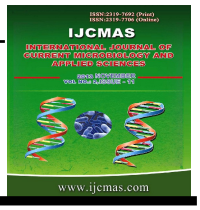
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 2 Number 11 (2013) pp. 419-428**
http://www.ijcmas.com



Original Research Article

Incidence of entomopathogenic *Bacillus* spp. in some plantations in Ibadan, Nigeria

T.A. Adelani-Akande[1, 2]*, O.E. Fagade[1] and B.I. Aderiye[2,3].

[1]Department of Microbiology, University of Ibadan, Ibadan, Nigeria
[2]Microbiology Unit, Biological Sciences Department, Landmark University, Omu Aran, Kwara State, Nigeria
[3]Microbiology Department, Ekiti State University, Ado-Ekiti, Nigeria
*****Corresponding author*

A B S T R A C T

Soil samples from some agricultural research plantations (IITA, CRIN, IAR&T, NIHORT and FRIN) in Ibadan metropolis, Nigeria were screened for the presence of entomopathogenic strains of *Bacillus* species. The loamy-sand and sandy-loam soil types were typical soil texture of these locations with soil pH ranging between 4.9 and 6.1. The loamy-sand soil type recorded the highest number of *Bacillus* spp. and the history of pesticides applied on the sampling locations did not seem to affect the distribution of *Bacillus thuringensis* and other entomopathogenic *Bacillus* spp. in the different soils. Eighty-two strains of *Bacillus* spp. were isolated with only seven of these being entomopathogenic. The seven strains of *Bacillus* spp. were toxic to *Anopheles* mosquito larvae exhibiting between 5% and 15% mortality within 72h.

Keywords

Bacillus spp.; loamy-sand; sandy- loam; entomopathogenesis; *Anopheles* mosquito larvae.

Introduction

Some *Bacillus* species have been recognized for their effectiveness against larvae of some major insects which are either plant pests such as *Spodoptera frugiperda, S. littoralis, Lymantria dispar, Drosophila melanogaster* (Al-Momani and Obeidat, 2012; Aly 2007; Broderick *et al.*, 2000; Manju *et al*., 2009; Valicente *et. al.*, 2005) or vectors of diseases such as *Anopheles gambiae, Culex* and *Aedes* (Gbehou *et. al*., 2010; Majambere *et al*., 2007). *Bacillus thuringiensis* (Bt.) and *Bacillus sphaericus* are well known but *B. sphaericus* is reported to be slow in its action on insect larvae and thus Bt. has been widely explored for its insecticidal ability (Chatterjee *et al*., 2007; Surendran and Vennison, 2011). In Africa and particularly Nigeria, malaria is wide spread and the *Plasmodium* parasite is transferred to humans by the female *Anopheles* mosquito. *Bacillus thuringiensis*, a Gram positive, toxin producing species of the genus *Bacillus*

has been widely known because of the insecticidal prowess resulting from its ability to produce parasporal protein bodies (Prescott *et al*., 2010). *B. thuringiensis* belongs to the group 1 bacilli which include *B. thuringiensis*, *B. cereus* and *B. anthracis*; however, *B. thuringiensis* does not cause food poisoning. *Bacillus thuringiensis* serovar *israelensis* is known for its efficiency against the larvae of mosquitoes (Charles and Nielson-Le Roux, 2000).

B. thuringiensis is able to produce an intracellular protein crystal during sporulation which is toxic to mosquitoes. The protein crystals so produced are solubilized in the mid-gut of the insect to form proteins called delta-endotoxins (Ammouneh *et al.,* 2011) that are toxic in low concentrations to insects that have specific receptors located in the mid-gut epithelium (Tyrell *et al.,* 1979). Although *B. thuringiensis* has no adverse effects on variety of vertebrates and invertebrates animals, however under certain conditions, it was reported to be pathogenic to earthworm *Lumbricus terrestris* (Zwahlen *et. al*., 2003). Isolation of *Bacillus thuringiensis* from soil used to be cumbersome however the development of better isolation techniques and media has enhanced research on *B. thuringiensis* (Travers *et al.,* 1987; Andrzejczak and Lonc, 2008).

Bacillus sphaericus has also been shown to be insecticidal to mosquito larvae (Charles and Nielson-Le Roux, 2000). Surendran and Vennison (2011) isolated *B. sphaericus* from soils obtained in different ecological zones in Devakottai, India and tested for larvicidal activity on *Culex quinquefasciatus.* They reported a significant level of variation in larvicidal activity of the *B. sphaericus* isolates.

Valicente *et al*. (2005) reported the wide spread of *Bacillus thuringiensis* in soils in Brazil indicating that its occurrence was higher in the West-Central and Southern regions of the country. Occurrence of *Bacillus thuringiensis* has also been revealed in various regions of the United States (Gbehou *et. al*., 2010) as well as in 12.5% of soil samples collected from various regions of Syria (Ammouneh *et al.,* 2011).

Studies on the use of *B. thuringiensis* and other entomopathogenic *Bacillus* spp. against mosquito larva include the report by Majambere *et al.* (2007) where the third instar larvae of *Anopheles gambiae* were susceptible to water-dispersible and corn granule formulations of the *Bacillus sphaericus* strain. In Brazil, Valicente *et al.* (2005) also documented the incidence of *B. thuringiensis* in soil using a modified form of Luria Bertani broth and its ability to effectively control the maize pest, *Spodoptera frugiperda* (fall armyworm). No further application of the chemical insecticide was required after the application of *B. thuringiensis*; this further confirmed the efficacy of this organism as a biocontrol agent. Ammouneh *et al*., (2011) confirmed the toxicity of *B. thuringiensis* to the larvae of *Ephestia kuehniella*, *Phthorimaea operculella*, and *Cydia pomonella* but those of *Culex quinqefasciatus* were not susceptible.

Biocontrol methods have proved to be safer compared to chemical control of pest (Majambere *et al*., 2007). This study therefore seeks to isolate and screen the different strains of *Bacillus* spp. obtained from some soil samples in Ibadan metropolis and document the occurrence of entomopathogenic *Bacillus* spp. within this region.

Materials and Methods

Sample Collection

Soil samples were collected from farms and forests located in research institutes within and around Ibadan, Oyo State of Nigeria namely: International Institute of Tropical Agriculture (IITA), Moniya; Cocoa Research Institute of Nigeria (CRIN), Idi Ayunre; Institute of Agricultural Research and Training (IAR&T), Moor Plantation; Nigerian Institute of Horticulture (NIHORT), Idi Ishin and Forestry Research Institute of Nigeria (FRIN), Idi-Ishin.

Soil samples were collected using soil auger, hand trowel, shovel and sampling bags as required in each location. The samples were collected within a range of 0 - 15 cm depth. The bulk sample collected from each farm/ forest was thoroughly mixed and a representative sample was taken for further analysis.

Table 1 shows details of the farming practices employed in each location. All the soil samples had been treated with one or more pesticides in the past couple of years except those obtained from the Forestry Research Institute of Nigeria (FRIN), Idi-Ishin, where there was a fire incidence in 2008. However, there were no indications that pesticides were applied on the plantation.

All the soil samples were treated with one type of pesticide or another except the FRIN soil samples. The soil samples from IAR & T were treated with different pesticides such as Karate, Kochem, Furadan, Paraquat, Primextra, Glyphosphate, depending on the type of crops planted (Table 1). At different sites in the cocoa plantation at CRIN, Ultimax, Ridomil Plus and Kocide were applied to the pods at the recommended doses. Soils from the cassava plantation at IITA and the vegetable farms at the Nigerian Institute of Horticulture (NIHORT) were also treated with one form of pesticide (not disclosed).

Sterilization and Media Preparation

All apparatus used were properly sterilized. The media were prepared according to the manufacturer's direction and sterilized by autoclaving at 121^{o}C and 1.05Kg/cm^{2} for 15 minutes.

Isolation of Bacteria from Soil Samples

Isolation was done using the modified method of Travers *et al.* (1987). A portion of each soil sample was spread evenly on aluminum foil and heated at 80^{o}C for 30 minutes in an oven. Serial dilution was carried out and samples were plated out on tryptone soy agar. The plates were incubated at 27^{o}C ± 2 for 24 h. The total viable bacteria count in each sample was determined. Pure cultures were obtained by streaking isolates on tryptone soy agar using a wire loop.

Morphological and Biochemical Characterization of isolates

Morphological and biochemical characteristics of the isolates were determined using the standard procedure described by Harrigan and McCance (1966).

Identification of Isolates

The isolates were identified according to Bergey's Manual of Determinative Microbiology, 8th edition (Buchanan and Gibbons, 1974) and compared with standard microbial cultures from our laboratory.

Table.1 Summary of crops and pesticide application on sampling sites

SAMPLE LOCATION	CROPS PLANTED	PESTICIDE APPLICATION
IITA, Moniya	Cassava	Yes
CRIN, Idi Ayunre	Cocoa	Ultimax, Ridomil Plus, Kocide
IAR & T, Moor Plantation	Maize, Pigeon pea/ local beans, yam/ cassava	Karate, Kochem, Furadan, Paraquat, Primextra, Glyphosphate
NIHORT, Idi Isin	Vegetable, Okra, water melon	Yes (except in one of the farms)
*FRIN, Idi Isin	Pine, *Gmelina*	Not Determined

*Fire incidence around 2008

Bioassay of Isolates on Mosquito Larvae

Newly emerging *Anopheles* larvae were harvested from stagnant water bodies at Ojoo, in Ibadan. Identification of the insect larvae was carried out at the Department of Zoology, University of Ibadan. The larvae were allowed to acclimatize in distilled water for 18h before the bioassay. The third and fourth instar of *Anopheles* larvae were used for the bioassay. Only seven isolates of the *Bacillus* spp. were screened for their ability to produce toxins harmful to larvae of *Anopheles* mosquito.

The bacteria isolates were grown in nutrient broth supplemented with $MnSO_4$ for 48 h at $27^oC \pm 2$ (Ammouneh *et al.*, 2011). The cells where harvested in a refrigerated centrifuge at 2,000 rpm for 15 min, later rinsed and adjusted with distilled water to a known concentration.
Twenty (20) third and fourth instar larvae of field collected *Anopheles* mosquito were introduced into 10 ml of known concentration of each isolate. The control contained only distilled water and the mosquito larva. The effect of each *Bacillus* spp. on the mosquito larvae was monitored over a period of 72 h at $27^oC \pm 2^oC$, checking for mortality at 12h, 18h, 24h, 36h, 42h, 48h and 72h.

Result and Discussion

Considering the soil texture, a very high proportion of sand (ca. 69.2% - 83.2%) was observed in all the samples compared to the quantity of silt (10% - 14%) and clay (6.8% - 16.8%) (Fig.1). The texture of the soil samples obtained from IITA, IAR&T and NIHORT were loamy-sand while those from CRIN and FRIN were of sandy-loam nature. Soil samples from IAR&T had the highest sand content (83.2%) while the CRIN soil samples had low sand content (69.2%). The percentage of silt (14.0%) and clay (16.8%) on the cocoa farm was relatively high compared to other soil samples [Fig. 1]. The soil sample from IAR&T had the least clay content (6.8%).

The pH of soil samples ranged between 4.9 and 6.1, mainly of acidic nature (Fig. 2). The soil sample obtained from the fruit and vegetable plantation (NIHORT) was the most acidic (4.9) while those samples collected from the cassava (IITA) and the cocoa farms (CRIN) were weakly acidic (pH 6.0 and 6.1 respectively).

Fig. 3 shows the total viable bacteria count (VBC) of the different soil samples. The IAR&T soil samples revealed the highest VBC of 1.87×10^3 CFU/ml while the CRIN soil samples recorded the least total

VBC (7.7 X 10^2 CFU/ml). The soil samples from IITA and IAR&T showed very high incidence of bacteria (values above 1.50 X 10^3 CFU/ml).

A total of eighty-two isolates of *Bacillus* spp. were obtained from all the soil samples, with sixty from the loamy-sand and twenty-two from the sandy-loam soil (Table 2). Very high number of isolates were obtained from the soil samples in IITA, IAR&T and NIHORT (73.1%). Soils from IITA and IAR&T had the highest incidence of *Bacillus* spp. (>50%) while those from CRIN and NIHORT recorded average occurrence of *Bacillus* spp. (less than 40%) and FRIN recorded very low incidence of *Bacillus* spp. Only few isolates (8.5%) of *Bacillus* spp. were recovered from the soil of the forest plantation (FRIN). For further work, only seven strains of *Bacillus* spp. were screened for entomopathogenesis.

All the *Bacillus* spp. displayed some degree of mortality within the first 12h of the bioassay. *Bacillus* sp. TAA1018 displayed 10% mortality within 12h, and this remained constant throughout the 72h bioassay (Table 3). The same trend was observed with *Bacillus* sp. TAA1024 which recorded a final mortality of 10%. After 12h of bioassay with *Bacillus* sp. TAA1019, a 10% mortality of *Anopheles* larvae was recorded, this was followed by a 5% increase after 36h.

The final percentage mortality after 72h was 15%. The *Bacillus* sp. TAA1022 obtained from CRIN recorded a percentage mortality of 5% within 12h and maintained the same potency until 72h of the bioassay. Within 12h of the bioassay, *Bacillus* sphaericus (TAA1025) and *Bacillus* sp. TAA1031 showed 5% mortality. Their potency increased to 10% mortality after 18h and the value remained constant until 72h. *Bacillus thuringiensis* (TAA1033) on the other hand revealed 5% mortality after 12h and after 24h there was an increase to 10%.

The control did not show any mortality during the first 42h of the bioassay but 5% mortality was recorded after 48h of the bioassay (Table 3). At the end of the 72h bioassay, only one of the *Bacillus* sp. TAA1022 displayed a 5% mortality, five *Bacillus* spp. including *Bacillus thuringiensis* TAA1018, *Bacillus thuringiensis* TAA1033 and *Bacillus sphaericus* TAA1025 revealed a percentage mortality of 10% while *Bacillus* sp. TAA1019 exhibited a mortality of 15%.

The incidence of *Bacillus* spp. varied with the soil texture of the different plantations. Similarly, the soil pH had an effect on the diversity of the microbes. But Lauber *et al.* (2008) stated that the soil texture, total carbon and extractable phosphorous differed significantly between land use types while noting that the soil pH did not vary significantly. It is not clear from this study if the application of the known pesticides affected the occurrence of *Bacillus* spp. However, it is evident that the outbreak of fire in 2008 at FRIN had an impact on the incidence of these microbes in the pine and *Gmelina* plantations.

Sixty *Bacillus* spp. were obtained from the loamy-sand while only twenty-two isolates were recovered from the sandy-loam soil. At the time of sampling, cassava was planted on all the sampling locations in IITA while yam and cassava were cultivated on one of the farms in IAR&T. The high incidence of *Bacillus* spp. in IAR&T, FRIN and IITA is likely due to high sand content in this soil samples.

Fig.1 Average proportion of sand, silt and clay in sampling locations

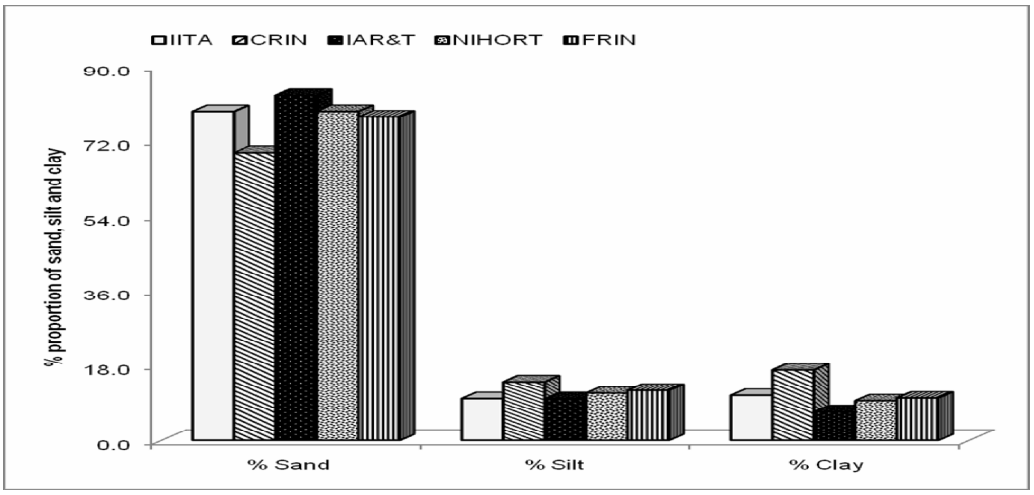


Fig.2 Average pH of soil samples

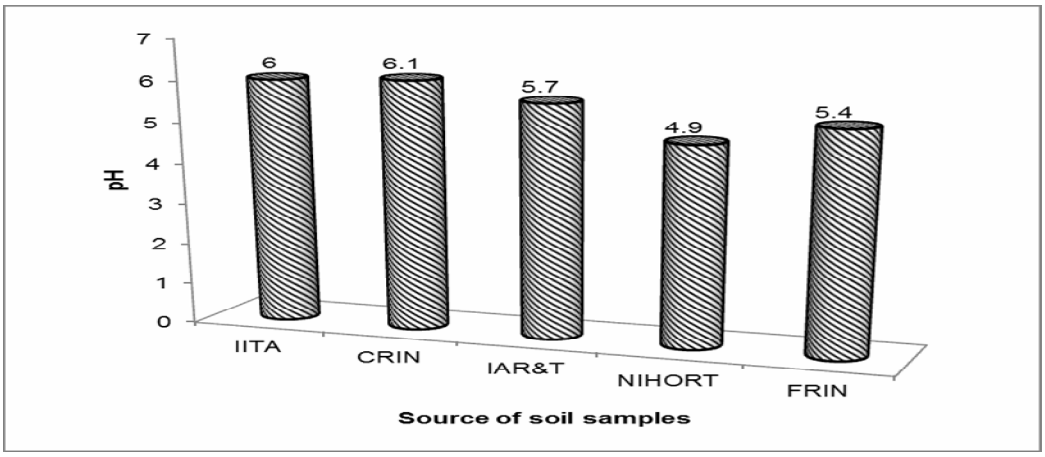


Fig.3 Total Viable Bacteria Count

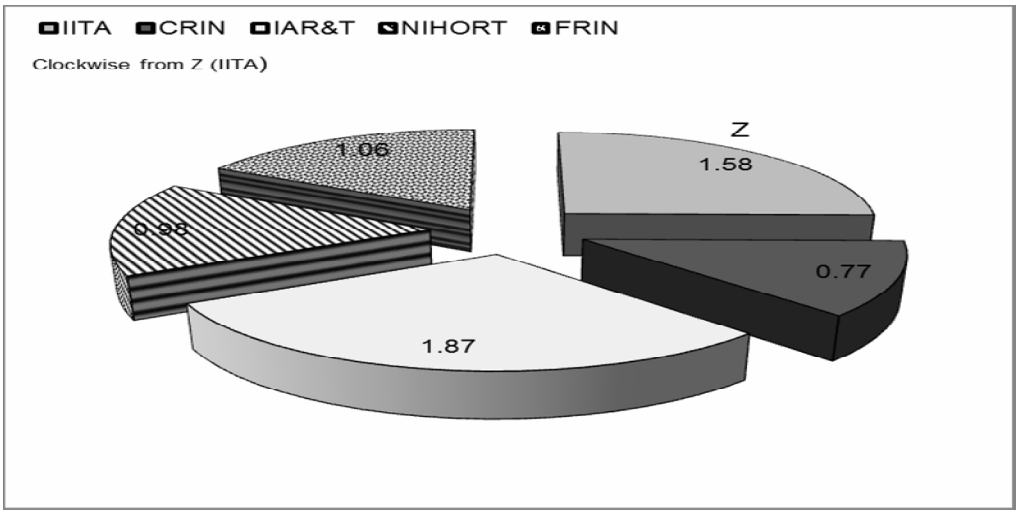


Table.2 Occurrence of *Bacillus* spp. in the plantations

Sample location	Soil Texture	No. of *Bacillus* spp.	% Occurrence in Soil types
IITA	Loamy-sand	22	26.8
CRIN	Sandy-loam	15	18.3
IAR&T	Loamy-sand	21	25.6
NIHORT	Loamy-sand	17	20.7
FRIN	Sandy-loam	7	8.5

Table.3 Percentage *mortality of seven strains of *Bacillus* spp. screened for entomopathogenesis

Location of isolate	*Bacillus* spp.	Percentage Mortality						
		12h	**18h**	**24h**	**36h**	**42h**	**48h**	**72h**
IITA	*Bacillus thuringiensis* TAA1018	10	10	10	10	10	10	10
IITA	*Bacillus* sp. TAA1019	10	10	10	15	15	15	15
CRIN	*Bacillus* sp. TAA1022	5	5	5	5	5	5	5
IAR & T	*Bacillus* sp. TAA1024	10	10	10	10	10	10	10
IAR & T	*Bacillus sphaericus* TAA1025	5	10	10	10	10	10	10
NIHORT	*Bacillus* sp. TAA1031	5	10	10	10	10	10	10
NIHORT	*Bacillus thuringiensis* TAA1033	5	5	5	10	10	10	10
CONTROL	-	0	0	0	0	0	5	5

*Average of 20 mosquito larvae were tested in each case

Chau *et al*. (2011) reported that the richness of bacterial species increased with the coarseness of the soil which was measured by the percentage of sand content of the soil.

The optimum pH for growth of *Bacillus thuringiensis* and *B. sphaericus* was reported to be between 5.5 - 7.5 (Kheseli *et al*., 2012; Khurshed, 2003; Saleh *et al*., 1969). Saleh *et al*. (1969) reported that the optimum growth of *B. thuringiensis* occurred at pH of 6.4 to 6.7. In this study a gradual reduction of the incidence of the organism occurred as the pH reduced to 4.4. Some entomopathogenic *Bacillus* species were obtained from some soil samples with pH ranging between 4.9 and 6.1. However, unlike Saleh *et al*. (1969) the detrimental effect of some strains of *Bacillus thuringiensis* occurred at pH of 4.9 and 5.1.

The acidic nature of the soil may also have affected the total viable bacteria count in the soil samples except for the samples collected from the cocoa farm which showed very low total VBC even though the pH of the soil tended towards neutrality (6.1). Rousk *et al*. (2009) reported a five-fold decrease in the bacterial growth and a five-fold increase in fungal growth with lower pH (tending towards pH 4). Lauber *et al*. (2008) on the other hand reported that the soil texture affected the composition of bacteria in soil.

It is possible that the type of crop planted affected the viable bacteria count in these locations because higher VBC was obtained in soils collected from the farm lands on which root and tuber crops had been grown. The IAR&T soil sample with the highest VBC (1.87 X 10^3 CFU/ml) was collected from various farms including the yam/cassava farm while the only crop cultivated on the IITA plantation was cassava with an average soil microbiota load of 1.58 X 10^3 CFU/ml.. The diversity and concentration of bacteria in CRIN soil samples was the least (7.7 X 10^2 CFU/ml) possibly due to the use of different pesticides on cocoa yearly.

Despite the use of pesticides on most of the sampling locations, the presence of *Bacillus* spp. was still reported in the IITA, IAR&T and NIHORT soil samples. One of the cocoa farms (CRIN) was not sprayed with any pesticide, yet some of the soil samples still showed the presence of some cells of *Bacillus* spp. Details of the pesticide (s) applied on the soil at the forest plantation were not made available. However, the low incidence of *Bacillus* spp. in the samples cannot be attributed to pesticide application.

B. thuringiensis, *Bacillus sphaericus* and some other entomopathogenic *Bacillus* spp. were obtained from four of the five locations sampled. The ratio of occurrence of *B. thuringiensis*, *Bacillus sphaericus* and the other entomopathogenic *Bacillus* species in the soil samples from the different locations was 4: 1. Our report affirms the finding of Valicente *et al*. (2005) that in Brazil *B. thuringiensis* was obtained in all the regions of the country. Only the FRIN soil samples collected from the pine and *Gmelina* plantations did not have any strain of entomopathogenic *Bacillus* spp. This however contradicts an earlier report on the incidence of *B. thuringiensis* in Virgin woods (DeLucca *et al*., 1981). The low incidence/isolation of *Bacillus* spp. from the FRIN soil samples may have been due to the fire incidence reported in 2008. The microbial flora of the forest location may have been destroyed by the fire leading to on set of a

new microbial community which may be devoid of entomopathogenic *Bacillus* spp. *Bacillus* sp. TAA1019 which exhibited the highest mortality (15%) may be a subspecies of *Bacillus thuringiensis* probably *B. thuringiensis var israelensis* which is documented in literature as one of the most potent strains of *Bacillus thuringiensis.* This however needs to be confirmed by molecular identification of this strain of *Bacillus* sp. TAA1019.

Use of biocontrol measures against both plant pests and disease vectors is popular in many countries because it is a safer alternative to chemical insecticides. The incidence of entomopathogenic strains of *Bacillus* spp. in soils within Ibadan metropolis and the larvicidal activities of these indigenous isolates against *Anopheles* larvae has been confirmed. Entomopathogenic strains of B*acillus* spp. should be exploited for large scale production of biopesticides in Nigeria; also, their use against vectors such as mosquitoes and other plant pests should be encouraged within the country.

References

Al-Momani, F., and Obeidat M. 2012. Abundance and Serotyping of pathogenic isolates of *Bacillus thuringiensis* isolated from Ajloun Forests. J. Biodiver.Ecol. Sci. 2(1): 16 - 21

Aly, N. A. H., 2007. PCR detection of *cry* genes in local *Bacillus thuringiensis* isolates. Australian. J.Basic Appl. Sci. 1: 461- 466.

Ammouneh, H., M. Harba, E. Idris and Makee, H. 2011. Isolation and characterization of native *Bacillus thuringiensis* isolates from Syrian soil and testing of their insecticidal activities against some insect pests. Turkish. J. Agricult. Forest.35: 421-431.

Andrzejczak, S., and Lonc, E. 2008. Selective isolation of *Bacillus thuringiensis* from soil by use of L-serine as minimal medium supplement. Poland. J. Microbiol. 57: 333- 335.

Broderick, N. A., R.M. Goodman, K.F. Raffa and Handelsman, J. 2000. Synergy Between Zwittermicin A and *Bacillus thuringiensis* subsp. *kurstaki* Against Gypsy Moth (Lepidoptera: Lymantriidae). Environ. Entomol.29(1): 101 - 107

Buchanan, R. E., and Gibbons N. E. 1974. Bergey's manual of determinative bacteriology. 8th ed. Baltimore: Williams and Wilkins Company.

Charles, J. F., and Nielsen-LeRoux, C. 2000. Mosquitocidal bacterial toxins: diversity, mode of action and resistance phenomena. Mem. Inst. Oswaldo Cruz, Rio de Janeiro 95: 201- 206.

Chatterjee, S. N., T. Bhattacharya, T.K. Dangar and Chandra, G. 2007. Ecology and diversity of *Bacillus thuringiensis* in soil environment. African. J. Biotechnol. 6 (13): 1587-1591.

Chau, J. F., A.C. Bagtzoglou and Willig, M. R. 2011. The Effect of Soil Texture on Richness and Diversity of Bacterial Communities. Environ. Forensics. 12: 333 - 341.

DeLucca, A. J., J.G. Simonson and Larson, A. D. 1981. *Bacillus thuringiensis* distribution in soils of the United States. Canadian. J. Microbiol. 27: 865- 870.

Gbehou, N.A., S.C. Houssou and Yilian, L. 2010. Effect of *Bacillus thuringiensis var. israelensis* (H-14) on *Culex*, *Aedes* and *Anopheles* larvae (Cotonou; Benin). Stem Cell. 1: 60- 67.

Harrigan, W. F., and McCane, M. 1966. Laboratory Methods in Microbiology, London: Academic Press Inc.

Kheseli, P. O., J.O. Ochanda and Okoth, S. A. 2012. Isolation and characterization of Bacillus thuringiensis from coastal intertidal brackish sediments and from farmlands in Machakos District Kenya. from

http://erepository.uonbi.ac.ke/handle/123456789/28372 retrieved 8th October, 2013

Khurshed, N., 2003. Studies on the locally isolated strains of *Bacillus* for their possible use as bioinsecticide. from http://eprints.hec.gov.pk/1513/1/1394.HTM retrieved 8th October, 2013

Lauber, C. L., M.S. Strickland, M. A. Bradford and Fierer, N. 2008. The influence of soil properties on the structure of bacterial and fungal communities across land-use types. Soil Biol. Biochem. 40: 2407- 2415.

Majambere, S., S.W. Lindsay, C. Green, B. Kandeh and Fillinger, U. 2007. Microbial larvicides for malaria control in the Gambia. Malaria. J. 6: 76.

Manju K., S. George, A.R.V. Kumar and Manjulakumari D. 2009. Distribution of *Bacillus thuringiensis* Berliner strains in the soils of different habitats and their activity against white grubs. Karnataka. J. Agricult. Sci. 22 (3): 628-630.

Prescott, L. M., J.P. Harley and Klein, D. A. 2010. Microbiology. 8th ed. New York: Mc Graw Hill.

Rousk, J., P.C. Brookes and Baath, E. 2009. Contrasting Soil pH Effects on Fungal and Bacterial Growth Suggest Functional Redundancy in Carbon Mineralization. Appl. Environ. Microbiol. 75: 1589 – 1596.

Saleh, S. M., R.F. Harris and Allen, O. N. 1969. Method for determining *Bacillus thuringiensis var thuringiensis* Berliner in soil. 1969. Canadian. J. Microbiol. 15: 1101-1104.

Surendran, A., and Vennison, S. J. 2011. Occurrence and Distribution of Mosquitocidal *Bacillus sphaericus* in soil. Academic. J. Entomol. 4 (1): 17-22.

Travers, R. S., P.A.W. Martin and Reichelderfer, C. F. 1987. Selective process for efficient isolation of soil *Bacillus* spp. Appl. Environ. Microbiol. 53: 1263 - 1266

Tyrell, D. J., L.I.Davidson, L. I. Bulla and Jr, Ramoska, W. A. 1979. Toxicity of parasporal crystals of *Bacillus thuringiensis* subsp. *israelensis* to mosquitoes. Appl. Environ. Microbiol. 38: 656- 658

Valicente, F. H., R.F. Zanasi, K.G. Boregas and Barretto, M. R. 2005. *Bacillus thuringiensis* in Brazil: geographical distribution and fermentation media for production. Proceedings of the 6th Pacific Rim conference on the biotechnology of *Bacillus thuringiensis* and its environmental impact, Victoria BC. Canada.

Zwahlen, C., A. Hilbeck, R. Howald and Nentwig, W. 2003. Effects of transgenic Bt. corn litter on the earthworm *Lumbricus terrestris*. Mole. Ecol. 12: 1077–1086.