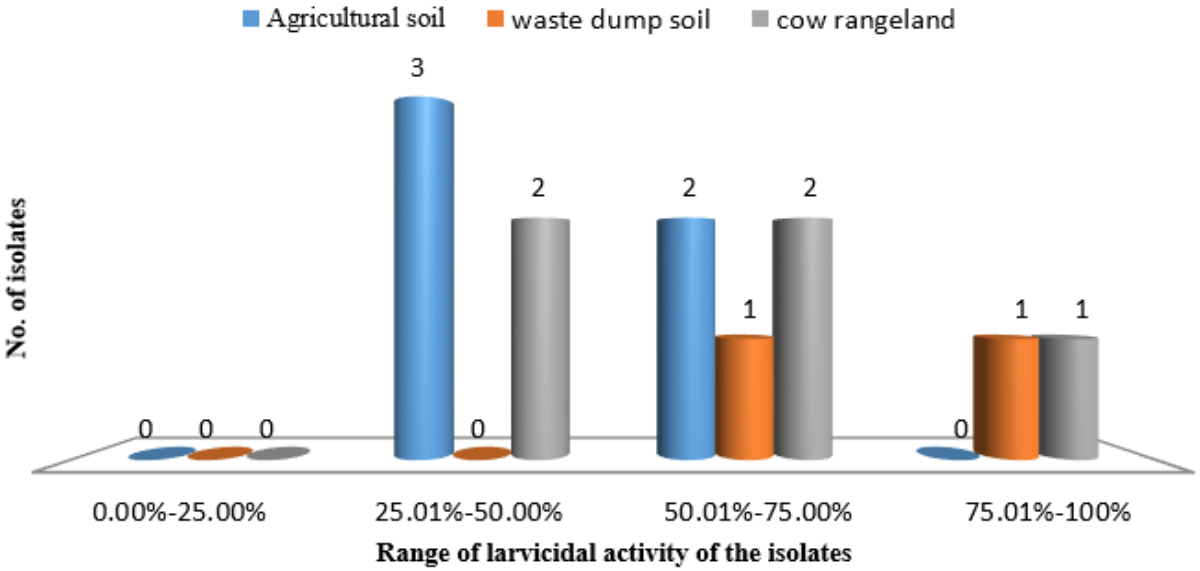

Figure 2: Larvicidal activity of the *B. thuringiensis* isolates from the three soil types against *Cx. quinquefasciatus* using 100 ppm spore and crystal mixture

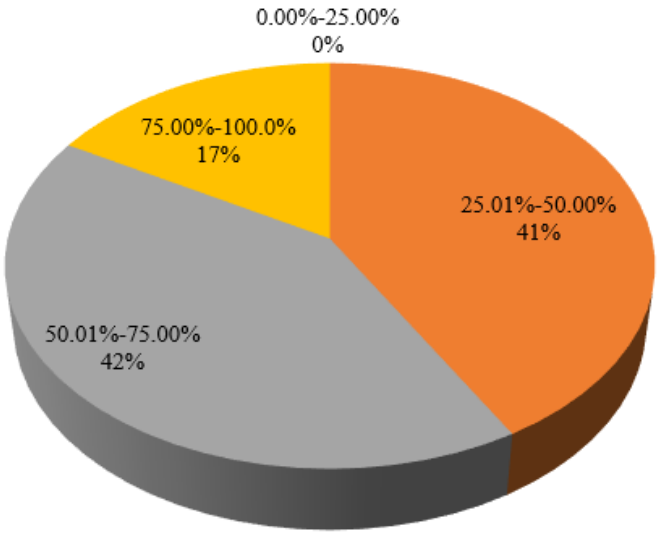

Figure 3: Distribution of larvicidal activity of 100 ppm spore and crystal mixture against *Cx. quinquefasciatus* larvae.

DISCUSSION

There is an increased research interest on alternative ecofriendly means of controlling insect. *B. thuringiensis* is known to produce toxic crystals that are potent to agricultural pests (Çetinkaya, 2002).

A total of 12 *B. thuringiensis* isolates were isolated from the 30 organic rich soil samples collected. This shows the relative abundance of *B. thuringiensis* in organic rich soil. This isolation rate (40.0%) is however low compared to the report of Adeyemo *et al*. (2018) who isolated 6 *B. thuringiensis* from 8 soil samples collected giving an isolation rate of 75.0%. This variation might be due to difference in the soil properties, season during which the samples were collected or human activities carried out on the soil.

In this study, the bioassay focused on the larval stage of mosquito which has been reported to be more effective as it reduces the number of emerging adult mosquitoes. So also, the low morbidity of larvae and the fact that are found confined to water bodies makes it easy to apply control and invention measures. Adult mosquitoes are highly mobile flying insect, as such, they can detect and evade many invention and control measures (Killeen *et al.*, 2002).

Larvicidal activity against larvae of *Cx. quinquefasciatus* was exhibited by spore crystal mixture of all the isolates at 100 ppm, 75 ppm and 50 ppm concentrations. This result reveals the entomopathogenic potential of *B. thuringiensis* isolates native to Zaria soil. This implies that all the isolates are potential biocontrol agent. Contrary to this observation, Ahmed *et al*. (2017) reported low percentage (14.71%; 10/68) of *B. thuringiensis* isolates with larvicidal active against *Cx. pipiens* larvae. Furthermore, finding by El-Kersh *et al*. (2012) revealed that most *B. thuringiensis* isolated from various regions of Saudi Arabia had no activity against larvae of *Cx. pipiens*. This observation might be attributed to the variation in susceptibility of species of *Culex* mosquito (*Cx. Quinquefasciatus* or *Cx. pipiens*) treated or difference in the number and type (shape) of parasporal crystals produced by the *B. thuringiensis* isolates screened.

A statistically significant decline in mortality of the larvae was observed with decline in the concentration of spore crystal mixture. This phenomenon is not unexpected, since higher concentration produce high effect or activity.

The 12 isolates of *B. thuringiensis* exhibited differences in their larvicidal activity against of *Cx. quinquefasciatus* larvae at the same concentration and exposure time, with isolate L3 exhibiting the highest larvicidal activity of 96.67% and isolate D2 exhibiting the lowest larvicidal activity of 33.33% mean mortality at 100 ppm concentration of spore crystal mixture. Differences in the larvicidal activity of the isolates might be attributed to difference in metabolic capability of the isolates (Ahmed *et al*., 2017), genes composition (*cry* and *cyt*) of the isolates (El-Kersh *et al.*, 2016) or complex interactions between various types crystal proteins (Ben-Dov, 2014).

This is similar to the report of Adeyemo *et al.* (2018) who also observed differences in larvicidal activity of isolates of *B. thuringiensis* against the mosquito larvae at the same period of time and same dilution factors.

The LC_{50} values of the isolates were estimated to be between 39.81 ppm and 148.73 ppm for isolate with the highest toxicity (L7) and isolate with the least toxicity (L1) respectively. Mortality was not observed in any of the control test tubes used after 24 hours. This confirms that mortality observed is due to exposure and ingestion to spore crystal mixture. This is contrary to the findings of Adeyemo *et al.* (2018) who observed 100 % mortality in control tubes after 24 hours.

All the *B. thuringiensis* isolates in this study exhibited promising larvicidal potential against *Cx. quinquefasciatus* larvae; this might be linked to the nature of the environment from which they were isolated. Soils rich in organic matter and containing host insects favour the occurrence and distribution of entomopathogenic *B. thuringiensis* isolates. Similar phenomenon was reported by Gonzalez *et al.* (2013).

CONCLUSION

Bacillus thuringiensis isolates with promising larvicidal activity against *Cx. quinquefasciatus* larvae were recovered from organic rich soil samples collected from Zaria. Hence, these isolates are promising biocontrol agents for *Cx. quinquefasciatus* at larval stage.

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