UNDER PEER REVIEW

Original Research Article

Assessment of the synergism of entomopathogenic organisms associated with *Zonocerus variegatus*

ABSTRACT

In this study, organisms pathogenic against short-horned grasshoppers (*Zonocerus variegatus*) were isolated and identified from their diseased insect population and used to challenge healthy population. *Zonocerus variegatus* were collected from cassava farmlands, fed with surface sterilized cassava leaves and distilled water allowed to acclimatize for two weeks. Grasshoppers were studied in their appearance and behaviour for abnormalities which include lethargy, colour change, abnormal outgrowths, reduced feeding and death. Organisms were isolated from the diseased and dead grasshoppers and they include *Salmonella typhii, Staphylococcus aureus, Pseudomonas aeruginosa, Staphylococcus epidermis, Klebsiella sp., Bacillus subtilis, Bacillus sphaericus,* and *Streptococcus feacalis* for bacteria. Fungal isolates obtained include *Geotrichum Sp, Penicillium Sp, Aspergillus niger, Aspergillus fumigatus, Beauveria bassiana* and *Fusarium sp.*

Pathogenicity tests were carried out for each of the bacterial and fungal species. One of the bacteria, *Bacillus subtilis* and two of the fungi *Beauveria bassiana* and *Aspergillus niger* were able to induce lethargic and morbid effects on the grasshoppers. These three organisms were re-isolated from the resulting cadavers to confirm entomopathogenicity. Re-infection using different concentrations of both the entomopathogenic fungi and bacterium were carried out on grasshoppers to determine the minimum lethal concentration (LD_{50}) required to kill 50 percent of the grasshopper population. *Beauvaria bassiana* was found to be pathogenic at concentration $\log_{10}^{-1}$ 4.69 which is equivalent to 5.0 $\times 10^4$sfu/ml. Lowest concentration of *Aspergillus niger* which was pathogenic against *Z. variegatus* was $\log_{10}^{-1}$ 8.90 which is equivalent to 8.0 $\times 10^8$sfu/ml. *Bacillus subtilis* was found to be pathogenic at the lowest concentration value of $\log_{10}^{-1}$ 6.77 which corresponds to a value of 6.0 $\times 10^6$cfu/ml. Insects were also challenged with mixtures of the organisms to determine possible synergistic effects and it was observed generally that there was greater entomopathogenic effect when a bacteria-fungi formulation was used compared to when a fungi-fungi formulation was used. However, none of the synergistic formulation was found to have greater entomopathogenic effect compared to the deaths recorded when single organisms were used to challenge the insects. Fungi and bacteria obtained in this study have entomopathogenic potential against grasshoppers and should be further studied, process of mass production should be developed and a suitable and efficient method of application should be devised.

Keywords: *Bacillus subtilis*, *Beauvaria bassiana,* entomopathogen, synergism

INTRODUCTION

UNDER PEER REVIEW

Insects are among the most diverse group of animals on the planet. They are arthropods having a chitinous exoskeleton, legs, eyes, and sometimes antennae. They sometimes act as vectors responsible for the transmition of disease-causing microorganisms from an infected person or animal to another (Pingel *et al.,* 2004). The number of extant species is estimated at between six and ten million (Vojtech *et al.,* 2002) and potentially represent over 90% of the differing metazoan life forms on Earth (Erwin and Terry, 1982). The life cycles of insects vary but most of them hatches from eggs. Insect growth is constrained by the inelastic exoskeleton and development involves a series of molts. The immature stages can differ from the adults in structure, habit and habitat and can include a passive pupal stage in those groups that undergo complete metamorphosis. Insects are important members of the ecosystem due to their unquantifiable economic importance. Their actions has rewritten men's history in times past and they have also helped in shaping our future

Entomopathogens refer to organisms which are capable of causing diseases in insects. 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 (Tanada and Kaya, 1993). 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 (Wittig, 1963).

JUSTIFICATION

While many insects such as silkworms, termite alates and bees are considered as advantageous to man and his environment, many are being resented because of the economic damages and health hazards they pose to man, plants and other animals. Insects regarded as pests include weevils due to the damaging effects they have on crops by feeding on sap, leaves or fruits, other ones such as termites also damage wooden structures. A few such as mosquitoes, lice and bed bugs bite humans and livestock, alive and dead, to feed on blood and some are capable of transmitting diseases to humans, pets and livestock.

Zonocerus variegatus are known to be voracious and destructive. In the process of feeding, they defoliate and destroy the stem bark of food crops especially at the end of the dry season. About 30 to 90 percent yield losses due to the infestation of *Z. variegatus* on maize and cowpea, respectively, had been reported on some farms in Africa (Langewald *et al.,* 1997). *Z. variegatus* had been designated as a major pest of the agro ecosystem by the National Agricultural Technical Committee of Nigeria since 1970 (Modder, 1986). Attempts to control these insects by using chemical insecticides and a host of other techniques have proved to be of limited efficiency. Some of these chemical insecticides have been proved to be detrimental to humans and his environment while the insect population sometimes develops resistance against the chemicals over a period of time. Thus, other effective and more environmentally-friendly ways are required to better control the insect population.

MATERIALS AND METHODS

UNDER PEER REVIEW

Methods:

Collection and stocking of Grasshopper (*Zonocerus variegatus*)

Grasshoppers were sourced from Wesley University Farm with the aid of nets traps. These insects were then taken to the laboratory. They were provided with fresh water leaves and sterile water. The grasshoppers were allowed to acclimatize in the cages for 2 weeks, after which they were fed with 20 g of surface sterilized cassava and water leaves as described by Dourou- pindou *et al.,* (1995) and Thomas *et al.,* (1997).

Observation of the insect population for diseased individuals

Insects' natural conditions 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 a long while 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

Isolation of Fungi from diseased Grasshoppers

Grasshopper 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 (Dourou-kpinduo *et al.,* 1995). After drying they were humid incubated in clean dessicators with silica gel at room temperature (Luz and Farques, 1998). 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 Sabourand Dextrose Agar (SDA) as described by Poinar and Thomas (1984). 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 25^0C for 72 hours.

Isolation of Bacteria from diseased Grasshoppers

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 37^0C for 24 hours. Colonies obtained were subsequently sub-cultured to obtain pure cultures.

Identification of isolated microorganisms

UNDER PEER REVIEW

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 (Fawole and Oso, 2001), Spore staining test (Barnett *et al.,* 2000), Catalase test (Harrigan, 1998), Coagulase test, Sugar fermentation test (Harrigan, 1998), motility test and citrate utilization test. Identification of fungi was done by placing mycelia in cotton blue lactophenol and observing under the microscope as described by Onions *et al*., (1995).

Preparation of spore suspensions for the infection of test insects

Each of the fungi was inoculated onto fresh PDA plates and incubated at 27°C for 14 days for sporulation to take place. Spores and conidia were washed from the plates using a 0.1% Tween 80 solution. Conidia suspensions were stored at 4°C before serial dilutions for the susceptibility test was done. The spores suspensions obtained were dispensed into aspirators capable of aerosolizing the organism (Balogun and Fagade, 2004).

Preparation of bacterial suspension for infection of insects

Bacterial suspensions were prepared from each of the isolated organisms. Each bacterium was inoculated into 50mls of sterilized broth. They were incubated at 37^0C 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 50mls of water and dispensed into special containers capable of aerosolizing the microbial suspensions Tanada and Kaya (1993)**.**

Infection of Grasshopper with microbial suspensions

New batch of apparently healthy grasshoppers 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 insects. Control experiment was set up by spraying separate populations of insects with sterile saline. This is in accordance with the methods of Balogun and Fagade, (2004) ; Drummond and Gladney (1969).

Selection of potential entomopathogens from the isolated organisms

Pathogenicity test was carried out on all the isolated organisms. Selection of entomopathogens was done by observing the organism able to cause infection in the insects in forms of morbidity and mortality characterized by death, reduced activities, lethargy and reduced feeding. Organisms which were able to exhibit morbid and mortal effects on the insects after pathogenicity were selected as suspected entomopathogens. The resulting insect cadavers were further grinded and organisms were isolated from it. The newly isolated organisms were identified to see whether it will also contain the initial microbe used for the infection

UNDER PEER REVIEW

Determination of the minimum microbial concentration needed for pathogenicity

The minimum microbial concentration needed for pathogenicity was determined by infecting the insects with various concentrations of the recorded entomopathogens. The least concentration showing pathogenic activity was calculated and recorded after observing for 120 hours.

Evaluation of the synergism among entomopathogens

Synergism between entomopathogens was determined by challenging insects of study with 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

Results

Isolation of Bacteria from diseased grasshoppers (*Zonocerus variegatus*)

Bacteria isolated from diseased *Z. variegatus* include *Salmonella typhii, Staphylococcus aureus, Pseudomonas aeruginosa, Staphylococcus epidermis, Klebsiella sp., Bacillus subtilis, Bacillus sphaericus,* and *Streptococcus feacalis.* These are shown in the table 1 below

Isolation of Fungi from diseased *Zonocerus variegatus*

After the appropriate incubation period, the microbes were identified using standard techniques. Fungi isolated from *Zonocerus variegatus* as shown in table 2 include *Geotrichum Sp, Penicillium Sp, Aspergillus niger, Aspergillus fumigatus, Beauveria bassiana, Fusarium sp*

Infection of *Zonocerus variegatus* with the isolated bacteria

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* discovered to be susceptible to only one which is *Bacillus subtilis*. These are shown in the figure 1 below where the organisms used are plotted against the number of dead insects

Infection of *Z. variegatus* with fungal isolates

Fungal isolates were used in the infection of the *Z. variegatus* used in this study. A total number of six fungi were used and these include *Aspergillus fumigatus, Aspergillus niger, Articulospora inflata, Beauvaria bassiana, Fusarium sp., Geotrichum sp., Penicillium sp.* out of which only *B. basianna* and *A. niger* showed marked lethal signs on the test insects used. This is represented in the figure 2 below where the organisms used for infection are plotted against the number of dead insects.

Determination of the minimum lethal concentration of pathogenic microorganisms required for pathogenicity in *Zonocerus variegatus*

UNDER PEER REVIEW

The minimum concentrations (LD_{50}) of *Beauvaria bassiana*, *Aspergillus niger* and *Bacillus subtilis* needed for pathogenicity were determined in *Zonocerus variegatus* through the selection of the lowest concentration of the organisms which is capable of causing diseases in the insects. *Beauvaria 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 *Aspergillus niger* which was pathogenic against *Z. variegatus* was $\log_{10}^{-1}$ 8.90 which is equivalent to 8×10^{8}sfu/ml. *Bacillus 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 3-5 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.

Evaluation of synergism among entomopathogens

Insects of study were challenged with more than one entomopathogen. *Zonocerus variegatus* was challenged with *Beauvaria bassiana* and *Aspergillus niger*, *Beauvaria bassiana* and *Bacillus subtilis, Aspergillus niger* and *Bacillus subtilis, Beauvaria bassiana, Bacillus subtilis* and *Aspergillus niger*. In the case of ternary, organisms used for synergistic infection include *Serratia marcescens* and *Pseudomonas aeruginosa, Serratia marcescens* and *Metarhizium anisopliae, Pseudomonas aeruginosa* and *Metarhizium anisopliae, Pseudomonas aeruginosa, Serratia marcescens* and *Metarhizium anisopliae*. These are shown in the figure below where the organisms used for infection are plotted against the number of deaths in insects of study and observations were made on day 5.

DISCUSSION

The occurrence of entomopathogens in nature as displayed in the findings of this study is in agreement with previous submissions by (Gillespie and Moorhouse, 1989; McCoy *et al.,* 1988; Roberts and Humber, 1981). *Fusarium* sp. and *Beauvaria bassiana* isolated from *Zonocerus variegatus* in this study agrees with the observation of Paraiso *et al.,* 1991. *Aspergillus flavus* and *A. niger* isolated in this study from *Zonocerus variegatus* have previously been recorded by Smit (1991) and Hernandez and Santiago (1997) with significantly frequent incidence rates.

Penicillium sp. isolated in this study had been reported by Poinar and Thomas (1984) from various insects populations. *Mucor* sp belongs to the family Mucoraceae. Lomer and Lomer (1996) had reported the isolation of members of this family from *Zonocerus variegatus*.

The occurrence of *Beauvaria bassiana* in various insects was initially reported by Inglis *et al.*, 200

Initial observation shows that locust cadavers infected with *Beauvaria basiana* were able to infect healthy control locust because of their closeness in proximity at the beginning of this experiment. This conforms to the submissions of Hemmati *et al.,* 2001 and Roy *et al.,* 2001 when they discovered that the Conidia of Hyphomycetes such as *Metarhizium* and *Beauveria* spp. are hydrophobic and passively spread from infected cadavers to healthy insects.

UNDER PEER REVIEW

Saikkonen *et al.,* 2004 stated that *B. bassiana* are inherently present in internal plant tissue as an adaptive protection against herbivorous insects. Since *Zonocerus variegatus* majorly feeds on foliage, it is most likely that they encounter this organism during their process of feeding. Elliot *et al.,* 2000 and White *et al.,* 2002 also made similar findings when they referred to *B. bassiana* as endophytic fungi which is an inherent plant-defending mutualist.

Posada and Vega in 2005 also reported the natural occurrence of *Beauvaria bassiana* in the leaf tissue of corn. In addition, its endophytic activity in cacao (*Theobroma cacao*), poppy (*Papaver somniferum*), coffee (*Coffea* spp.) and tomato (*Lycopersicon esculentum*) were all reported in 2006 by Quesada- Moraga *et al,*.

Butt *et al.,* 2001 stated that Entomopathogenic fungi are usually identified based on the growth on insect cadavers. This is further affirmed in this study with the growth of *Beauvaria bassiana* on the cadavers of *Zonocerus variegatus*

The virulence of *B. bassiana* on *Zonocerus variegatus* can be attributed to its proven ability to produce proteases, chitinases, and lipases. All of these are enzymes which can degrade insect cuticle and thus lead to their sicknesses and eventual death. This previous findings were made by Charnley and St. Leger, 1991; Charnley, 2003.

B. bassiana has also been shown to degrade the chitin covering of insects facilitated by a combination of mechanical force and enzymic degradation (Zacharuk, 1970; Hassan and Charnley, 1989).

This study also reveals high lethality being recorded for grasshoppers infected with *B. bassiana.* This suggests that its spores are lethal to *Z. variegatus* and could cause significantly high mortality in population of grasshoppers. This agreed with Johnson *et al,*. (1992) who reported a highly significant level of mortality in *B. bassiana* treated grasshoppers in Mali. This further suggests that it has great potentials as a microbial control agent of *Z. variegatus*.

This study also shows the occurrence of several entomopathogens such as *Beauveria bassiana*, *Metarhizium anisopliae*. This occurrence agrees with earlier findings by Sri-Arunotai *et al.,* 1975; Swingle and Seal, 1931 and Sikorowski, 1985. They all worked on entomopathogens and they reported the natural occurrence of *Beauveria bassiana, Metarhizium anisopliae,* and *Paecilomyces* sp. and the bacterium *Serratia marcescens* in various insects' populations.

This study has implicated *Bacillus subtilis* as a potential entomopathogens which is capable of causing lethargic effects which subsequently result in the death of most of the study grasshoppers used. This is in accordance with the findings of Lawrence and Joe who reported various *Bacillus* sp. as potential entomopathogens which have been used extensively for the control of insect and pests populations in domestic, crops, forests, and the aquatic environment.

UNDER PEER REVIEW

This claim was further established by established by Mir, (1991) during his studies on the potencies of *Bacillus* sp. entomopathogens in controlling mosquitoes' population in developing countries.

In addition, Backman *et al.,* (1997) also described some naturally occurring strains of *Bacillus subtilis* as having innate insecticidal properties which is useful in biocontrol activities.

The emergence of marked lethal signs in insects used after 48 hours of infection by bacterial entomopathogens especially in the case of *Zonocerus variegatus* further confirms that bacterial pathogens of insects requires at least 48 hours of incubation period before the onset of morbid manifestations. This is in agreement with the works of Hajek, (2004).

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. This findings agree with the experiments carried out on synergistic usage of 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 same entomopathogens are used. Synergistic virulence to *Cyclocephala* spp. was observed in combinations of entomopathogenic nematodes with *Paenibacillus popilliae* (Thurston *et al.,* 1993, 1994), or with *Bacillus thuringiensis* Berliner subspecies *japonensis* (Koppenhöfer and Kaya, 1997; Koppenhöfer *et al.,* 1999). Although the fungi-bacterial formulation yielded more insect deaths compared to fungi-fungi formulation, it was noticed that none of the synergistic formulation was found to have greater entomopathogenic effect compared to the deaths recorded when single organisms were used to challenge the insects.

References

Backman, P. A.; Wilson M. and Murphy, J. F., (1997). Bacteria for Biological Control of Plant Diseases in Rechcigl and Rechcigl, eds., Environmentally Safe Approaches to Crop Disease Control. pp. 95-109. CRC Press.

Balogun, S. A. and Fagade, O. E., (2004). Entomopathogenic fungi in population of *Zonocerus variegatus* (l) in Ibadan, Southwest, Nigeria. African Journal of Biotechnology Vol. 3 (8), pp. 382-386.

Charnley, A. K. and St. Leger R. J., (1991). The role of cuticle-degrading enzymes in fungal pathogenesis in insects. p. 267–287. In: "Fungal Spore Disease Initiation in Plants and Animals" (E.T. Cole, H.C. Hoch, eds.). Plenum Press, New York, 555 pp.

Charnley, A. K., (2003). Fungal pathogens of insects: cuticle degrading enzymes and toxins. Adv. Botanical Res. 40: 241–321.

UNDER PEER REVIEW

Elliot, S. L., Sabelis, M.W. and Janssen A., (2000). van der Geest LPS, Beerling EAM, Fransen J. Can plants use entomopathogens as bodyguards? Ecol. Lett. **3**, 228–235.

Gillespie, A. T. and Moorhouse, E. R., (1989). The use of fungi to control pest of agricultural and horticultural importance. In Biotechnology of Fungi for Improvement of Plant Growth, J. M. Whipps and R. D. Lumsdon, eds. London: Cambridge University Press.

Hajek, A., (2004). Natural enemies: an introduction to biological control. Cambridge University Press, Cambridge, UK.

Hassan A. E. M. and Charnley A. K. (1989). Ultrastructural study of the penetration by *Metarhizium anisopliae* through Dimilin-affected cuticle of *Manduca sexta*. J. Invertebr. Pathol. 54: 117–124.

Hemmati, F., Pell, J. K., McCartney, H. A. and Deadman, M. L., (2001). Airborne concentrations of conidia of *Erynia neoaphidis* above cereal fields. *Mycol Res*, **105**, 485–489.

Inglis, G. D., Goettel, M. S., Butt, T. M. and Strasser, H., (2001). Use of hyphomycetous fungi for managing insect pests. In: Butt TM, Jackson CW, Magan N (Eds.), *Fungi as Biocontrol Agents: Progress, Problems and Potential.* CABI International/ AAFC, Wallingford, United Kingdom, pp.23-69.

Johnson, D. J., Goettel, M. S., Bradeley, C., Van Der Paauw, H. and Maiga, B., (1992). Field trials with the entomopathogenic fungus *Beauveria bassiana* against grasshoppers in Mali in July 1990. In Biological Control of Locusts and Grasshoppers (Lomer C.J. and Prior C. (eds) Proceedings of a workshop held in cotonou, CAB Int. Wallingford, pp. 296 – 310.

Luz, Christian and Farques, Jacques, (1998). Factors affecting conidia production of *Beauveria bassiana* from fungus – Killed cadavers of *Rhodnius prolixus*. J. Inv Pathol. 72: 97 – 103.

McCoy, C. W., Samson, R. A. and Boucias, D. G., (1988). Entomogenous fungi. In Handbook of Natural Pesticides, Boca, Raton, Fla: *Mr ic* Press. Vol. 5, Microbial Insecticides, Part A, Entomogenous Protozoa and Fungi, C. M. Ignoffo and N. B. Mandava, eds.

Mir S. Mulla, (1991). Biological Control of Mosquitoes with Entomopathogenic Bacteria. Proceedings of the 4th national vector control symposium. Pp 93-104.

Moorhouse, E. R., Gillespie, A. T. and Charnley, A. K., (1993). Laboratory selection of Metarhizium sp. Isolates for control of vine weevill larvae (*Otiorhynchus sulcatus*). J. Invertebr Pathol. 62: 15-21.

Poinar, O. G. and Thomas, M. G., (1984). Laboratory guide to insect pathogen and parasites. Plenum press. New York and London.

UNDER PEER REVIEW

Posada, F. and Vega, F. E. (2005). Establishment of the fungal entomopathogen Beauveria bassiana (Ascomycota : Hypocreales) as an endophyte in cocoa seedlings (Theobroma cacao). *Mycologia,* **97**, 1195–1200.

Roy, H. E., Pell, J. K. and Alderson, P. G., (2001). Targeted dispersal of the aphid pathogenic fungus *Erynia neoaphidis* by the aphid predator *Coccinella septempunctata*. *Biocontrol Sci Technol,* **11**, 99–110.

Saikkonen, K., Wali, P., Helander, M. and Faeth S. H., (2004). Evolution of endophyte-plant symbioses. *Trend Plant Science,* **9**, 275–280.

Smit, J. J. (1991). CILSS/DEPV; The permanent Interstate Committee for Drought control in the Sahel and the Department of Crop Protection Training Niger. In Biological control of Locusts and Grasshoppers. Proceedings of a Workshop held at IITA Cotonou, Republic du Benin (Lomer CJ, Prior C) (Eds), pp. 30 – 37.

Tanada, Y. and Kaya, H. K., (1993). *Insect pathology.* Academic Press, San Diego.

Thomas, M. B., Blandford, Simon and Lomer, C. J., (1997). Reduction of feeding by the variegated grasshoppers. *Zonocerus variegates* following infection by the fungal pathogen *Metarhizium flavoviride*. Biocontrol Sci. Tech. 7: 327 – 334.

White, J. F., Belanger, F., Meyer, W., Sullivan, R. F., Bischoff, J.F. and Lewis, E.A., (2002). Clavicipitalean fungal epibionts and endophytes—development of symbiotic interactions with plants. *Symbiosis*, **33**, 201–213.

Zacharuk, R. Y., (1970). Fine structure of the fungus *Metarhizium anisopliae* infecting three species of larval Elateridae. III. Penetration of the host integument. J. Invertebr. Pathol. 15: 372–396.

Table 1: Name and characteristics of bacterial isolates obtained from Grasshopper

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
Morphological Characteristics								
Gram reaction	-	+	-	+	-	+	-	+
Cell Shape	Rods	Cocci	Rods	Cocci	Rods	Rods	Rods	Rods
Motility	Motile	Non-motile	Motile	Non-motile	Non-Motile	Motile	Motile	Motile
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
Probable Organism	*Salmonella typhi*	*S. aureus*	*P. aeruginosa*	*S. epidermis*	*Klebsiella sp*	*B. subtilis*	*Bacillus sphaericus*	*Strep. feacalis*

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

UNDER PEER REVIEW

UNDER PEER REVIEW

Table 2: Fungi isolated from diseased *Zonocerus 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*

UNDER PEER REVIEW

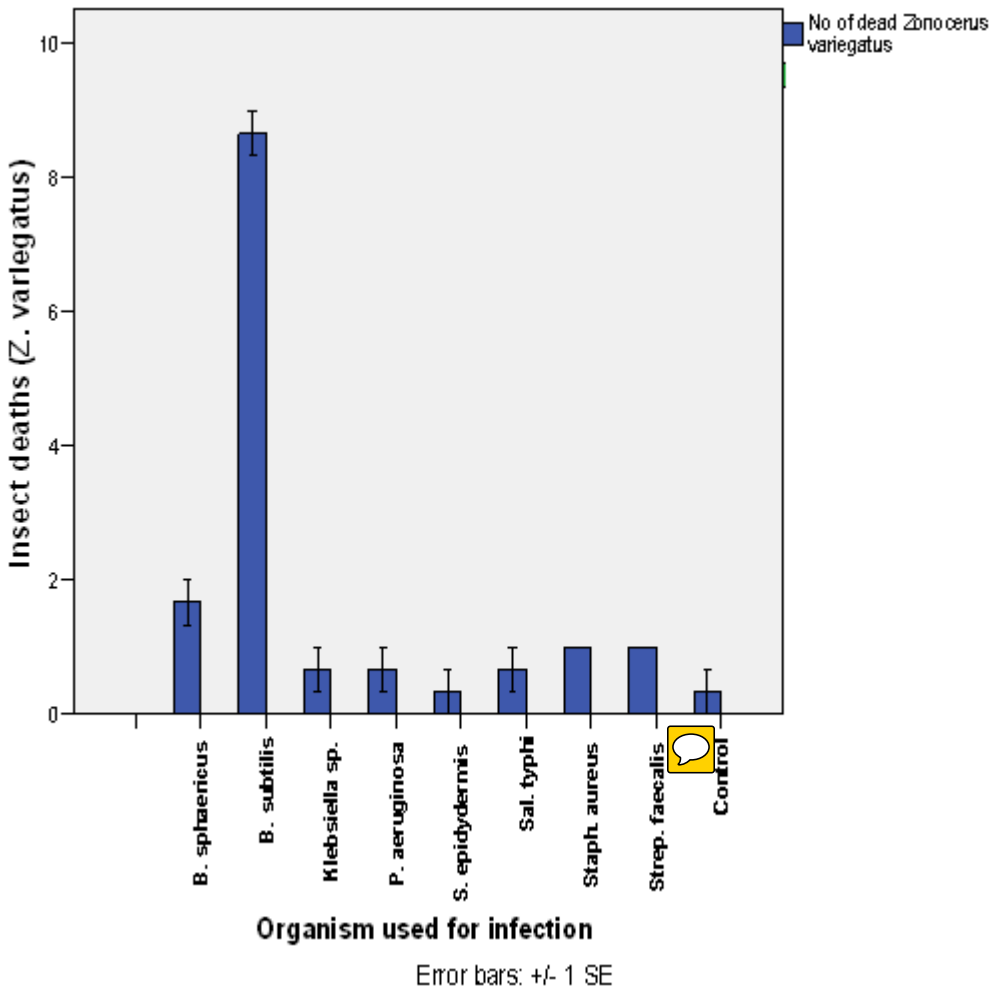

Figure 1: Infection of *Z. variegatus* with bacterial isolates

UNDER PEER REVIEW

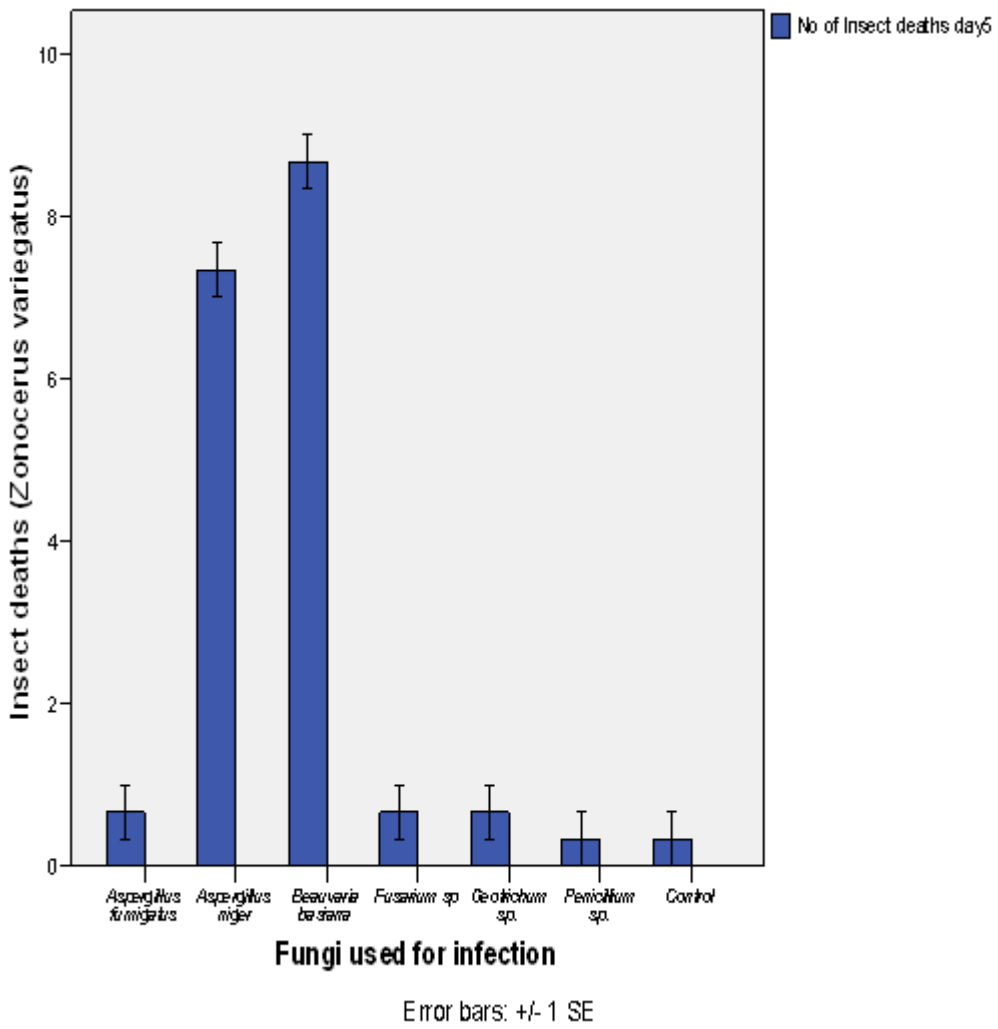

Figure 2: Infection of *Z. variegatus* with fungal isolates

UNDER PEER REVIEW

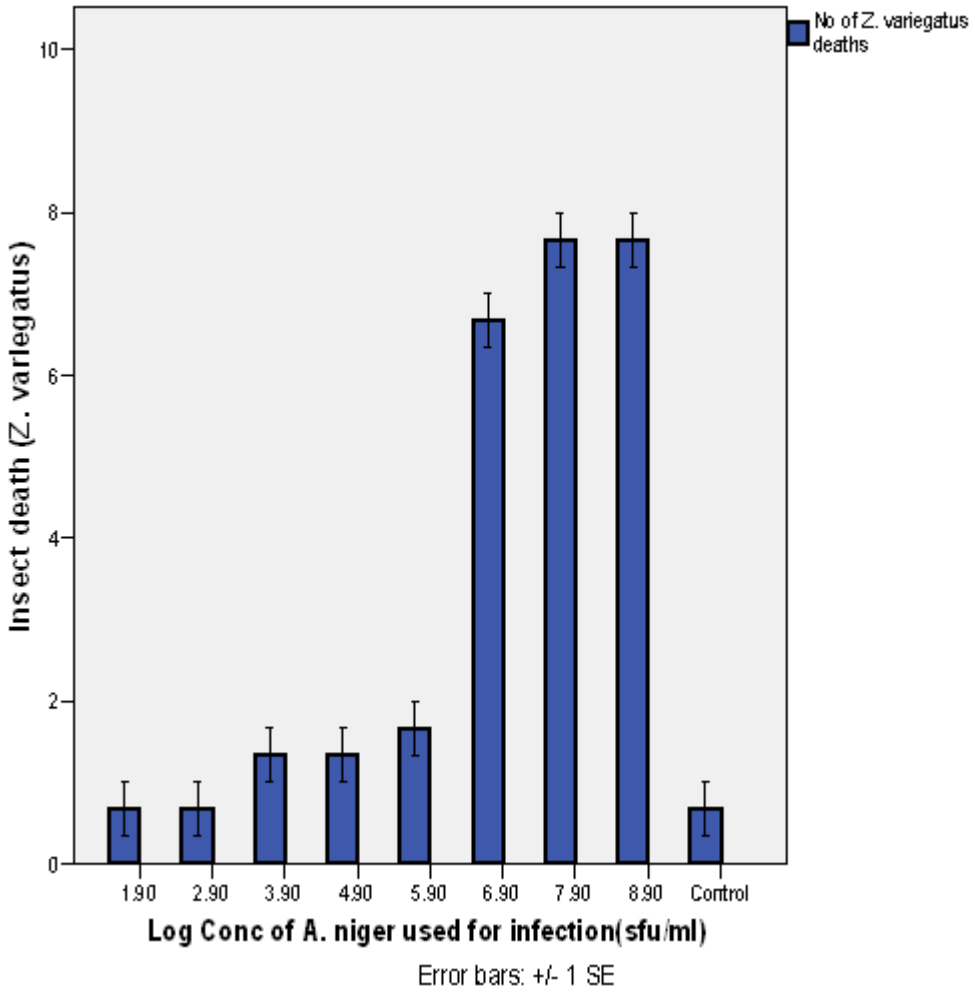

Figure 3: Determination of the MLC of *A. niger* required for pathogenicity in *Z. variegatus*

UNDER PEER REVIEW

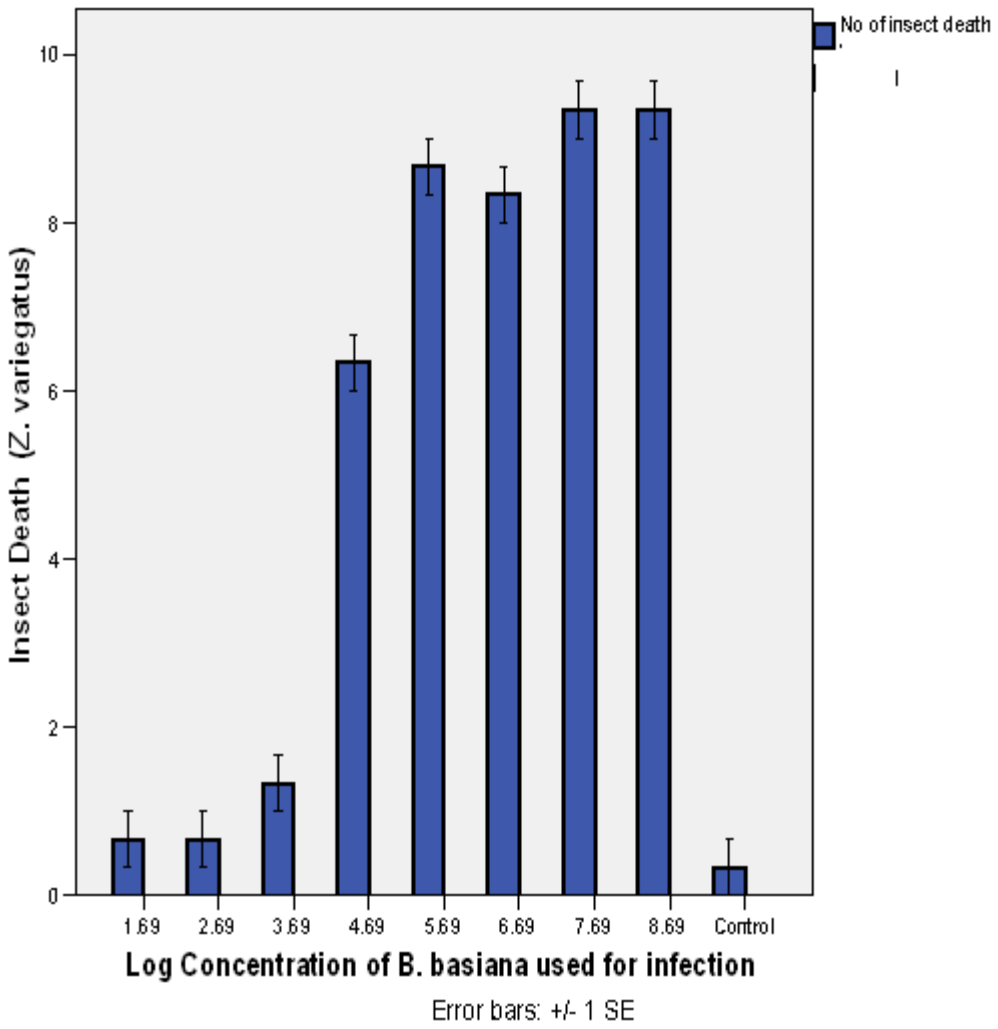

Figure 4: Determination of the MLC of *B. bassiana* required for pathogenicity in Z. variegatus

UNDER PEER REVIEW

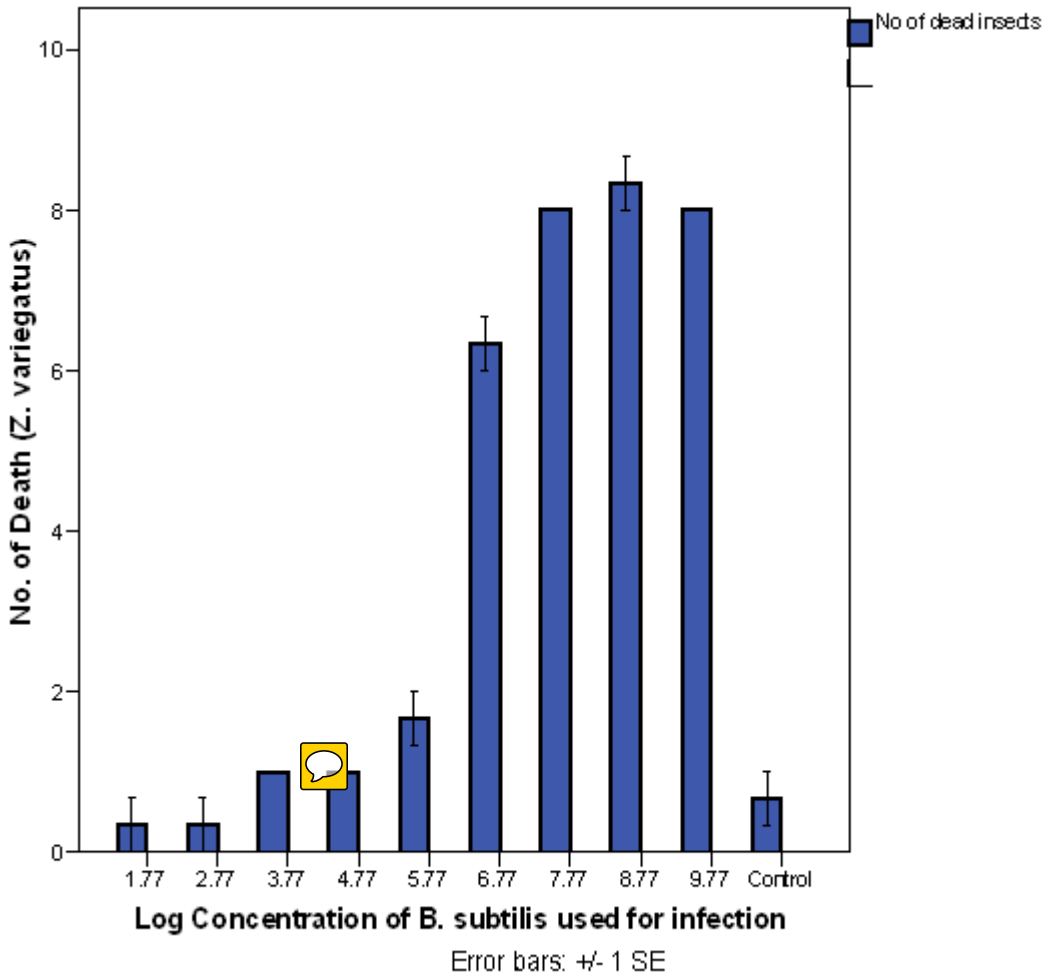

Figure 5: Determination of the MLC of *B. subtilis* required for pathogenicity in Z. variegatus

UNDER PEER REVIEW

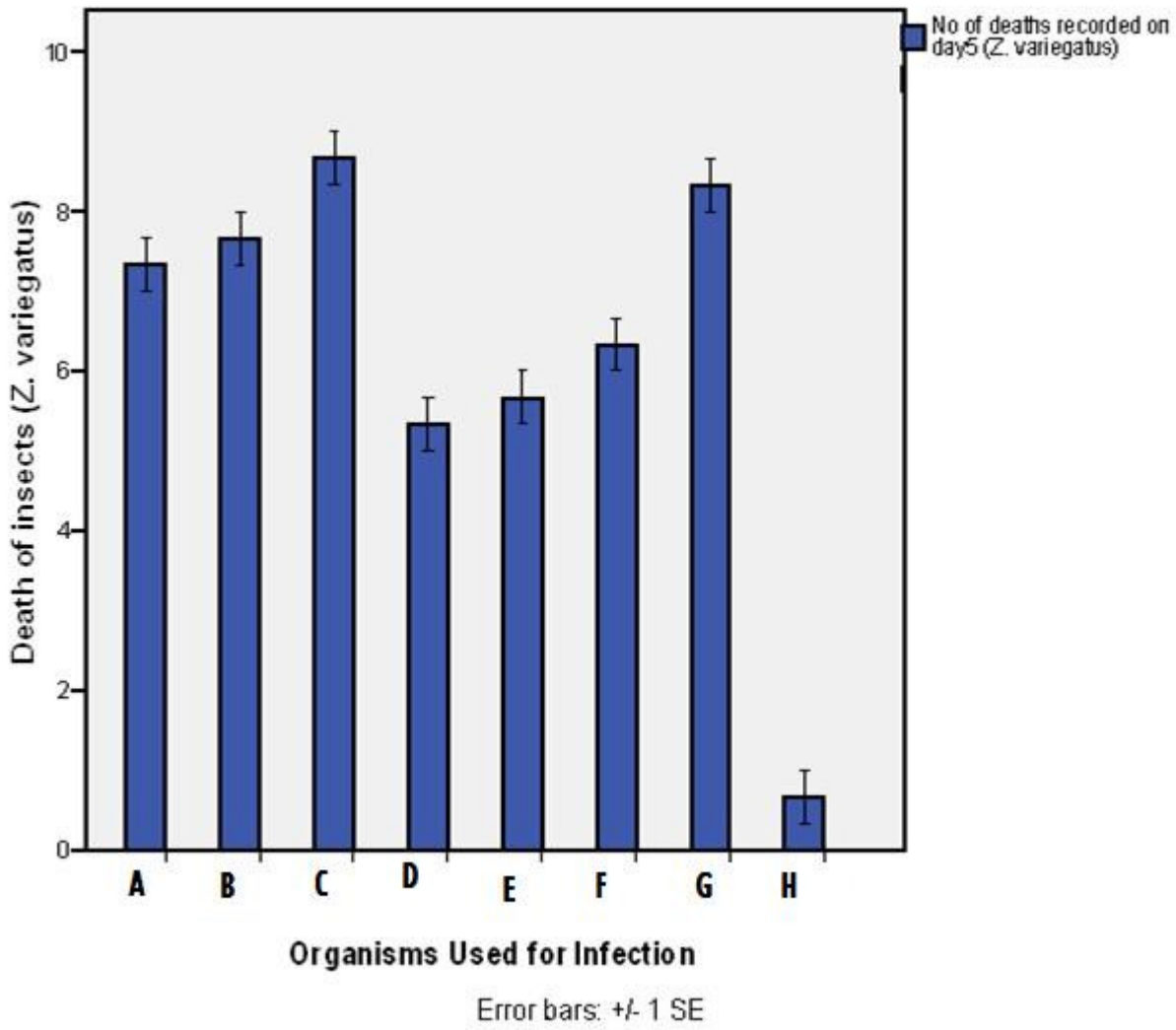

Figure 6: Synergism test on entomopathogens of *Z. variegatus* for day 5

Key: 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