

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 the diseased *Z. variegatus* population. The organisms isolated from the diseased insects were then used to challenge a new healthy population of the same species. *Z. variegatus* were collected from cassava (*Manihot esculenta*) farmlands and allowed to acclimatize for two weeks. They were studied for onset of disease symptoms. Organisms were isolated from the diseased and dead *Z. variegatus*. Bacterial isolates obtained include *Salmonella typhi*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Staphylococcus epidermis*, *Klebsiella sp.*, *Bacillus subtilis*, *Bacillus sphaericus*, and *Streptococcus faecalis*. 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 isolated bacterial and fungal species. One of the bacteria which is *B. subtilis* and two of the fungi which are *B. bassiana* and *A. niger* were able to induce lethargic and morbid effects on the grasshoppers. Re-infection using different concentrations of both the entomopathogenic fungi and bacterium were carried out on grasshoppers to determine the minimum lethal concentration required to kill 50 percent of the grasshopper population (LD_{50}). *B. bassiana* was found to be pathogenic at concentration $\log_{10}^{-1} 4.69$ which is equivalent to 5.0×10^4 spore forming units per milliliter (sfu/ml). Lowest concentration of *A. niger* which was pathogenic against *Z. variegatus* was $\log_{10}^{-1} 8.90$ which is equivalent to 8.0×10^8 sfu/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 6.0×10^6 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. Synergistic evaluation however showed no significant increase in insect deaths when compared to the usage of single organisms. Fungi and bacteria obtained in this study which possess entomopathogenic ability may be further studied. The process of mass production should be further developed and a suitable and efficient method of applying the entomopathogens should be devised.

Keywords: *Bacillus subtilis*, *Beauveria 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

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SAMPLE ABSTRACT:

Aims: Here clearly write the aims of this study.
Sample: To correlate platelet count, splenic index (SI), platelet count/spleen diameter ratio and portal-systemic venous collaterals with the presence of esophageal varices in advanced liver disease to validate other screening parameters.
Study design: Mention the design of the study here.
Place and Duration of Study: Sample: Department of Medicine (Medical Unit IV) and Department of Radiology, Services Institute of Medical Sciences (SIMS), Services Hospital Lahore, between June 2009 and July 2010.
Methodology: Please write main points of the research methodology applied. **Sample:** We included 63 patients (40 men, 23 women; age range 18-75 years) with liver cirrhosis and portal hypertension, with or without the medical history of gastrointestinal bleeding. Clinical as well as hematological examination (platelet count) and ultrasonography (gray as well as color Doppler scale including splenic index and splenorenal/pancreaticoduodenal collaterals) was done besides upper GI endoscopy for esophageal varices. Platelet count/spleen diameter ratio was also calculated.
Results: Kindly make sure to include relevant statistics here, such as sample sizes, response rates, P-values or Confidence Intervals. Do not just say "there were differences between the groups".
sample: Out of 63 patients, 36 patients with small varices (F1/F2) and 27 with larger (F3) varices were detected on endoscopy. Significant increase in mean splenic index from low (86.7 +/- 27.4) to high (94.7 +/- 27.7) grade varices was documented. Opposite trend was found with platelets (120 ... [1])

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insecticides[3]. 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 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. The number of extant species is estimated at between six and ten million and potentially represent over 90% of the differing metazoan life forms on Earth. The life cycles of insects vary but most of them hatch 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 have rewritten men's history in times past and they have also helped in shaping our future [4].

3. 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]. Chemicals used in controlling these insects are dangerous to non-targets in the environment [6] while these insects are also resistant to some of these chemicals [7]. Therefore, methods of control must be environmentally friendly and effective against these organisms thus necessitating the usage of entomopathogens. *Z. variegatus* will be collected from *M. esculenta* farmlands, organisms will be isolated from the diseased ones, pathogenicity test will be carried out on all isolated organisms to determine those with entomopathogenic potentials and synergistic tests will be done on all the entomopathogens.

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4. 2. MATERIALS AND METHODS

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 2 weeks during which they were fed with surface sterilized fresh cassava and water leaves [8-10][8][9][10].

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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*

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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 dessicator with silica gel at room temperature [12]. Cadavers with luxuriant fungal growth were regarded as being positive while cadavers without fungal growth were regarded as negative. The sporulating fungi on cadavers were isolated in pure culture on Sabourand 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°C~~ 25°C for 72 hours [9].

2.4 Isolation of Bacteria from diseased *Z. variegatus*

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

2.5 Identification of isolated microorganisms

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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*

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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 and sterile glass rods [13]. Conidia suspensions were stored at 4°C before serial dilutions for the susceptibility test was done. The ~~spore~~ spores suspensions obtained were dispensed into aspirators capable of aerosolizing the organism [9].

2.7 Preparation of bacterial suspension for infection of *Z. variegatus*

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Bacterial suspensions were prepared from each of the isolated organisms. Each bacterium was inoculated into 50mls of sterilized broth. They were incubated at ~~37°C~~ 37°C 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 [11].

2.8 Infection of *Z. variegatus* with microbial suspensions

New batch of apparently healthy *Z. variegatus* in groups of ten per treatment 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 [9, 17].

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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. 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].

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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. 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 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.

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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 (ANOVA) and Duncan's Multiple Range Test for the estimation of means. The 't' value will be tested at 95% confidence interval.

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3. RESULTS

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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 below.

3.2 Isolation of Fungi from diseased *Z.variegatus*

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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.*

3.3 Infection of *Z. variegatus* with bacterial isolates

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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 *B. subtilis*. These are shown in the figure 1 below where the organisms used are plotted against the number of dead insects.

3.4 Infection of *Z. variegatus* with fungal isolates

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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*, *Articulosporainflata*, *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. This is represented in the figure 2 below where the organisms used for infection are plotted against the number of dead insects.

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

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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 insects. *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 8×10^8 sfu/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 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.

3.6 Evaluation of synergism among entomopathogens

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Insects of study which were grouped in tens were challenged with more than one entomopathogen. *Z.variegatus* was challenged with *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 figure 6 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 to allow maximum time for insect deaths.

6.4. 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].

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 nematode and 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 among both fungal agents. Both fungal agents might be competing for the insect cadaver as substrate thus secreting enzymes which may be inhibiting the activity of each other.

7.5. CONCLUSION

A. Nigerniger, *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 bio-pesticides 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.

8. ACKNOWLEDGEMENT

Our appreciation goes to Mr. Ajibare of Wesley University Ondo for the trapping, sorting and identification of the *Z. variegatus* used in this study.

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9. REFERENCES

1. De Faria, M. R., and Wright, S. P., (2007). Mycoinsecticides and Mycoacaricides: A comprehensive list with worldwide coverage and international classification of formulation types. *Biological Control* 2007: 43: 237-256.
2. Wright, M. S., Osbrink, W. L. and Lax A. R., (2002). Transfer of entomopathogenic fungi among formosan subterranean termites and subsequent mortality. *J. Appl Ent.* 126: 20-23.
3. Pu, X Y., Feng, M. G., Shi C. H., (2005). Impact of three application methods on the field efficacy of a *Beauveria bassiana*-based mycoinsecticide against the false-eye leafhopper, Empoasca vitis (Homoptera: Cicadellidae) in tea canopy. *Crop Prot* 24:167–175.
4. Banjo, A. D., Lawal, O. A., and Songonuga, E. A. (2006). The nutritional value of fourteen species of edible insects in southwestern Nigeria. *African Journal of Biotechnology*. Vol. 5 (3), pp. 298-301,
5. Langewald, J., Thomas, M. B., Dourou-Kpindou, O. K. and Lomer, C. J., (1997). Use of *Metarhizium flavoviride* for control of *Zonocerus variegatus*. A model linking dispersal and secondary infection from the spray residue with mortality in caged field samples. *Entomologia Experimentalis of Applicata* 82: 1 – 8.
6. Lomer, C. J., Bateman, R. P., Johnson, D. L., Langewald, J and Thomas M., (2001). Biological Control of Locusts and Grasshoppers. *Annu Rev Entomol* 46: 667 702.
7. Shi X. G., Zhu Y. K., Xia X. M., Qiao K., Wang H. Y., Wang K. Y., (2012). The mutation in nicotinic acetylcholine receptor $\beta 1$ subunit may confer resistance to imidacloprid in *Aphis gossypii* (Glover).- *Journal of Food, Agriculture and Environment*, 10 (2): 1227-1230.
8. Dourou-Kpindou, O. K., Godonou, I., Houssou, A., Lomer, C. J. and Shah, P. A., (1995). Control of *Zonocerus variegatus* by ultra-low volume application of an oil-formulation of *Metarhizium Flavoviride* conidia. *Biocontrol Sci. Technol.* 5: 131 – 139
9. 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.
10. Thomas, M. B., Blandford, Simon and Lomer, C. J., (1997). Reduction of feeding by the variegated grasshoppers. *Zonocerus variegatus* following infection by the fungal pathogen *Metarhizium flavoviride*. *Biocontrol Sci. Tech.* 7: 327 – 334.
11. Tanada, Y. and Kaya, H. K., (1993). *Insect pathology*. Academic Press, San Diego.
12. 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.
13. Mohammadbeigi, A., and Port, G., (2013). “Efficacy of *Beauveria Bassiana* and *Metarhizium Anisopliae* against *Uvarovistia Zebra* (Orthoptera: Tettigoniidae) via Contact and Ingestion.” *Intl J Agri Crop Sci.* Vol., 5 (2), 138-146.
14. Poinar, O. G. and Thomas, M. G., (1984). Laboratory guide to insect pathogen and parasites. Plenum press. New York and London.
15. Fawole, M. O. and Oso, B. A., (2001). Laboratory manual in Microbiology. 3rd Edition. Pp.102-105.

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- 16. Barnett, J. A.; Payne, R.W. and Yarrow, D., (2000).** Yeast: Characteristics and identification. 3rd Edition, Cambridge university press, Cambridge, England, pp.111- 125.
- 17. Drummond, R. O. and Gladney W. J., (1969).** Further evaluation of animal systemic insecticides. *Journal of Medical Entomology* 6(4): 934-6.
- 18. 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.
- 19. Saikkonen, K., Wali, P., Helander, M. and Faeth S. H., (2004).** Evolution of endophyte-plant symbioses. *Trend Plant Science*, 9, 275–280.
- 20. 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.
- 21. 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.
- 22. 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.
- 23. Charnley, A. K., (2003).** Fungal pathogens of insects: cuticle degrading enzymes and toxins. *Adv. Botanical Res.* 40: 241–321.
- Croft, B., (1990).** Arthropods Biological Control Agents and Pesticides. John Wiley and Sons., New York.
- 24. Mir S. Mulla, (1991).** Biological Control of Mosquitoes with Entomopathogenic Bacteria. Proceedings of the 4th national vector control symposium. Pp 93-104.

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
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	<i>Salmonella typhi</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>S. epidermidis</i>	<i>Klebsiella sp</i>	<i>B. subtilis</i>	<i>Bacillus sphaericus</i>	<i>Strep. faecalis</i>

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

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Table 2: Name and characteristics offungalisolates obtained from diseased *Z.variegatus*

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Isolat	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.	<i>Geotrichum sp.</i>
FG2	Varying coloration from Yellowish green to dark green with a cream coloured base	Septate Hyphae, occurrence of stigmata, conidiophores are hyaline.	<i>Penicillium sp.</i>
FG3	Initial whitish to yellow luxuriant growth which later bears black dust-like conidia.	Microscopic evaluation shows upright single conidiophores with sphere-like swellings at the ends. Swellings which bears phallides are also seen at the apex.	<i>Aspergillus niger</i>
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.	<i>Aspergillus fumigatus</i>
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.	<i>Beauveria bassiana</i>
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.	<i>Fusarium sp.</i>

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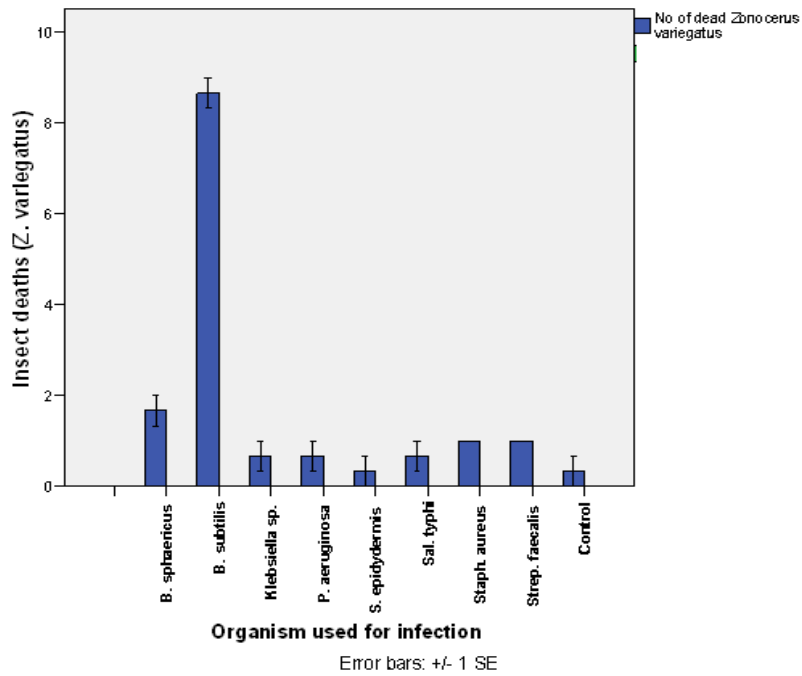


Figure 1: Infection of *Z. variegatus* with bacterial isolates

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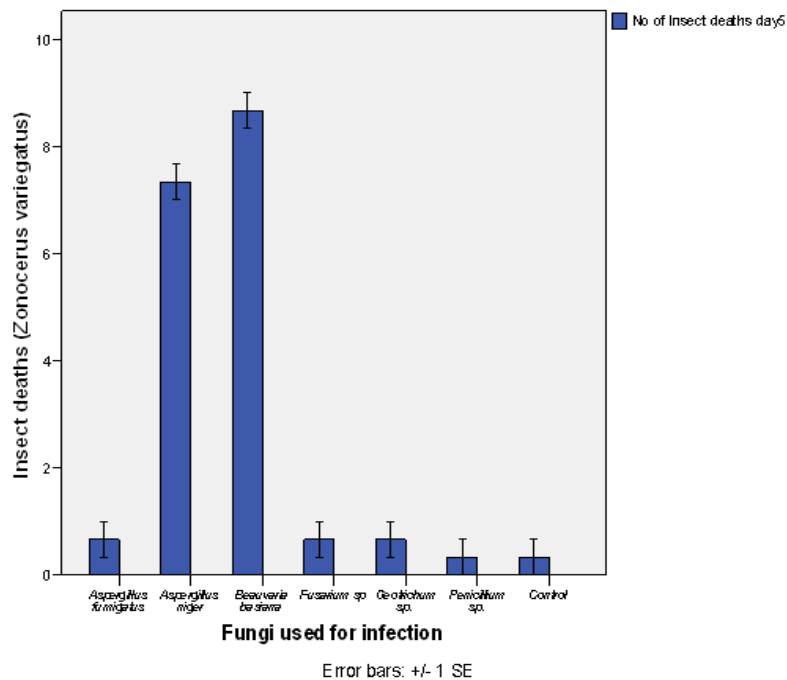


Figure 2: Infection of *Z. variegatus* with fungal isolates

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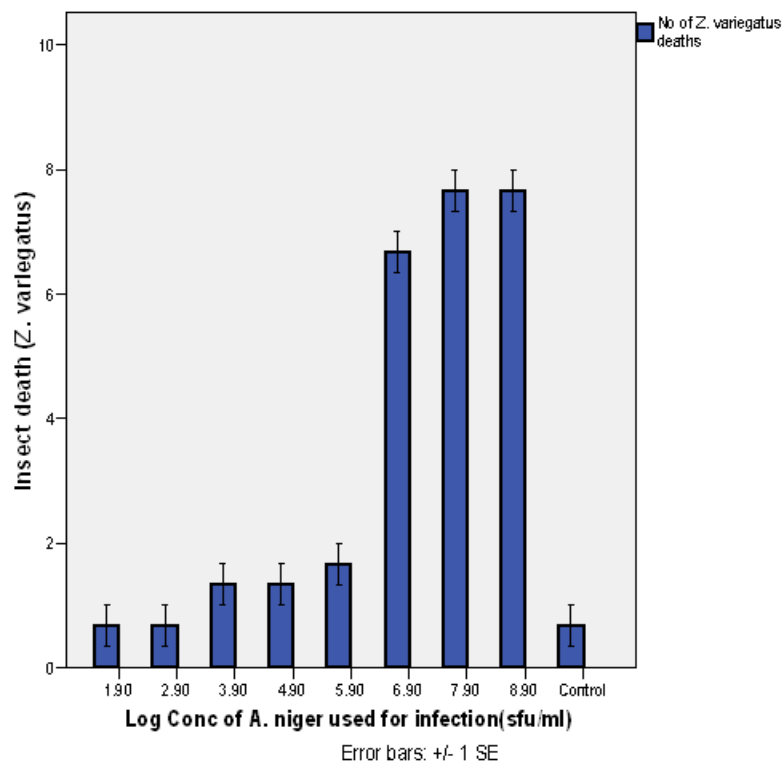


Figure 3: Determination of the MLC of *A. niger* required for pathogenicity in *Z. variegatus*

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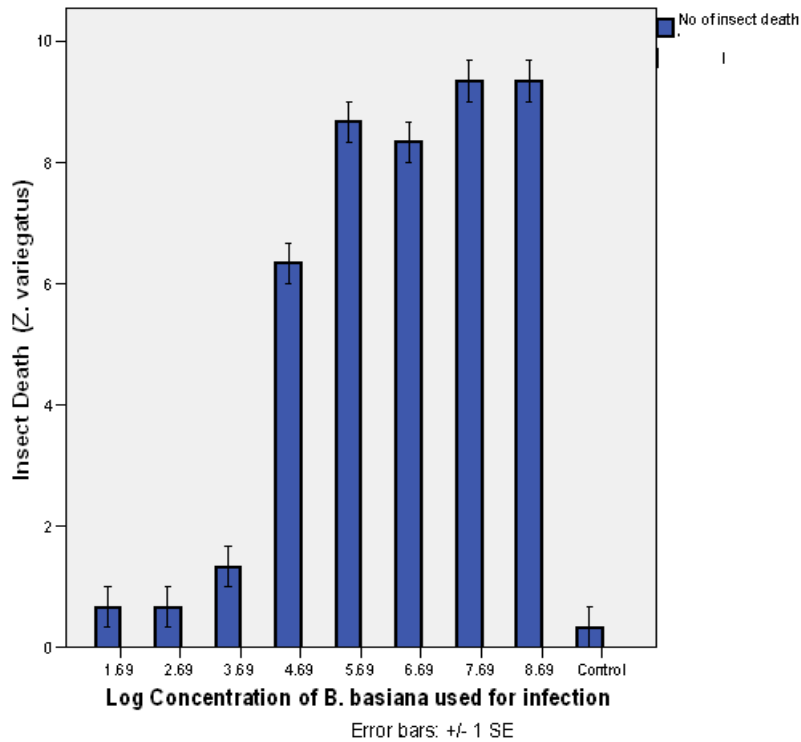


Figure 4:- Determination of the MLC of *B. bassiana* required for pathogenicity in termites

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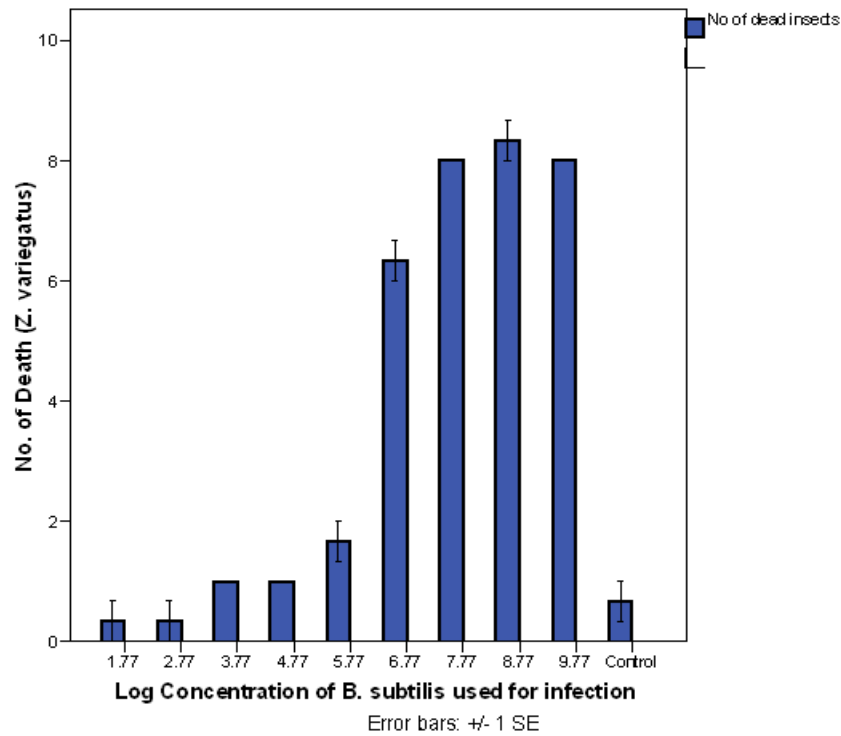


Figure 5: Determination of the MLC of *B. subtilis* required for pathogenicity in *Z. variegatus*

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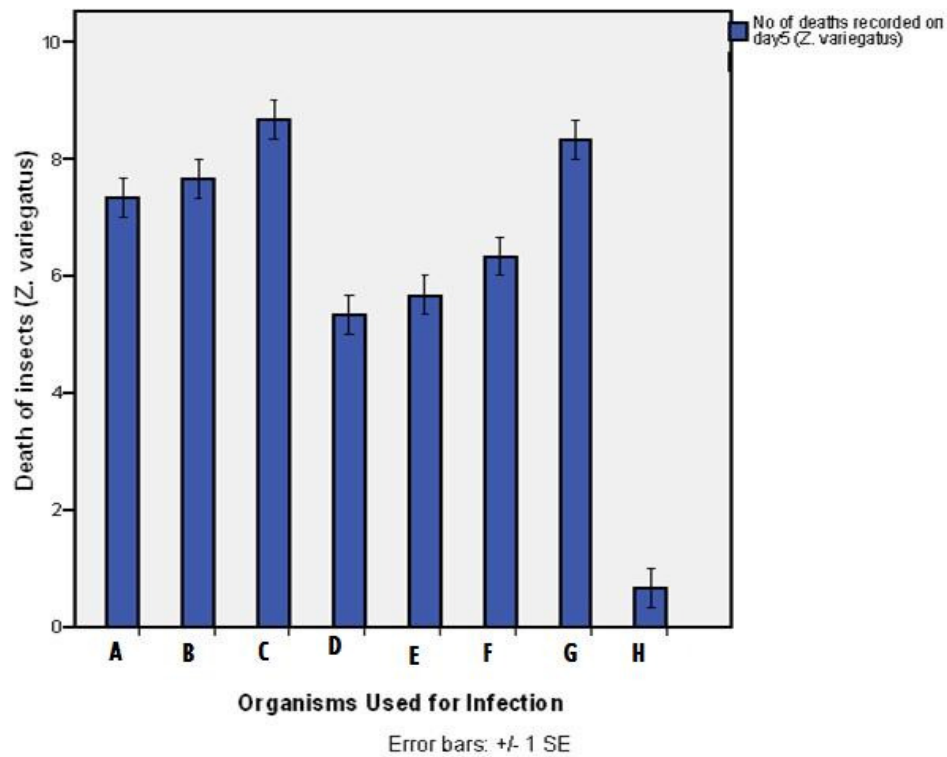


Figure 6: Synergism test on entomopathogens of *Z. variegatus* for day 5

Key: A: *Aspergillus niger*, B: *Bacillus subtilis*, C: *Beauveria 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*

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ABSTRACT (ARIAL, BOLD, 11 FONT, LEFT ALIGNED, CAPS)

Different sub-sections, as given below, should be used.

SAMPLE ABSTRACT:

Aims: Here clearly write the aims of this study. Sample: To correlate platelet count, splenic index (SI), platelet count/spleen diameter ratio and portal-systemic venous collaterals with the presence of esophageal varices in advanced liver disease to validate other screening parameters.

Study design: Mention the design of the study here.

Place and Duration of Study: Sample: Department of Medicine (Medical Unit IV) and Department of Radiology, Services Institute of Medical Sciences (SIMS), Services Hospital Lahore, between June 2009 and July 2010.

Methodology: Please write main points of the research methodology applied. Sample: We included 63 patients (40 men, 23 women; age range 18-75 years) with liver cirrhosis and portal hypertension, with or without the medical history of gastrointestinal bleeding. Clinical as well as hematological examination (platelet count) and ultrasonography (gray as well as color Doppler scale including splenic index and splenorenal/ pancreaticoduodenal collaterals) was done besides upper GI endoscopy for esophageal varices. Platelet count/spleen diameter ratio was also calculated.

Results: Kindly make sure to include relevant statistics here, such as sample sizes, response rates, P-values or Confidence Intervals. Do not just say "there were differences between the groups". sample: Out of 63 patients, 36 patients with small varices (F1/F2) and 27 with larger (F3) varices were detected on endoscope. Significant increase in mean splenic index from low (86.7 +/- 27.4) to high (94.7 +/- 27.7) grade varices was documented. Opposite trend was found with platelets (120.2 +/- 63.5 to 69.8 +/- 36.1) and platelets/ splenic diameter ratio (1676.7 to 824.6) declining significantly. Logistic regression showed splenic collaterals and platelets are significantly but negatively associated with esophageal varices grades.

Conclusion: Non-invasive independent predictors for screening esophageal varices may

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