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Biosafety of *Beauveria bassiana* as a biopesticide: no effects on sex hormones in experimental rats

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Abstract

Background: Although biological control is regarded as a safer and more sustainable alternative to chemical pesticides for insect pest management, the effect of biocontrol agents on the endocrine system of non-target organisms remain inadequately characterized. This study investigates the hormonal responses of Wistar rats as a non-target mammalian model following exposure to the entomopathogenic fungus Beauveria bassiana.

Materials and methods: The fungus was isolated from diseased variegated grasshopper. Veen's media was used to isolate the entomopathogenic fungus from the insect cadavers. Spore suspensions of the fungus were prepared used to infect another batch of the insect to confirm entomopathogenicity. The inoculum was standardized and injected into experimental rats intraperitoneally. Rats were observed for a period of 7days before being sacrificed. Blood was collected into heparin tube through ocular puncture and analysed for selected reproductive hormones. The rats were examined to ascertain they are in uniform reproductive cycle to minimize the spiking or reduction in hormonal concentration.

Results: Results showed a slight change in some hormonal levels in challenged rats compared to rats within the control group. Progesterone levels were 6.67±1.16a ng/mL and 6.33±0.58a ng/mL in rats within the test and control groups respectively. Testosterone levels were 3.40±0.20a ng/mL and 3.50±0.17a ng/mL respectively in the test and control groups. Follicle Stimulating Hormone level were 2.00±0.10a mIU/mL and 1.93±0.12a mIU/mL while Lieutenizing Hormone were 16.40±0.10a and 16.43±0.06a in rats within the test and control groups respectively. Despite the reduction, the values were still within the acceptable range with the exception of testosterone.

Conclusion: The study showed that usage of B. bassiana as an entomopathogenic fungus in the formulation of biopesticides may pose less risk to the handler and the environment upon its deliberate release or accidental exposure

Keywords: entomopathogens, biopesticides, FSH, LH, progesterone, testosterone

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Introduction

Microbial toxicology is a scientific discipline involving the study of structure and mechanism related to the toxic effects of microbial agents and the toxins they produce [1, 2]. It is a specialized branch of toxicology that focuses on the interactions between microorganisms and toxins. It involves the study of how microorganisms produce toxins, how these toxins affect human health and the environment, and how microbial activity can influence the toxicity of various substances. This discipline encompasses technology advances in research related to microbiological aspects of toxicology. Microbial toxins are toxins produced by microorganisms, including bacteria, algae, viruses and fungi. They can be endotoxins which are produced as components of the outer membrane which are released when bacteria die and their cell walls break apart. They can also be exotoxins secreted by bacteria during microbial growth and metabolism [3]. Microbial toxins are important virulence determinants responsible for microbial pathogenicity and/or evasion of the host immune response. These toxins can exert their adverse effects by disrupting cellular membranes, inhibiting protein synthesis, interfering with signal transduction

pathways and triggering immune responses. Microbial toxins can equally have significant effects on the endocrine system, disrupting hormonal balance and function. Some microbial toxins can interfere with the synthesis or secretion of hormones. Toxins can also bind to hormone receptors, either mimicking the hormone's action (agonist effect) or blocking the hormone's action (antagonist effect). Some toxins interfere with intracellular signalling pathways that are activated by hormones, leading to inappropriate cellular responses [4].

Before any drug, substance or agent can be certified for usage, there must be toxicological analysis of its effects upon its use so as not to exert adverse effects on the human population and its environment [5]. While a lot of work has been carried out on the toxicological potentials of medically important microbial strains, not a lot has been done on environmental strains due to the assumption that environmental strains usually use in biological control are largely benign [6-10]. Once an organism is released into the environment, its behaviour can no longer be controlled. Thus, a previously benign organism can become pathogenic after release. Even the few toxicological work carried out largely involve the evaluation of the histopathological and haematological parameters. There is a less common work been done on hormonal evaluation. Biocontrol agents like *B. bassiana* are usually considered as benign. However, certain strains of the fungus is known to produce a variety of toxic secondary metabolites, including beauvericin, bassianolide, and tenellin, which have cytotoxic and ionophoric properties. Beauvericin in particular can disrupt calcium homeostasis in mammalian cells, leading to apoptosis and cellular dysfunction. Such disruptions can affect gonadal cells thus impairing the synthesis of sex steroids like testosterone, estrogen and progesterone [11]. In order to prevent a potential plague especially in the case of *B. bassiana*, the toxicological effects have to be confirmed before its usage can be encouraged.

Methods

Collection of *Z. variegatus*

Z. variegatus were sourced from cassava Farm using sweep nets [12]. They were transferred to the laboratory, provided with fresh water leaves and sterile water allowed to acclimatize and watched for the onset of disease symptoms. Individuals exhibiting symptoms such as lethargy, colour change, abnormal outgrowths and reduced feeding rate were separated from the population for maceration and subsequent isolation of microorganisms [13-16].

Isolation of *B. bassiana* from insect cadavers.

Cadavers of *Z. variegatus* were removed from the cages and surfaced sterilized by generously rinsing in 5 percent sodium hypochlorite, followed by 75 percent ethanol. Cadavers were then further rinsed in plenty of sterile distilled water. The cadavers were then left to dry out naturally for 48 hours [13], transferred to desiccators for humid incubation at room temperature as described by Luz and Farques[17]. Sporulating cadavers as shown in Figure 1 were regarded as being positive for the growth of *B. bassiana*. The sporulating fungi on cadavers were inoculated onto Veen's medium for the isolation of *B. bassiana* and incubated at 25 °C for 72 hours [18]. Veen's medium was prepared by dissolving 5 g of Peptone, 10 g of glucose and 6 g of agar into 500 ml of distilled water. The pH was adjusted to 6.3 with 1M of HCL and then autoclaved for 20 minutes at 120 °C. The medium was allowed to cool to 60°C after which 0.5ml of streptomycin, 0.5ml of tetracycline, 0.5 ml of dodine and 2.5 ml of cyclohexamide were added [19].

Figure 1. The growth of *B. bassiana* on *Z. variegatus* cadaver.

Further identification of sporulating fungi

Two drops of cotton blue lactophenol were placed on a clean grease-free microscopic slide and a small piece of mycelium from the Veen's media was removed with sterile inoculating needle, transferred onto the stain on the slide and covered with clean slip as described by Fawole and Oso [20]. The identification of molds were done by comparisons of the observed morphological characteristics beneath the microscope in accordance with standard methods.

Confirmation of entomopathogenicity of *B. bassiana*

B. bassiana was inoculated onto fresh Potato Dextrose Agar (PDA) plates and incubated at 27 °C for 14 days for sporulation to take place. Spores and conidia were harvested from these plates with a 0.1 percent Tween 80 solution. Conidia stock suspensions were stored at 4 °C until being used. The spores suspensions obtained were dispensed into plastic aspirators. New batch of apparently healthy grasshoppers *Z. variegatus* were infected with the suspension. Control experiment was set up by spraying separate populations of insects with sterile saline [21-23].

Collection and rearing of wistar rat for hormonal analysis

Male rats were used for testosterone analysis while female rats were used for progesterone, follicle stimulating hormone as well as luteinizing hormones analysis. The parent rats were purchased from the animal house of the Department of Physiology, University of Ibadan, Nigeria. The rats were housed in suitable cages which allow the free flow of air and also contain wood shavings as beddings.

Each sample group contains seven rats each. The rats were bred in the animal house of the University and kept under standard conditions in a well-ventilated room at temperature of 26.0 ± 2.0°C. Rats experienced conditions of 12 hours light/dark cycle for 5weeks and fed with a standard rodent pellet and water. Pellets were purchased from certified Top feed mills outlet in Ondo City. After the acclimatization period the rats were used for the experimental work. All experiments were carried out in accordance with guidelines of Experimental Animal Ethic Committee, refrigerated to preserve the integrity of cells and prevent enzyme denaturation [24].

Preparation of spore suspensions for the infection of wistar rats

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 with 0.1 percent Tween 80 solution and sterile glass rods. The number of active spore and conidia was determined and adjusted for subsequent inoculation into the experimental animals [25].

Inoculation of *B. bassiana* into albino rat

Laboratory animals were injected with 1ml of the prepared microbial suspension after being adjusted to 106 sfu/ml using a spectrophotometer. The pure cells and conidia of fungi were injected into each rat intraperitoneally using the 1ml syringe [26]. Rats were watched for seven days before being sacrificed and their blood harvested for hormonal analysis

Collection of blood from infected wistar rat

The albino rats were made to fast overnight and sacrificed after seven days. The rats were put into air-tight jar containing diethyl ether and were slightly anaesthetized. Blood samples were collected from the overnight fasted rats under anaesthesia via tail and ocular vein puncture using heparin bottle immersed in ice-cold water. The clot was removed by centrifuging at 4000 rpm for 15 minutes; the resulting supernatant which was the serum was collected into plain tubes and labelled accordingly for hormonal test [27].

Hormonal assay on infected animals

Testosterone

The serum testosterone concentration was quantitatively determined using the direct human testosterone enzyme immunoassay (EIA) kit as described by the manufacturer's protocol which adopted the principle of Tietz[28] with modifications from Nnamah et al. [29]. The testosterone EIA is based on the principle of competitive binding between testosterone HRP conjugate for a constant amount of rabbit anti-testosterone.

The number of desired coated wells in the holder was secured. 10 µl of standards, specimen and controls were dispensed into appropriate wells. 100 µl of testosterone-HRP conjugate reagent was dispensed to each well and mixed thoroughly for 30 seconds and incubated at 37°C for 90 minutes for the standards, specimen and control. The microwells were rinsed and flicked for 5 minutes with washing buffer. 100 µl of 3,3',5,5'-Tetramethylbenzidine (TMB) reagent was dispensed into each well, mixed gently for 5 seconds and incubated at room temperature for 20 minutes inside a dark chamber. The stop solution is added to terminate enzymatic reactions and stabilize color development allowing for accurate measurement. The blue colour completely turned to yellow and the absorbance was read at wavelength of 450 nm with a micro titre well reader within 15 minutes of the preparation.

Follicle stimulating hormone

The serum FSH was quantitatively determined using the direct human serum follicle stimulating enzyme immunoassay (EIA) kit as described in the manufacturer's protocol, which adopted the principle of Tietz[28].

Microplate wells for each serum reference, control and samples to be assayed were in duplicate. 25 µl of each calibrators, control serum and samples were pipetted into appropriate wells. 100 µl conjugate was pipetted into each well for control and sample, except blank and incubated on a thermoshaker for 30 minutes at 37°C. The wells were washed 5 times with 300 µl of working washing solution per well and tapped firmly against absorbance paper to ensure that it is dry. 100 µl of TMB substrate was pipetted into each well at timed interval and incubated for 30 minutes at room temperature in a dark place. 150 µl of stopping reagent was pipetted into each well and mixed gently for 5-10 seconds. The plate was read on microplate reader at 450 nm within 20 minutes after.

Luteinizing hormone

The serum LH was quantitatively determined using the direct human serum luteinizing enzyme immunoassay (EIA) kit as described in the manufacturer's protocol, which adopted the principle of Tietz [28].

Microplate wells for each serum reference, control and samples to be assayed were in duplicate. 25 µl of each calibrators, control serum and samples were pipetted into appropriate wells. 100 µl conjugate was pipetted into each well for control and sample serum, except blank and incubated on a thermoshaker for 30 minutes at 37°C. The wells were washed 5 times with 300 µl of working washing solution per well and tapped firmly against absorbance paper to ensure that it is dry. 100 µl TMB substrate was pipetted into each well at timed interval and incubated for 30 minutes at room temperature in a dark place. 150 µl of stopping reagent was pipette into each well and mixed gently for 5-10 seconds, the plate was read on microplate reader at 450 nm within 20 minutes after addition of the stopping reagent.

Progesterone

The serum progesterone was quantitatively determined using microplate immunoenzymometric assay kit as described in the manufacturer's protocol, which adopted the principle of Tietz [28]. To 0.025 ml of each calibrator, control and serum samples were pipetted into microplate wells. 0.10 ml of conjugate was pipetted into each well and the micro-plate was swirled gently for 20-30 seconds to mix and incubated for 60 minutes at room temperature, the content of the micro-plate was decanted and 0.30 ml

washing solution was added repeatedly four times. 0.10 ml of TMB Substrate was added and incubated for 25 minutes at room temperature in a dark place. 0.15 ml of stopping reagent was pipetted into each well. The plate was read on microplate reader at 450 nm within 20 minutes after.

Statistical Analysis

The data obtained from the hormonal assays were subjected to statistical analysis to compare the means of groups as well as the standard deviation. T-Test was applied to compare the test and control groups. $P < 0.05$ was considered to be significant

Results

Isolation of *B. bassiana* from *Z. variegatus*

B. bassiana was isolated from the cadavers of the insects using the Veen's media. The fungus has a whitish muscadine growth when re-cultured on SDA. It was kept on slants for further analysis while a new subculture was prepared to incubate for 14 days for sporulation and subsequent harvest to take place. The microscopic feature of the fungus is described in Table 1.

Hormonal assay on infected animals

Rats injected with *B. bassiana* showed physical and stress-related symptoms including slightly hunched posture, raised fur and reduction in the rate of feeding. The Hormonal assay as represented in Table 2, generally showed a slight reduction in the level of the hormones assayed for in infected rats group compared to the rats in the control group with the exception of the follicle stimulating hormone which was hardly affected in rats infected with *B. bassiana*. The highest difference was noticed in the progesterone level which reduced in the rats within the test group compared to those in the control group.

Discussion

The isolation of *B. bassiana* from *Z. variegatus* as demonstrated in this study further affirmed that Beauveria bassiana is a well-known entomopathogenic fungus that infects a wide range of insect hosts. The grasshopper species used in this study is a significant agricultural pest in Africa, causing extensive damage to crops [30]. The use of *B. bassiana* as a biological control agent has been studied due to its ability to naturally infect and kill *Z. variegatus* thus decimating the numbers of insect pest that may destroy crops and cause significant damage to food crops.

The infection of *Z. variegatus* by spraying the insects with the spore suspensions of Beauveria bassiana also supports the submissions by Balogun and Fagade[31] that the entomopathogenic fungus infects the insect through the contact of the chitinous cuticle with the conidia (spores) of the fungus. The spores adhere to the cuticle of the grasshopper, germinate, and penetrate the insect's body, eventually leading to its death. The fungus proliferates within the host and exhibits its presence by producing a whitish muscardine growth as noticed on the grasshopper cadavers (plate 1) used in this study thus producing toxins and disrupting physiological processes (Goettel and Inglis[21]).

Table 2. Effect of entomopathogens on hormonal levels in experimental rats

Hormone	*B. bassiana*	Control
Progesterone (ng/mL)	6.67±1.16a	6.33±0.58a
Testosterone (ng/mL)	3.40±0.20a	3.50±0.17a
Luteinizing (ng/mL)	16.40±0.10a	16.43±0.06a
Follicle Stimulating (mIU/mL)	2.00±0.10a	1.93±0.12a
Active Straight Leg Raise	1.96±0.73	2.39±0.59*

The virulence of *B. bassiana* on *Z. variegatus* can be attributed to its proven ability to produce proteases, chitinases, and lipases. The insect cuticle contains a waxy layer composed mainly of lipids, which acts as a hydrophobic barrier preventing water loss and pathogen entry. All of these are enzymes that can work in synergy to degrade insect cuticle thereby providing an entry point for the fungal hyphae which eventually lead to their sicknesses and subsequent death [32]. Aside this, *B. bassiana* has also been shown to degrade the chitin covering of insects facilitated by a combination of mechanical force and enzymic degradation [33].

Behavioural signs exhibited by rats inoculated with B. bassiana including the hunched posture, piloerection and reduction in food intake reflects the progression of infection as well as the immune response of the host [34].

Although the Hormonal assay which showed a difference in progesterone levels in rats challenged with *B. bassiana* and unchallenged rats. However, the change in progesterone level is still within the acceptable range for female rats in the estrus phase. A slight drop was only noticed in the values of Luteinizing hormone in both test and control rats. The decrease in the values of luteinizing hormone is also still within the normal range of female rats within the estrus phase while the slight change in follicle stimulating hormones is well within the normal range for female rats within the puberty age range. A reduction in the testosterone level of rats challenged with *B. bassiana* is only slightly below the baseline level for male rats that are not

Table 1. Examination of B. bassiana under the microscope

Isolate	Cultural characteristics	Microscopic examination	Suspected Organism
VM1	Powdery mycelia which is whitish to pale yellow.	Conidia are hyaline, short and globose or ovoid in shape Conidiogenous cells are flask-shaped, rachiform, proliferating and aggregating into sporodochia.	*Beauveria bassiana*

engaged in sexual activities.

Since progesterone is a steroid hormone (hormone of pregnancy) [35] which plays an important role in the preparation for and maintenance of pregnancy [36], the likely hood of the occurrence of gestational anomaly in the administration and usage of *B. bassiana* may be unlikely since the values obtained in the test and control rats group are still within the acceptable rate.

Hormonal assay showed that testosterone level was slightly reduced in rats challenged with B. bassiana below the baseline values. Since testosterone is involved in health and general well-being and the prevention of osteoporosis [37], insufficient levels of testosterone may arise from the repeated accidental exposure to these microorganisms when used as biopesticides. However, testosterone reduction can be supplemented in such cases through the usage of testosterone boosting therapeutics.

Levels of follicle stimulating hormone was relatively higher to what was obtained in the control rat group but both values are still within the acceptable range. This enzyme is needed to regulate follicular growth and maturation of ovaries, trigger ovulation in females [38]. There is a reduced likelihood that subsequent use and exposure of females to the entomopathogenic strain may affect or prevent the maturation of the ovaries and even affect subsequent implantation of a foetus after conception

LH is a crucial gonadotropin produced by the anterior pituitary gland which plays a significant role in the regulation of reproductive functions. Impaired LH production may lead to a decrease in testosterone synthesis thus resulting to reduced libido, delayed ovulation, erectile dysfunction, and infertility. Insufficient LH during adolescence can also delay the development of secondary sexual characteristics [38]. Although the LH levels within rat in the test group is slightly lower compared to those within the control group, it is unlikely that the reduction will result to a major impairment of the reproductive functions because the reduction falls within acceptable LH levels within the rats.

Conclusion

This study shows that the exposure of wistar rats to B. *bassiana* within a 7-day period has no major effects on their reproductive hormones thus highlighting and further emphasizing the safety of these agents when employed as biocontrol agents. These results is a good indication that it might be safe to integrate these agents in pest management practices without adversely affecting non-target mammalian species, including humans. However, further research especially on long-term studies, varied environments and extended exposure are necessary to better understand the broader impacts of these agents on reproductive health so as to further affirm their safety across different environmental and biological contexts.

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