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Assessing the efficacy and mass production of fungal entomopathogens associated with *Macrotermes bellicosus*

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Omoya, F. O.[1] and Kelly, B. A.[2]*

[1]Department of Microbiology, Federal University of Technology, Akure, Nigeria.
[2]Biological Sciences Department, Wesley University, Ondo, Nigeria.

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ABSTRACT

***Macrotermes bellicosus* (Family: Termitidae, Order: Isoptera) adult workers were harvested from the farm of Federal University of Technology, Akure, Nigeria. They were allowed to acclimatized in the laboratory and were observed for the onset of disease symptoms. Fungi were isolated from diseased *M. bellicosus* using generalized and dodine based media. Isolated fungi were investigated for their lethality on the insects. The minimum concentration of the fungal entomopathogen (LD_{50}) required to kill at least 50 percent of the test insects was determined. Mass production of the entomopathogens was evaluated on selected organic by-products. Analyses were carried out in triplicates and results were plotted on bar charts with the error bars indicating the standard deviation from the average values. The results obtained show that *Metarhizium anisopliae* was able to cause disease in worker termites used. The minimum lethal concentration (LD_{50}) of the fungi required to induce pathogenicity was 5.0×10^5 spore forming units per milliliters. Mass production of the microorganism carried out by inoculating the entomopathogen onto organic by-products also showed the growth of *M. anisopliae* on maize shaft, rice water and brewer's mash. *M. anisopliae* can be a suitable biocontrol agent for the control of *M. bellicosus* in Ondo state, Nigeria. It also shows that the organism has the potential to be mass produced using cheap organic by-products.**

INTRODUCTION

Entomopathogenicity is derived from the Greek word *'entomon'* which refers to insects and 'pathogenic' which means 'disease-causing'. Therefore, entomopathogenic organisms can be explained to be those microbes which possess disease causing abilities in insects (Van Zyl and Malan, 2014), arachnids and other arthropods (Goble et al., 2010). Examples include fungi such as *Beauveria bassiana* (Hypocreales: Clavicipitaceae), *Lecanicillium* sp. (Hypocreales: Cordycipitaceae), *Metarhizium* sp. (Hypocreales: Clavicipitaceae). Other examples include viruses such as the *Cydia pomonella granulovirus* (Baculovirus) and nematodes such as *Steinernema feltiae* (Rhabditida: Steinernematidae) and *Phasmarhabditis hermaphrodita* (Rhabditida: Rhabditidae) (Copping, 2009).

Insects (from Latin 'insectum') refers to crawling and flying arthropods possessing a chitinous exoskeleton, a three-part body (head, thorax, and abdomen), jointed legs, compound eyes and two antennae. Officially, they are the largest group of animals (Tsakas, 2010). They occupy countless habitats due to their biological adaptations and are known to play various significant roles in the places they occupy (Chapman, 2013).

While many insects are of immense benefits including acting as pollinators, serving as food source for humans

*Corresponding author. E-mail: kellytunde@yahoo.com, kellybabatunde@wesleyuni.edu.ng. Tel: +2348038599386.

(entomophagy) and other animals, others are resented because of the damages they cause and health hazards they pose to humans, animals and plants. They are involved in transmission of malaria, yellow fever, typhus, plague, dengue, various forms of encephalitis, relapsing fever, river blindness, filariasis, sleeping sickness and other debilitating diseases (Wojciechowsk et al., 2016). Some insects also defoliate and act as vector in the transmission of certain diseases of plants (Wright et al., 2002).

Earlier literatures described Termites (Isoptera) as having about 2,600 species from 7 families (Abe et al., 2000). More recent literatures described a total of 12 families and more than 3000 species (Krishna et al., 2013). They are related in taxa to wood roaches (Klass et al., 2008). They cause great damage to wooden structures in buildings as a result of their wood-eating habits. Their ability of remaining hidden until after extensive damage has been done is one of their unique features. Termites do not limit themselves to wood; they also damage paper, cloth, carpets and other cellulosic materials. Just like some other insects, various human activities have equally facilitated the movement of wood-eating termites between continents (Cornelius and Osbrink, 2001).

As a result of the damages caused by these insects, it becomes imperative to reduce and control their population where and when necessary. Unfortunately, chemical pesticides used in controlling these insects are dangerous and lethal to non-targets and beneficial animals in the environment (Mondal et al., 2012). Extensive usage of these chemicals can also alter the soil microbiota. Runoffs from farmlands where these chemicals have been used on can also contaminate ground water sources thus rendering it unsuitable for human consumption and inhabitable for aquatic life. Cases of mammalian sterility have also been widely reported from the prolong usage of chemical pesticides (Lomer et al., 2001).

Furthermore, insects have built resistance to many of these chemicals while the ones they are still potent are not easily transferrable among the insects (Silva et al., 2012). Residues which are toxic to humans are frequently discovered in food crops treated with insecticides (Shi et al., 2012). As a result, this study is aimed at researching into microorganisms lethal to *M. bellicosus* as potential agents capable of being used to biologically control termite infestation as a safer and environmentally friendly alternative.

MATERIALS AND METHODS

Construction of termite containment and stocking of termites

Termite containments were fabricated in accordance with standard designs. A rubber cage having a length of 50 cm, breadth of 35 cm and depth of 25 cm was specifically provided for the termites to limit their access to cellulose. Containments were covered with wire nettings to prevent their escape and to also allow for adequate air passage. Containments were kept in dark enclosed areas. Termites were sourced from the university farm using termite traps which consist of perforated plastic buckets filled to the brim with layers of moistened thick papers and plywood. The bucket was covered and buried inside a hole dug in the ground. The depth of the hole dug was equivalent to the height of the bucket such that the top of the bucket levels with the surface of the ground. Worker termites were allowed to infest the trap after 10 days and harvested by carefully removing the paper layers and picking the termites on the surface. The termites were brought to the laboratory where they acclimatized. Sterilized wood twigs and cardboards wetted with sterile water were served to them as a source of food. They were left to acclimatize for one week.

Observation of the insect population for diseased individuals

Insects' natural conditions were closely simulated in the laboratory. A temperature range between 26 to 28°C was maintained with a relative humidity of 65%. The cages were placed in dark enclosures. 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 abnormal outgrowths were separated from the population for maceration and subsequent isolation of microorganisms.

Preparation of media for isolation

Two different solid culture media were used in this study to facilitate the isolation different organisms which may be responsible for the morbid conditions in the insects. These include Potato Dextrose Agar and Veen's media. They were prepared according to manufacturer's specification and sterilized.

Preparation of dodine-based for the isolation of entomopathogenic fungi

Sabouraud dextrose agar (SDA) (32.5 g) was dissolved in 500 mL of distilled water. 1 mL of dodine was added to inhibit the growth of opportunistic fungi. The medium was mixed and autoclaved for 20 min at 120°C. The medium was allowed to cool down to around 60°C. 500 µL of chloramphenicol and 500 µL of streptomycin sulphate

were added to the medium. The medium was then dispensed into Petri dishes.

Isolation of fungi from diseased termites

Termite cadavers were surfaced sterilized in 5% sodium hypochlorite and 75% ethanol solution. They were afterwards rinsed in plenty of sterile water. Cadavers were left to dry for 48 h (Dourou-Kpindou et al., 1995) and incubated in clean desiccators at room temperature as described by Luz and Farques (1998). They were then macerated inside mortar and pestle. This was followed by the addition of sterile distilled water to obtain the macerate which was then diluted serially and the appropriate diluent was then plated on the prepared media under a sterile hood and incubated at 25°C for 72 h.

Purification, identification and preservation of isolated fungi

Each different spore forming units were further purified by taking some mycelia growth with the aid of a wire loop and transferring to the centre of a fresh media. Identification was done morphologically on culture plates and microscopically on slides. Preparation of slides using lactophenol, observation of the fungal mycelia under the microscope and identification was done using Richard A. Humber manual for identification of fungi and Fawole and Oso Microbiology laboratory manual (Fawole and Oso, 2001). All pure cultures obtained were streaked on double strength agar slants in triplicates, incubated and kept refrigerated for further analysis.

Preparation of spore/conidial 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 harvested from these plates by washing with 0.05% tween 80 solution using sterile glass rods. Conidia stock suspensions were stored at 4°C before being dispensed into special plastic aspirators.

Infection of termites with fungal suspensions

The spores and conidia obtained from washing were dispensed into sterile aspirators. The aspirators are formed from polypropylene with a leak proof screw cap which is attached to a long tube that extends into the container. The screw cap contains a spring and a manual nebulizer which works when pressed. Pressing the screw cap expels 1 ml of the solution in a misty form while the spring ensures the cap returns to its initial position. This was used in spraying the insects. Control experiment was set up by spraying separate populations of insects with sterile 0.05% tween 80 solution.

Selection of entomopathogens from the isolated organisms

Pathogenicity test was carried out on all the fungi isolated from the diseased termites. This was done by infecting fresh batches of apparently healthy worker termites with each of the fungi isolated from the previous cadavers. Each fresh batches were obtained from the bucket trap earlier described. The termites were watched for 5 days after infection. Control experiment was conducted by spraying a separate batch of termites with sterile 0.05 tween 80 solution. Each batch of both test and control termites consisting of 20 individuals. The fungus which is able to exhibit morbid and mortal effects on the insects after the pathogenicity test were selected as suspected entomopathogens (Mohammadbeigi and Port, 2013).

Mass production of the entomopathogenic fungi into liquid form using brewer's mash, maize shaft and rice water

Brewer mash was collected from the International Breweries Plc, Ilesha, Osun State, Nigeria. The wet mash was sundried for seven days to prevent enzyme denaturation and further microbial degradation. The dried spent grain was grounded into powder using high speed blender and sieved to obtain the finest particles. Maize shaft and rice water were collected from the catering department of Wesley University, Ondo, Ondo state, Nigeria. The maize shaft was sun dried for seven days to prevent microbial spoilage, further blended to obtain finer particles and sieved while the rice water was immediately transferred into laboratory freezers. The sieved maize shaft and dried mash were weighed and 100 g was suspended in 1 L of water. A litre of the rice water was also measured and dispensed into flasks. All of these were sterilized and allowed to cool. The cooled liquid substrates were then inoculated with a loopful of the entomopathogenic fungi mycelium and incubated accordingly.

Preparation of powdered talc-based formulation for *M. anisopliae*

The fungal isolate was initially grown in each of the broth for seven days using the incubator shaker. After their growth and susbsequent sporulation, the broths in the

Table 1. Names and characteristics of fungi isolated from diseased termites.

Isolate	Cultural characteristics	Microscopic examination	Suspected organism
TSDA1	Massive mycelial growth, with a dense cottony growth. Colour is initially white and turns greyish	Presence of stolons, presence of sporangiophores, formation globuse and columellate sporangia	*Rhizopus stolonifer*
TSDA2	Initially yellow but subsequently becomes bright to dark yellow-green with age to greenish black	Spreading yellow-green, colonies, rough-walled stipes, mature vesicles bearing phialides over their entire surface and conspicuously echinulate conidia	*A. flavus*
TPDA1	Surface is powdery and subsequently turned crusty, dark herbage green	Conidia in chain form, globose conidia	*M. anisopliae*
TPD2	Violet colouration, rapid growing, nonspreading and often wrinkled	Hypahe shows septation, presence of stigmata, conidiophores are hyaline	*Penicillium* sp.
TPDA3	Black to dark Brown coloration, fast growing and heavily sporing	Microscopic evaluation shows upright single coniodophores with globose at the ends. Swellings which bears phallides are also seen at the apex	*A. niger,*
TSDA3	Whitish fluffy growth on PDA which subsequently turns to greenish black	Hyphae shows septation and also bear conidiophores	*A. inflata*

flasks were thoroughly mixed using magnetic stirrer to dislodge spores and conidia from the mycelia mass. Resulting suspension were filtered using sterile muslin cloth. Talc was mixed with the filtrate which has the dislodged spores and conidia of the entomopathogenic fungi in the ratio 2:1. Carboxymethyl cellulose which functions as an adhesive was weighed and 5 g was added. Resulting mixture was dried in a shade under aseptic conditions for a period of 72 h (Senthilraja et al., 2010). The clumps were powdered and stored in polypropylene bags. The total viable count of spores inside the freshly prepared and the dried finished talc based product was calculated by plating the finished product on suitable media to determine the loss in viability.

RESULTS

Isolation and identification of Fungi from diseased termite

Fungi isolated from diseased termites include *Rhizopus stolonifer, Aspergillus flavus, M. anisopliae, Penicillium* sp., *Aspergillus niger.* All of these are listed in Table 1.

Infection of termite with fungal isolates

A total of six fungi were used in the infection of termites in this study. They include *Articulospora inflata, Rhizopus sp, A. flavus, M. anisopliae, Penicillium* sp. and *A. niger.* Out of all of these, only *M. anisopliae* showed marked lethal signs on the test insects used. This is represented in the Table 2.

Determination of the minimum lethal concentration (LD_{50}) of *M. anisopliae* required for pathogenicity in termites

The minimum lethal concentration (LD_{50}) of *M. anisopliae* needed for pathogenicity was determined in termites through the selection of the lowest concentration of the organism capable of causing death in at least 50 percent of the insects. The probit analysis was carried out and the logarithmic value for the LD_{50} is 1.22. The antilogarithmic value is 12.2 and the corresponding concentration falls on 5.0×10^5 sfu/ml. Other lower concentrations were unable to give clear signs of pathogenicity. This is represented in Figure 1 where the logarithm mortality is plotted against the probit values.

Growth and mass production of *M. anisopliae* on organic by product

Mass production of the *M. anisopliae* on the solidified and liquid organic by product shows that the growth of *M. anisopliae* was greatly supported by the spent brewer's mash and rice water. Slight growth was also noticed on maize shaft. Growth on the broth by product was estimated to be 5×10^6, 8×10^4, 7×10^7 and 6×10^8 sfu/ml respectively for parboiled rice water, maize shaft, spent brewer's mash and standard media (Potato dextrose broth) respectively (Table 3).

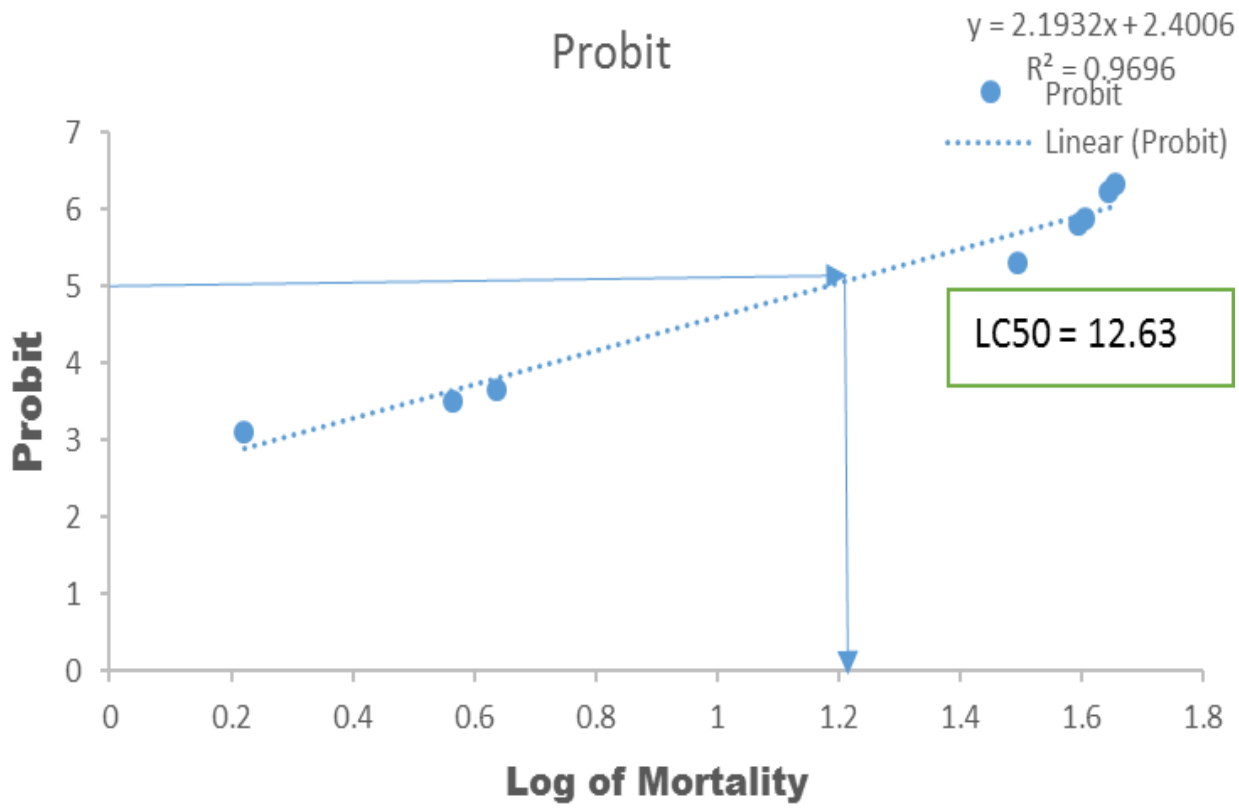

Figure 1. Determination of the LD_{50} of *M. anisopliae* require for pathogenicity in termites.

Table 2. Infection of termites with isolated fungi.

Organism used for infection	No. of deaths (Day 1)	No. of deaths (Day 2)	No. of deaths (Day3)	No. of deaths (Day 4)	No. of deaths (Day 5)
A. inflata	0±0.00[a]	0.33±0.58[a]	0.67±0.58[a]	0.67±0.58[a]	1.33±0.58[a]
A. flavus	0±0.00[a]	0.67±0.58[a]	1.33±0.58[a]	1.33±0.58[a]	1.33±0.58[a]
A. niger	0±0.00[a]	1.33±0.58[a]	2.67±0.58[b]	3.67±0.58[b]	3.33±0.58[b]
M. anisopliae	0.67±0.58[b]	5.67±0.58[b]	7.33±0.58[c]	13.33±0.58[c]	17.67±0.58[c]
Penicillium sp.	0±0.00[a]	0.33±0.58[a]	1.00±0.00[a]	1.00±0.00[a]	1.33±0.58[a]
Rhizopus sp.	0±0.00[a]	0.33±0.58[a]	0.67±0.58[a]	0.67±0.58[a]	1.33±0.58[a]
Control	0±0.00[a]	0.33±0.58[a]	0.33±0.58[a]	0.67±0.58[a]	1.00±0.00[a]

Mean ± SD in the same row with homogenous superscript are not significantly different (p>0.05).

Table 3. Total viable conidial count of fungi in formulated and standard broth media.

Organism	Broth used			
	Maize (sfu/ml)	Mash (sfu/ml)	Rice (sfu/ml)	Standard (sfu/ml)
M. anisopliae	8×10^4	7×10^7	5×10^6	6×10^8

Rice water		Maize shaft		Brewer's mash		Standard media (Potato dextrose broth)	
Initial Conc.	Final Conc	Initial Conc.	Final Conc	Initial Conc.	Final Conc.	Initial Conc.	Final Conc
6×10^5	9×10^4	7×10^4	9×10^4	8×10^6	5×10^6	5×10^8	4×10^8

Preparation of talc-based formulation of entomopathogenic fungi

The initial concentration of the talc formulation containing the entomopathogenic fungi is 6×10^5, 7×10^4, 8×10^6, 5×10^8 sfu/g in the glass jar respectively for rice water, maize shaft, brewer's mash and the standard media (potato dextrose broth). After the talc based formulation which took 120 h, there was a slight reduction in the concentration of the organism. Final concentration reads 9×10^4, 5×10^4, 5×10^6, 4×10^8 sfu/g in the glass jar respectively for rice water, maize shaft, brewer's mash and the standard media (potato dextrose broth) (Table 3).

DISCUSSION

From this study, deductions can be made that *M. anisopliae* isolated from the diseased *M. bellicosus* possess the ability of causing infection and eventual death in termites by contact under laboratory conditions. Fungal entomopathogens like *M. anisopliae* has been established as parasites and pathogens of many insect species in nature (Herlinda, 2010). Many literatures reported the presence of *Metarhizium* sp. and other entomopathogenic fungi on over two hundred insect hosts belonging to many orders in various habitats (Eilenberg et al., 2007). Previous researches in different locations has also reported *M. anisopliae* to be effective in the suppression of soil borne pests like termites, crickets, locusts, brown plant hopper in rice, pyrilla, spittle bug in sugarcane and root grubs. They are regarded as natural insect regulators (Blackwell, 2010), they are known to exhibit high virulence to some insects species and have been documented to manifest in terms of their outgrowths on the insect cuticles and subsequent mummification of such affected insects cadavers (Mohammadbeigi and Port, 2013).

The lethal activity of *M. anisopliae* on *M. bellicosus* as described in this study can be attributed to the ability of most strains of the organism to produce enzymes such as proteases, chitinases and lipases in addition to chemical toxins such as destruxin, bavericin, and efrapeptins (Herlinda, 2010). These enzymes can degrade insect cuticle through a combination of mechanical force and enzymic degradation and after the breakage of the insect cuticle, the organism eventually penetrates the insect hemolymph thus lead to their sickness and eventual death (Charnley, 2003).

Previous researches had also submitted that *M. anisopliae* is effective in the suppression of agricultural pests (Balogun and Fagade, 2004). Occurrence of plant association has also been documented for *M. anisopliae* (Hu and St. Leger, 2002) and this unique association occurs below ground in the rhizosphere which possibly explains the association of the organism with termites used in this study since termites mostly reside in their caste which usually occupies larger areas below the ground surface (Bais et al., 2006). *M. anisopliae* has equally been reported in various soils from widely differing climatic zones and have entomopathogenic activity against a range of insect pests (McCoy et al., 1988). Synergism between *M. anisopliae* and other entomopathogens have also been recorded on both laboratory and field studies using biopesticides based on fungi-bacteria (Bt) formulation against the stem borer and leaf folder of rice (Shahid et al., 2003). This was documented to have reduced the population of these pests effectively both in laboratory and in the field compared to when biopesticides based on one pathogen is being used.

This study suggests that water from parboiled rice, brewer's mash and maize shaft may be able to serve as a utilizable carbon source and support the growth of *M. anisopliae.* Similar studies on "the Mass Production, Yield, Quality, Formulation and Efficacy of Entomopathogenic *M. anisopliae*" also showed that rice grains is able to support the growth of the fungi (Ibrahim et al., 2015). Fogal et al. (1986) also described a simple mass production technique for *M. anisopliae* using growth media such as wheat bran, sorghum grains, boiled rice grains, coffee husk and coconut water. Findings from recent studies also prove that the brewer's mash can serve as a media for the growth of other fungal entomopathogens. Brewer's mash has also been used for the growth of *B. bassiana* (Masoud et al., 2013).

Talc-based formulation of *M. anisopliae* prepared during this study shows that there were reduction in the active conidia and spores after the formulation was dried. The reduction in *M. anisopliae* shows that drying the conidia of *Metarhizium* results to loss in viability. It shows *Metarhizium* conidia might require slower drying, 5–9 days, (Hong et al., 2000; Jaronski and Jackson, 2012) compared to other entomopathogenic fungi that has been dried using the method employed in this study.

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