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Synergism of Entomopathogenic Organisms Associated with *Zonocerus variegatus*

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Authors' contributions

This work was carried out in collaboration between both authors. Author FOO designed the study, wrote the protocol and managed the literature searches. Author BAK wrote the first draft of the manuscript, managed the analyses of the study and performed the statistical analysis. Both authors read and approved the final manuscript.

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ABSTRACT

Aim: Entomopathogenic organisms were investigated for their lethal ability, minimum lethal concentration and synergy against caged short-horned grasshoppers (*Zonocerus variegatus*).
Place and Duration of Study: Study was carried out in the Department of Biological Sciences, Wesley University, Ondo, Nigeria between March and August, 2016.
Methodology: Adult *Z. variegatus* of both sexes were caught by sweep nets from the university cassava (*Manihot esculenta*) farm, divided into batches of 10, fed and observed for the onset of disease symptoms such as lethargy, colour change, abnormal outgrowths and death. Organisms were isolated from the diseased insects. Pathogenicity test was carried out on all the isolated organisms. Synergism was examined amongst the organisms by challenging the insects with more than one entomopathogens at the same time.
Results: Duncan's Multiple Range Test was used for the estimation of means. The 't' value was

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tested at 95% confidence interval. 10 insects were included in each sample batch and each test were carried out in triplicates (n = 3), *Bacillus subtilis, Beauvaria bassiana* and *Aspergillus niger* were able to induce pathogenicity on the insects. *B. bassiana* caused death in 8.67±0.58 insects, *A. niger* was lethal on 7.33±0.58 while *B. subtilis* was lethal on 8.67±0.29. The minimum lethal concentration (MLC) of the organisms when used to challenge the insects are 5.0 $\times 10^4$ spore forming units per milliliter (sfu/ml) for *B. bassiana* as it causes death in 6.33±0.58 out of the 10 insects used. 8.0 $\times 10^8$ sfu/ml of *A. niger* causes death in 6.67±0.58 and 6.0 $\times 10^6$ cfu/ml for *B. subtilis.* Synergistic test carried out shows greater entomopathogenic effect when a bacteria-fungi formulation was used compared to when a fungi-fungi formulation was used. '*A. niger* - *B. bassiana*' formulation caused 5.33 ± 0.58^b death while the formulation using *A. niger* – *B. subtilis* and *B. bassiana* – *B. subtilis* caused 6.33 ± 0.58^c and 6.67 ± 0.58^{cd} lethal cases respectively.

Conclusion: *B. subtilis, B. bassiana* and *A. niger* isolated from diseased *Z. variegatus* in this study possess entomopathogenic ability. They can be further studied for the enhancement of their entomopathogenicity, biosafety, most suitable method of delivery and mass production.

Keywords: Bacillus subtilis; Beauvaria bassiana; entomopathogen; synergism; grasshopper.

1. INTRODUCTION

Entomopathogens refer to organisms which are capable of causing diseases in insects [1]. They include fungi, nematodes, bacteria and viruses. They are all widespread in the natural environment and cause infections in many pest species. Entomopathogens are regarded as potentials in the generation of safer and cleaner selective insecticides [2]. Many pathogens have been isolated from insects and arachnids of major economic importance and they are regarded as potential candidates for development into microbial insecticides [3]. Insects are among the most diverse group of animals on the planet. They are arthropods having a chitinous exoskeleton. They are sometimes beneficial as they pollinate many plants, assist in bioconversion and also serve as food to many – a practice known as entomophagy. However, they sometimes act as vectors responsible for the transmission of disease-causing microorganisms from an infected person or animal to another [4].

1.1 Justification

While many insects are of immense benefits, others are resented because of the economic damages and health hazards they pose to man, animals and plants. Insects regarded as pests include *Z. variegatus* due to the damage they do to crops especially by defoliating it thus leading to about 30 to 90 percent yield losses especially in Africa [5]. Chemical pesticides used in controlling these insects are dangerous to non-targeted beneficial animals in the environment. These chemicals can also alter the soil microbiota. Runoffs from farmlands sprayed with these chemicals can also contaminate water bodies thus making it unfit for human consumption and unsafe for aquatic life. Cases of mammalian toxicity have also been widely reported from the usage of chemical pesticides. [6] In addition, many of these insects have also developed resistance to some of these chemicals [7]. Therefore, methods of control must be environmentally friendly, safe to nontargets and effective against these organisms thus necessitating the usage of entomopathogens. *Z. variegatus* was collected from *M. esculenta* farmlands, organisms were isolated from the diseased ones, pathogenicity test were carried out on all isolated organisms to determine those with entomopathogenic potentials and synergistic tests were also conducted on all the entomopathogens.

2. MATERIALS AND METHODS

2.1 Collection and Stocking of *Z. variegatus*

Z. variegatus were sourced from Wesley University Farm with the aid of nets traps. These insects were then taken to the laboratory. The grasshoppers were allowed to acclimatize in the cages for two weeks during which they were fed with surface sterilized fresh cassava and water leaves [8-10].

2.2 Observation of the *Z. variegatus* Population for Diseased Individuals

Z. variegatus natural conditions such as foliage beddings and temperature were closely simulated in the laboratory. They were subjected to 'near-natural' treatment under laboratory

conditions. They were left to acclimatize to the new environmental conditions for two weeks after which individuals showing morbid and mortal symptoms in the form of death, reduced activities, lethargy, colour change and reduced feeding rate were separated from the population for maceration and subsequent isolation of microorganisms [11].

2.3 Isolation of Fungi from Diseased *Z. variegatus*

Z. variegatus cadavers aseptically removed from the cages were surfaced sterilized in 5% sodium hypochlorite and 75% ethanol solution. They were further rinsed in plenty of sterile distilled water and left to dry for 48 h [8]. After drying they were humid incubated in clean dessicators with silica gel at room temperature [12]. Cadavers with luxuriant fungal growth were regarded as being positive while cadavers without fungal growth are regarded as negative. The sporulating fungi on cadavers were isolated in pure culture on Sabouraud Dextrose Agar (SDA) [13,14]. Cadavers were further macerated to isolate other fungal pathogens which may be present within the insect. The macerate was emptied into a test tube containing sterile water, serial dilution was carried out on the macerate and the appropriate dilutions were plated on Potato Dextrose Agar and incubated at 25℃ for 72 hours [9].

2.4 Isolation of Bacteria from Diseased *Z. variegatus*

Bacteria were isolated from the grasshoppers by plating the macerate obtained from their cadavers on standard media. Cadaver maceration was done inside sterilized mortar and homogenized using sterile pestle. Macerate was reconstituted using sterile saline and diluted serially. The plates were incubated at a temperature of 37℃ for 24 hours. Colonies obtained were subsequently sub-cultured to obtain pure cultures.

2.5 Identification of Isolated Microorganisms

Identification of bacteria was done on fresh cultures to ascertain their identities. It comprised of both the cultural and biochemical characterization which included Gram's staining reaction, Spore staining test Catalase test, Coagulase test, Sugar fermentation test, motility test and citrate utilization test. Identification of fungi was done by placing mycelia in cotton blue lactophenol and observing under the microscope [15,16].

2.6 Preparation of Spore Suspensions for the Infection of *Z. variegatus*

Each of the fungi was inoculated onto fresh PDA plates and incubated at 27℃ for 14 days for sporulation to take place. Spores and conidia were washed from the plates using a 0.1% Tween 80 solution and sterile glass rods [13]. Conidia suspensions were stored at 4℃ before serial dilutions for the susceptibility test was done. The spores' suspensions obtained were dispensed into aspirators capable of aerosolizing the organism [9].

2.7 Preparation of Bacterial Suspension for Infection of *Z. variegatus*

Bacterial suspensions were prepared from each of the isolated organisms. Each bacterium was inoculated into 50 mls of sterilized broth. They were incubated at 37℃ for 24 hours. The resulting broth cultures were centrifuged to separate and obtain their cells from the broth. The cells were further washed by reconstituting with sterile saline and centrifuging again. The cells and spores obtained were reconstituted by adding 50 mls of water and dispensed into special containers capable of aerosolizing the microbial suspensions [11].

2.8 Infection of *Z. variegatus* with Microbial Suspensions

New batch of apparently healthy *Z. variegatus* were collected from the cassava farm and monitored for two weeks to acclimatize and ensure they are not already carrying pathogens from the field. They were divided into groups of ten per treatment. They were infected with all of the organisms obtained to determine those that will be responsible for pathogenicity. The cells and spores obtained from centrifugation and washing were dispensed into sterile aspirators. This was used in spraying the adult insects. Control experiment was set up by spraying separate populations of insects with sterile saline In cases where any of the insects comes down with any form of health anomalies either within the control batch or during the acclimatization of the whole population batch, the whole group is discarded [9,17].

2.9 Selection of Potential Entomopathogens from the Isolated Organisms

Pathogenicity test was carried out on all the isolated organisms including six fungi and eight bacteria. Each of the organisms isolated from the diseased *Z. variegatus* was used to infect healthy batch of the insects to determine the ones that are capable of causing diseases. Selection of entomopathogens was done by observing the organism able to cause infection in *Z. variegatus.* Such organisms manifest their entomopathogenicity in *Z. variegatus* by causing death, reduced activities, lethargy, unusual growth and reduced feeding. Organisms which were able to exhibit morbid and mortal effects on the insects after pathogenicity were selected as potential entomopathogens [13].

2.10 Determination of the Minimum Microbial Concentration Needed for Pathogenicity

The minimum microbial concentration needed for pathogenicity was determined by infecting *Z. variegatus* with nine varying concentrations of the recorded entomopathogens. This was done in triplicates with each batch that was infected containing ten insects. The least concentration showing pathogenic activity was calculated and recorded after observing for 120 hours.

2.11 Evaluation of the Synergism among Entomopathogens

Synergism between entomopathogens was determined by challenging *Z. variegatus* with the minimum lethal concentration of different entomopathogens at the same time. Results were compared to those obtained when insects were challenged with just an entomopathogen. Formulations having the highest degree of synergism will be recorded as positive for synergism compared with the groups infected with one organism and the control which are uninfected.

2.12 Experimental Replication and Statistical Analysis of Data

All experiments were carried out in triplicates and data obtained were subjected to statistical analysis of variance and Duncan's Multiple Range Test for the estimation of means. The 't' value will be tested at 95% confidence interval.

3. RESULTS

3.1 Isolation of Bacteria from Diseased *Z. variegatus*

Bacteria isolated from diseased *Z. variegatus* include *S. typhii, S. aureus, P. aeruginosa, S. epidermidis, Klebsiella sp., B. subtilis, B. sphaericus,* and *S. feacalis.* These are shown in the Table 1. Isolation of bacteria was repeated three times from ten insects using various differential, selective and general media due to the low frequency of occurrence of the bacterial entomopathogen.

3.2 Isolation of Fungi from Diseased *Z. variegatus*

After the appropriate incubation period, the microbes were identified using standard techniques. Fungi isolated from *Z. variegatus* as shown in Table 2 include *Geotrichum sp., Penicillium sp., A. niger, A. fumigatus, B. bassiana, Fusarium sp.* Isolation of fungi was also repeated three times from eight insects using selective and general including media due to the low frequency of occurrence of the fungal entomopathogen.

3.3 Infection of *Z. variegatus* with Bacterial Isolates

All eight bacteria isolated from *Z. variegatus* macerate were used in the infection for the determination of possible entomopathogens. Observations were made on the infected insects for signs of disease or morbid symptoms. Out of all the bacteria used for the infection, *Z. variegatus* was discovered to be susceptible to only one which was *B. subtilis*. It caused death in 85 percent of the insects used. This is shown in figure one below where the organisms used is plotted against the number of dead insects. Insect death depicted on graph was calculated by finding the average number of dead insects from the three groups of insects used for the study.

3.4 Infection of *Z. variegatus* with Fungal Isolates

Fungal isolates were used in the infection of the *Z. variegatus*. A total number of six fungi were used and these include *A. fumigatus, A. niger, Articulospora inflata, B. bassiana, Fusarium sp., Geotrichum sp., Penicillium sp.* out of which only *B. basianna* and *A. niger* showed marked lethal

signs compared with the control and the other organisms used for infection on the test insects used. Insect death depicted on graph was calculated by finding the average number of dead insects from the three groups of insects used for the study.

Table 1. Name and characteristics of bacterial isolates obtained from *Z. variegatus*

Laboratory code	Gssa1	Gmsa1	Gna1	Gmsa2	Gna2	Gna3	Gna4	Gmac1
Cultural characteristics								
Pigment	Greyish	Cream White	Green	Greyish	Greyish	White	White	White
Shape	Circular	Circular	Irregular	Circular	Undulate	Irregular	Irregular	Circular
Elevation	Flat	Raised	Flat	Raised	Raised	Flat	Flat	Raised
Surface	Moist	Moist	Wet	Moist	Dry	Dry	Dry	Moist
Edge	Entire	Entire	Irregular	Entire	Rhizoid	Rhizoid	Rhizoid	Entire
Spore formation	-	-	-	-	-	+	+	-
Biochemical characteristics								
Catalase	+	+	+	+	+	+	+	+
Coagulase	+	+	-	-	-	-	+	+
Oxidase	-	-	+	-	-	-	-	-
Indole	-	-	-	+	-	-	-	-
Urease	-	-	+	+	+	-	+	-
Sugar fermentation test								
Glucose	AG	AG	AG	AG	AG	A	-	AG
Galactose	AG	AG	-	-	AG	AG	A	-
Sucrose	-	AG	AG	AG	A	A	-	-
Lactose	-	AG	-	-	AG	-	-	AG
Mannitol	AG	AG	AG	-	A	A	-	AG

Key; AG = Acid and Gas production, A = Acid production, - = Negative, + = Positive

Table 2. Name and characteristics of fungal isolates obtained from diseased *Z. variegatus*

Isolate	Cultural characteristics	Microscopic examination	Suspected organism
FG1	White fluffy White and brown fluffy.	Mycelium is whitish and septate. Conidiophores are absent. Conidia is hyaline, short cylindrical with truncated ends, formed by segmentation of hyphae.	*Geotrichum sp.*
FG2	Varying coloration from Yellowish green to dark green with a cream coloured base	Septate Hyphae, occurrence of stigmata, conidiophores are hyaline.	*Penicillium sp.*
FG3	Initial whitish to yellow luxuriant growth which later bears black dust-like conidia.	Microscopic evaluation shows upright single coniodophores with sphere-like swellings at the ends. Swellings which bears phallides are also seen at the apex.	*Aspergillus niger*
FG4	Wooly appearance on media with a greyish to black colouration surround by white cottony mycelia.	Evaluation shows uniseriate and columnar conidial heads with the phialides limited to the upper two thirds of the vesicle and curving to be roughly parallel to each other.	*Aspergillus fumigatus*
FG5	Powdery mycelia which appears whitish to a dullish yellow.	Conidia are hyaline, short and globose in appearance. Luxuriant growth of conidiogenous cells which are flask-shaped. Cells further grows to form sporodochia.	*Beauveria bassiana*
FG6	Whitish aerial mycelium which turns purple.	Conidiophores are short and single. Macroconidia are curved slightly with a pointed tip, non-septate, ellipsoidal to cylindrical and straight.	*Fusarium sp.*

3.5 Determination of the Minimum Lethal Concentration of Pathogenic Microorganisms Required for Pathogenicity in *Zonocerus variegatus*

The minimum concentrations (LD_{50}) of *B. bassiana*, *A. niger* and *B. subtilis* needed for pathogenicity were determined in *Z. variegatus* through the selection of the lowest concentration of the organisms which is capable of causing diseases in about fifty percent of the ten insects infected per batch. *B. bassiana* was found to be pathogenic at concentration $\log_{10}^{-1}$ 4.69 which is equivalent to 5×10^4 sfu/ml. Lowest concentration of *A. niger* which was pathogenic against *Z. variegatus* was $\log_{10}^{-1}$ 8.90 which is equivalent to 8×10^8sfu/ml. *B. subtilis* was found to be pathogenic at the lowest concentration value of $\log_{10}^{-1}$ 6.77 which corresponds to a value of 6×10^6 cfu/ml. These are represented in the figures three to five below where the different logarithm concentrations used for infection is calculated and plotted against the number of mortals which was obtained after 120 hours of infection.

3.6 Evaluation of Synergism among Entomopathogens

Insects of study were challenged with more than one entomopathogen. *Z. variegatus* was challenged with minimal lethal concentrations of entomopathogens of *B. bassiana* and *A. niger*, *B. bassiana* and *B. subtilis, A. niger* and *B. subtilis, B. bassiana, B. subtilis* and *A. niger*. These are shown in the Fig. 6 where the organisms used for infection are plotted against the number of deaths in insects of study and observations were made on day five to allow maximum time for insect deaths. Results show that pathogenicity induced from the usage of *B. subtilis* with either *A. niger* or *B. bassiana* caused more insects' deaths compared to when the combination of *A. niger* and *B. bassiana* were used. A combination of all three entomopathogens however yielded higher mortalities compared to when two entomopathogens were used.

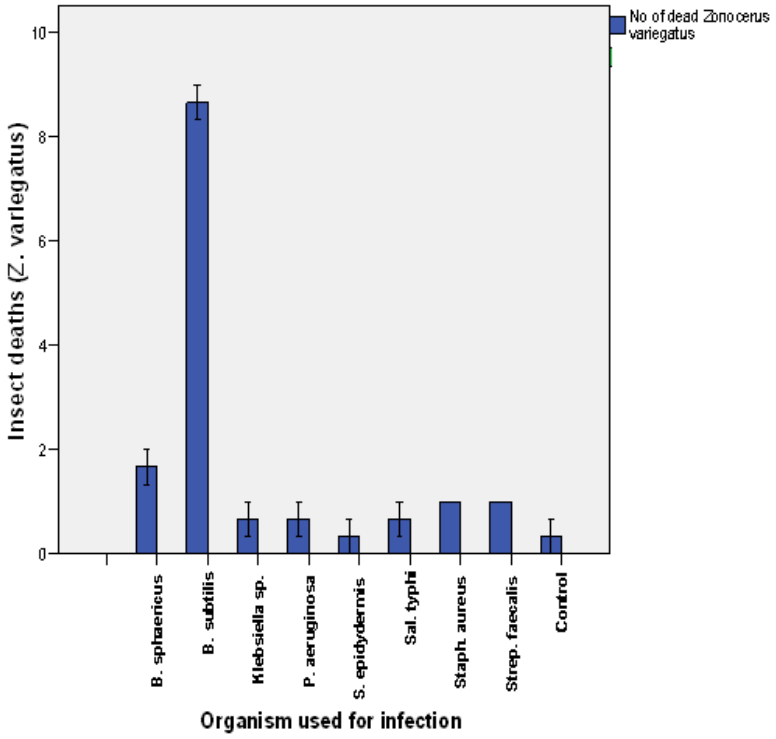

Fig. 1. Infection of *Z. variegatus* with bacterial isolates

Legend: Data depicted on Y-axis represents insect death (mean) ± STD
Data depicted on X-axis represents organisms (bacteria) used for infection of the insects n = 10
Experiments were repeated three times

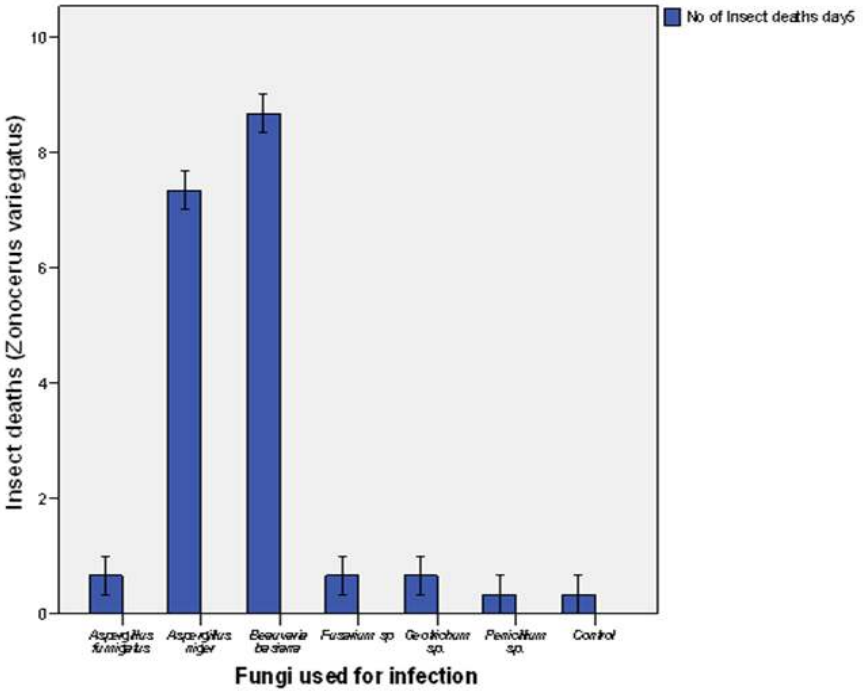

Fig. 2. Infection of *Z. variegatus* with fungal isolates

Legend: Data depicted on Y-axis represents insect death (mean) ± STD
Data depicted on X-axis represents organisms (fungi) used for infection of the insects n = 10
Experiments were repeated three times

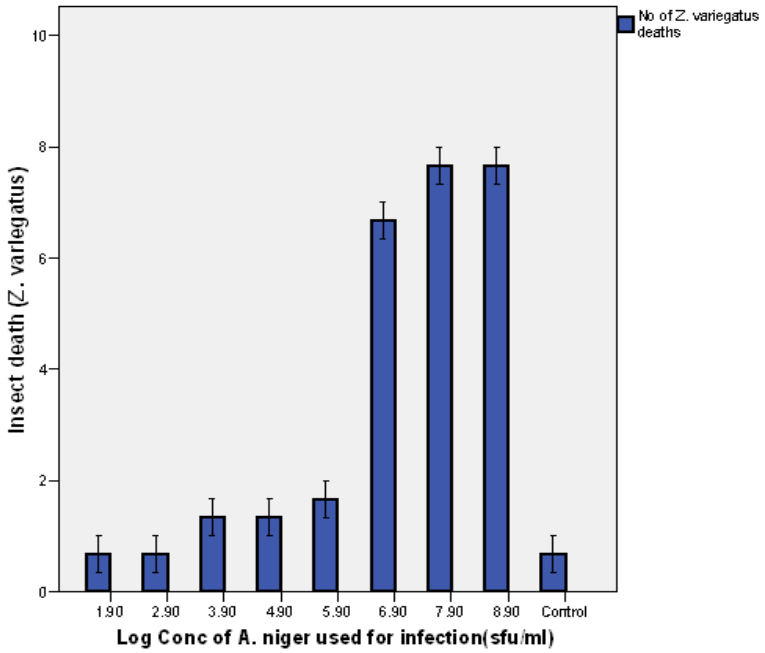

Fig. 3. Determination of the MLC of *A. niger* required for pathogenicity in *Z. variegatus*

Legend: Data depicted on Y-axis represents insect death (mean) ± STD
Data depicted on X-axis represents the different logarithmic concentrations of organism (A. niger) used for infection of the insectsn = 10
Experiments were repeated three times

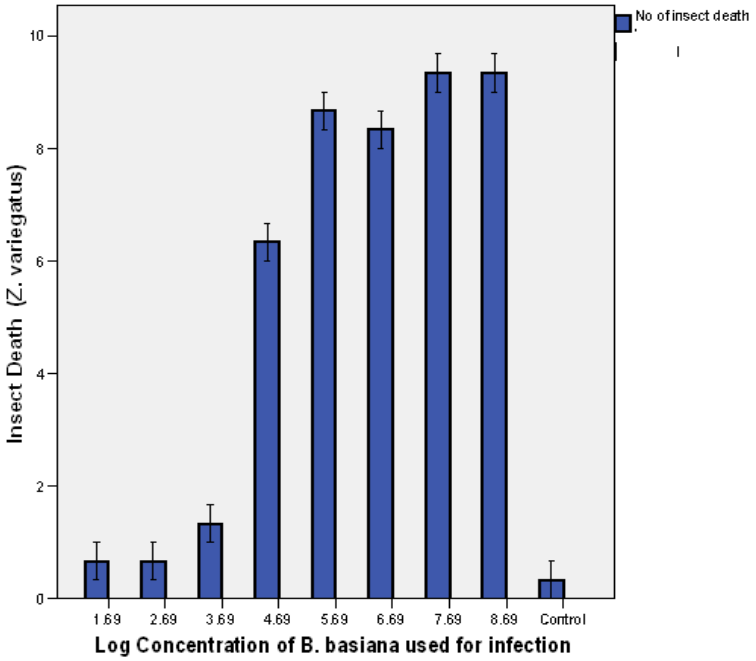

Fig. 4. Determination of the MLC of *B. bassiana* required for pathogenicity in termites

Legend: Data depicted on Y-axis represents insect death (mean) ± STD
Data depicted on X-axis represents the different logarithmic concentrations of organism (B. bassiana) used for infection of the insectsn = 10 Experiments were repeated three times

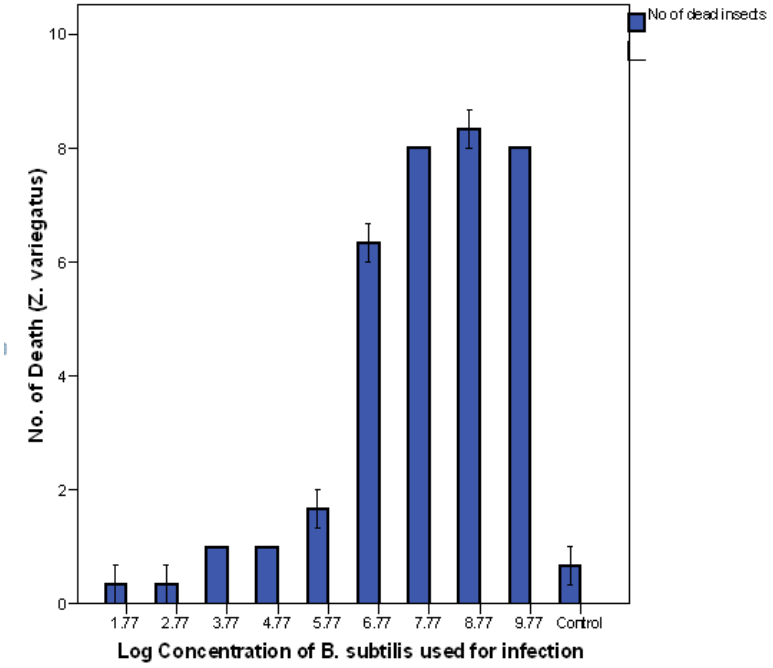

Fig. 5. Determination of the MLC of *B. subtilis* required for pathogenicity in *Z. variegatus*

Legend: Data depicted on Y-axis represents insect death (mean) ± STD
Data depicted on X-axis represents the different logarithmic concentrations of organism (B. subtilis) used for infection of the insects n = 10, Experiments were repeated three times

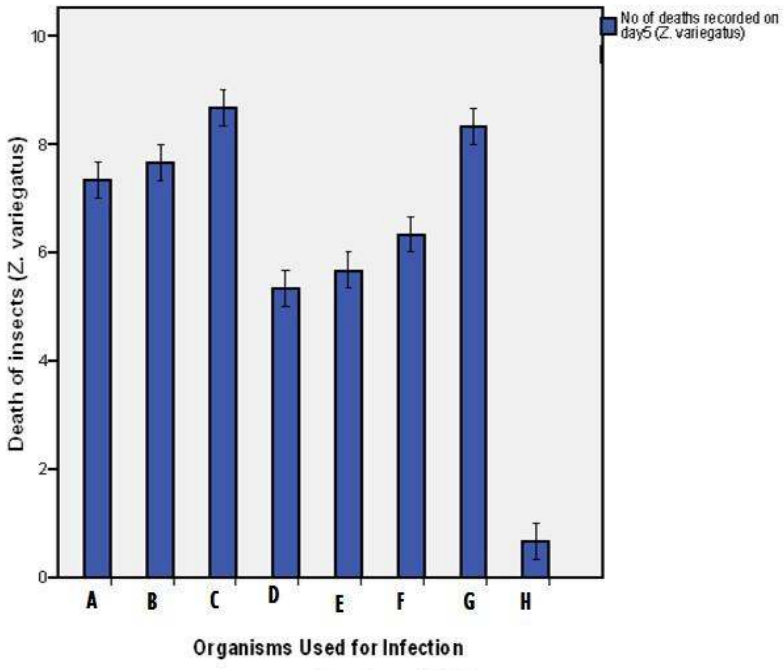

Fig. 6. Synergism test on entomopathogens of *Z. variegatus* for day 5

Legend: A: Aspergillus niger, B: Bacillus subtilis, C: Beauvaria bassiana, D: B. bassiana and A. niger, E: B. subtilis and A. niger, F: B. bassiana and B. subtilis, G: B. bassiana, A. niger and B. subtilis, H: Control
Data depicted on Y-axis represents insect death (mean) ± STD
Data depicted on X-axis represents the different formulations of organisms used for the infection of the insects
n = 10, Experiments were repeated three times

4. DISCUSSION

B. bassiana was pathogenic to the *Z. variegatus* used in this study. Similar studies reported that *B. bassiana* are inherently present in internal plant tissue as an adaptive protection against herbivorous insects [18,19]. Since *Z. variegatus* majorly feeds on foliage, it is most likely that they encounter this organism during their process of feeding. Similar findings also consider *B. bassiana* as endophytic fungi which functions as an inherent plant-defending mutualist [20,21]. *Penicillium sp.* isolated in this study has also been initially reported in a similar study [14].

Entomopathogenic fungi cause diseases in insects and they are usually identified based on their growth on insect cadavers. This study similarly shows that infected *Z. variegatus* became diseased and this was followed by the growth of *B. bassiana* on the cadavers [13].

Many strains of *B. bassiana* are able to produce enzymes such as proteases, chitinases, and lipases [22,23]. The virulence of *B. bassiana* on *Z. variegatus* can be due to this ability because enzymes like chitinases can degrade the chitinous insect cuticle - thus leading to their sicknesses and eventual death.

Occurrence of bacterial entomopathogens such as *S. marcescens* and *B. subtilis* is displayed in the findings of this study. *Bacillus sp*. are generally known as potential entomopathogens which have been used extensively for the control of insects and pests' populations. *Bacillus sp*. works sometimes by paralyzing the guts of infected insects through the production of certain proteins [24].

Minimum lethal concentration (LD_{50}) required for pathogenicity in *Z. variegatus* was lowest for *B. bassiana* with a concentration of 5.0×10^4. LD_{50} for *B. subtilis* is 6.0×10^8 while *A. niger* has the highest LD_{50} which is 8.0×10^8.

It was generally observed in this study that higher insect deaths were recorded with the usage of a mixture bacterial and fungal entomopathogens compared to what was obtained when the combination of two bacterial or two fungal entomopathogens were used.

Previous studies carried out on synergism using nematodal entomopathogens in addition with other entomopathogens have been reported in a number of studies to yield higher virulence compared to the results obtained when the same category of entomopathogens are used. The reduced synergistic activity noticed between the two fungi might be due to a form of competition where both fungal agents are competing for the insect chitinous exoskeleton as substrate thus secreting enzymes which may be inhibiting the activity of each other.

5. CONCLUSION

A. niger, B. bassiana and *B. subtilis* strains isolated in this study have been demonstrated to have insecticidal ability on *Z. variegatus*. They can be further studied and formulated into biopesticides after necessary toxicological assessment might have been done on the strain. In addition, cheap substrates which can support the growth of these organisms can also be used to cultivate them for mass production.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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