Journal of Advances in Microbiology

4(4): 1-9, 2017; Article no.JAMB.34610
ISSN: 2456-7116

Yield of *Metarhizium anisopliae* Conidia on Four Tropical Cereals and *in vitro* Evaluation of Virulence against Cowpea Weevil, *Callosobruchus maculatus* (Fabricius) (Coleoptera: Chrisomelidae)

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Authors' contributions

This work was carried out in collaboration between all authors. Author OAB designed the study, performed the statistical analysis, wrote the protocol, and the first draft of the manuscript. Authors AAO and MJF managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JAMB/2017/34610
Editor(s):
(1) Pongsak Rattanachaikunsopon, Department of Biological Science, Faculty of Science, Ubon Ratchathani University, Thailand.
Reviewers:
(1) Ali Sevim, Ahi Evran University, Turkey.
(2) Ana-Maria Andrei, Research Development Institute for Plant Protection, Romania.
(3) Aba-Toumnou Lucie, University of Bangui, Central African Republic.
Complete Peer review History: http://www.sciencedomain.org/review-history/20381

Original Research Article

Received 1st June 2017
Accepted 18th July 2017
Published 5th August 2017

ABSTRACT

Aims: To evaluate conidia yield of entomopathogenic fungus, *Metarhizium anisopliae* Net 275.86DC on different tropical cereal substrates and evaluate its virulence against the bruchid weevil, *Callosobruchus maculatus in vitro*.
Methodology: The fungus was cultured on Sabouraud Dextrose Agar (SDA) and incubated at ambient temperature (mean daily temperature=30°C) for 14 days. Radial extension was measured daily along pre-marked orthogonal axes and used to estimate growth rate. Conidia from 14 days SDA culture were scraped into 10 mL Reverse Osmosis (RO) water containing 0.02 µl of surfactant, Tween 80. Ten gramms sub-samples of cereal grains; maize, rice, sorghum and millet were washed and soaked in RO water overnight at 4°C. Thereafter, they were transferred into 250 mL glass jars with micro-porous lid in triplicates and autoclaved at 121°C and 15 psi for 30 minutes. The jars were left for 2-3 hours to cool and the water activity (a_w) of the substrates were determined, followed by

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inoculation with 1 µl of 1.0 x 10^4 conidia mL^{-1} of prepared seed inoculum. Incubation was done in the dark for 14 days in equilibrated environment (ERH>98%). Conidia were harvested by pouring RO water containing 0.02-0.05% Tween 80 unto the substrates in the jars followed by vortexing. Pure conidia were recovered by vacuum filtration and concentrated into 10 mL suspension using a centrifuge. Conidia concentration (conidia mL^{-1}=Yield) was determined using improved Neubauer haemocytometer under x40 objective of light microscope. Three concentrations, 1.0 x 10^5, 1.0 x10^6, 3.9 x10^6 conidia mL^{-1}were tested for virulence against *C. maculatus* using *in vitro* bioassay system with adult insects and the median lethal time, LT_{50} of the fungus was determined.

Results: The growth rate on SDA media was 2 mm day^{-1} and the yield on millet was significantly the highest, 5.53 x 10^6 conidia mL^{-1}. Conidia concentrations from maize, sorghum and rice substrates were 4.73 x 10^5, 2.8 x 10^5 and 1.3 x 10^5 conidia mL^{-1} respectively. The yields from maize and sorghum were not significantly ($P>0.05$) different. The fungus was virulent against C. maculatus, LT_{50}=1.6 days at 3.9 x10^6 conidia mL^{-1}.

Conclusion: Some of the tropical cereals could be adopted for mass production of virulent conidia of the entomopathogenic fungi at relatively low cost of production.

Keywords: Cereals; entomopathogenic fungus; bioassay; virulence; conidia yield.

1. INTRODUCTION

Cowpea, *Vigna unguiculata* is considered the most important of all indigenous African legumes. The edible seeds represent a cheap alternative source of dietary protein, minerals and vitamins, thus occupying a prominent position in the nutrition of Nigerians [1,2] Annually, significant quantitative and qualitative losses of cowpea due to entomological pest attacks are incurred in the field, after harvest and during storage., *Callosobruchus maculatus*- a field-to-store pest, with pestiferous larvae and adult, is capable of causing 100% damage of seeds where control measures are not adequately applied [3], and has been recognized as a major threat to food security in in Nigeria [4]. Apart from direct feeding and contamination of seeds with frass, excrement, larvae and pupae cocoons, their integument has been reported to contain carcinogenic methoxyl-quinines, which cannot be denatured by boiling [5].

Control of *C. maculatus* in large scale post-harvest crop processing chain and storage largely relies on synthetic chemical pesticides, the use of which are of significant environmental concerns [6]. Repeated and indiscriminate use of chemical pesticides have been reported as one of the causes of development of resistant strains of pest species [7]. Unfortunately, strains of *C. maculatus* which showed moderate to strong resistance to some of the registered and commonly used pesticides have been found in Nigeria [8]. In addition, pest problems may be severer in most agro-ecological regions in Sub-Saharan Africa which have been predicted as potential hot-spots for climate change [9,10]. While climate change could have serious effects on agriculture as a result of changes in rainfall patterns, pest and disease development and shortage of water for irrigation, the impact of agrochemicals on global warming is significant [11]. In view of these, we suggest the need for development of environment friendly Integrated Pest Management (IPM) approaches for cowpea, through adoption of new and sustainable biocontrol innovations such as the use of fungal biocontrol agents, which are capable of reducing dependence on chemical insecticides.

Use of entomopathogenic fungi for pest control is considered as one of the available safe options from ecological standpoint. Anamorphs of Hypocreales, especially *Beauveria bassiana*, *Metarhizium anisopliae and Isaria farinosa* have been tested against important horticultural pests in several studies [12-14] and some have been developed into commercially available biocontrol agents (BCAs) [15]. The strain of *M.anisopliae* (275.86DC) being reported in this study had previously been cultured on Sabouraud Dextrose Agar (SDA) and tested against the tomato whitefly, *Bemisia tabaci* under field conditions in Southwestern Nigeria [13]. It is also capable of establishing as an endophyte in sorghum roots when applied to the rhizosphere of seedlings [16].

Large quantities of stable and infective conidia are required for development of entomopathogenic fungi into commercially available BCAs. At industrial scale, conidia are produced using solid substrate fermentation (SSF) systems in bioreactors, which are designed for 'low volume and high value' expensive products [17], biopesticides may therefore be unaffordable by farmers in low

income countries. To create access of farmers to biocontrol products, high quality propagules which are stable and infective under ambient conditions must be amenable to large scale production using inexpensive local substrates. The aims of this study therefore, are to: (a) compare yield of conidia of *M. anisopliae* on relatively cheap tropical cereals and (b) test virulence of conidia against *C. maculatus in vitro.*

2. MATERIALS AND METHODS

2.1 Study Area and Source of Experimental Materials

The study was carried out in the Crop Protection Laboratory Unit, Ekiti State University Nigeria (7°31N and 5°13E). The entomopathogenic fungus, *M. anisopliae* (Net 275.86 DC) was originally isolated from insect and generously provided by Professor Tariq. Butt, Swansea University, UK. Indigenous cowpea landrace (Ife Brown variety) was supplied by the Ekiti State University Teaching and Research Farm. Initial *C. maculatus* stock were sieved from infested cowpea purchased from open market stocks in Nigeria and subsequently reared on cowpea in the laboratory.

2.2 Rearing of *C. maculatus*

Batches of 20 unsexed adult insects were introduced into transparent rectangular plastic box (L x B x H; 12 x 10 x 8 cm^3) containing 100 g of cowpea and covered with muslin held in place using a rubber band. The box was replicated three times and arranged into laboratory cupboards (30 x 25 x15 cm^3) alongside a beaker containing 1 litre of sterile distilled water, to equilibrate the environment to >98% Equilibrium Relative Humidity (ERH) at 25°C. This was done to provide a relatively high humidity environment in favour of insect oviposition and subsequent reproduction. After 3 days, the initial stock of adult insects were sieved out and the cowpea which contained eggs of the insects were left for 2 weeks or until emergence of adults.

2.3 Fungal Culture and Preparation of Seed Conidia

One centimeter Agar plugs of actively growing culture of *M. anisopliae* was transferred into standard sterile Sabouraud Dextrose Agar media (Sigma-Aldrich, SDA, 0.995 a_w) in 9 cm Petridishes. Triplicate plates were inoculated and incubated at ambient temperature inside a sealable polythene bag. Conidia from 3 weeks old cultures were gently brushed off into Universal bottles using Camel brush. Ten mLs sterile de-ionized water containing 0.02% Tween 80 as a surfactant was added to the conidia and vortexed for one minute. The conidia suspension was serially diluted and standardized to1.0x 10^7 conidia mL^{-1} [18].

2.4 Measurement of Fungal Growth Rate at Ambient Temperature

Prepared sterile SDA plates were inoculated at the center with 5 µl of conidia suspension containing 1.0 x 10^7 conidia mL^{-1}using Micropipette (Eppendorf, 2-20 µl). The plates were sealed with Parafilm to prevent moisture loss from the agar surface and incubated at ambient temperature in the dark. After 24 hours, colony diameter was measured along pre-marked perpendicular lines from the center of the dish and this continued daily for 14 days or until the surface of the plate was fully covered. Radial extension values (mm) against the period of growth (days) were fitted into a linear model to estimate growth rate [13,18].

2.5 Production of *M. anisopliae* Conidia on Cereals

Twenty five mL sterile distilled water was added to 10 g subsamples of 4 different cracked cereal grains (Igbemo rice-Nigerian local landrace, maize, sorghum and millet) in 150 mL glass jars with micro-porous lids and maintained at 4°C for 24 hours. Excess water that was not imbibed by the grains was decanted and the jars were autoclaved at 121°C and 15 psi for 30 minutes. The sterile cereal substrates were allowed to cool for 2-3 hours before they were inoculated with 1 µl of 10^4 conidia mL^{-1} of prepared seed inoculum. The jars were separately arranged inside transparent plastic boxes (50 x 30 x 20 cm^3) which was equilibrated using 500 mL of sterile distilled water. Incubation was done at ambient temperature for 14 days at 12:12 hours alternating light and darkness. Thereafter, 40 mL of water containing 0.05% Tween 80, was added into each jar followed by stirring with glass rod and vortexing for 15 minutes. Conidia suspension was poured into standard bottles and the process of extraction was repeated three times to ensure thorough extraction. Conidia were separated from mycelial contaminants by vacuum filtration and concentrated by centrifugation (1500 rpm for 25 minutes). Estimation of conidia concentration was done

using a haemocytometer under x400 magnification of light microscope.

2.6 Bioassay Procedure

Conidia from 14 days old *M. anisopliae* culture grown on millet were used to assess virulence. Three conidia concentrations; 1 x 10^5, 1 x 10^6 and 3.9 x 10^6 conidia mL^{-1} were prepared through serial dilution using Reverse Osmosis (RO) water containing 0.02% Tween 80. Fifteen centimeter disposable Petri-dishes were modified by cutting 1 cm^2 rectangular ventilation hole on the lid. The hole was covered with muslin cloth which could prevent insects from escaping and held in place with a masking tape. A filter paper was placed inside each Petridish to serve as an absorbent material for excess inoculum suspension. Cowpea pods were dipped into the prepared conidia suspensions and kept in the dish at the rate of 5 pods $dish^{-1}$. Cowpea samples for the control were dipped in RO water containing 0.02% Tween 80, and each treatment was replicated 3 times. Thereafter, 10 unsexed adult *C. maculatus* were introduced into each Petridish. The Petridish-systems were arranged into plastic box (Size = 40 x 30 x 20 cm^3) and equilibrated with 500 mLRO water kept in a glass beaker, to create the humid environment necessary for infection to occur, and cumulative mortality of the insects were recorded daily. The median lethal time, LT_{50} was calculated for each conidia concentration.

2.7 Statistical Analysis

Mortality data was not corrected for natural mortality since there was less than 10% mortality in the control. The LT_{50} was based on quantitative values without statistical comparison. Sporulation data was log transformed (Log_{10} conidia mL^{-1}) and tested for compliance with the assumptions of parametric statistical tests; equality of variance of the means was examined using the Leven's Test and normality of the data was analysed using the Shapiro-Wilks statistical analysis. Thereafter, significant difference in sporulation rates was analysed using the Analysis of Variance (ANOVA) procedure. Where ANOVA showed significant difference, a Post-Hoc test was done to separate the means using Tukey-Kramer's Honestly Significant Difference (HSD). All statistical analysis were performed using the SPSS-21, Statistical package, US.

3. RESULTS

3.1 Growth Rates at Ambient Temperature

Fig. 1 shows the typical graph of radial extension against growth period for estimation growth rate. The regression lines had R^2 values >0.98 and the estimated growth rate was approximately 2.0 mm day^{-1}. The minimum temperature recorded during the period of growth was 28.1°C while the maximum was 31.9°C and the mean daily temperature for the entire period of growth was approximately 30°C. (Fig. 2).

3.2 Yield of Conidia on Cereals

The yields of conidia on the four cereal substrates differed significantly [F (2, 8) = 0.43, P = 0.001]. Conidiation on millet was significantly the highest (concentration = 5.53 x 10^6 mL^{-1}) while the lowest, 1.3 x 10^5 conidia mL^{-1} was obtained from rice. The concentration of conidia extracted from maize and sorghum were 4.73 x 10^5 and 2.8 x 10^5 conidia mL^{-1} respectively and they were not significantly different from each other (Fig. 3).

3.3 Virulence of Fungus against *C. maculates*

Fig. 4 shows mortality trends of *C. maculatus* exposed to three concentrations of *M.anisopliae* conidia. The fungus was virulent against the insect at the three conidia concentrations and the onset of mortality was after 24 hours of exposure. At 5 days, 100% mortality of the insect population occurred in all the treatments compared with <10% mortality in the control. The LT_{50} at 1.0 x 10^5 and 1.0 x 10^6conidia mL^{-1} was 2 days while the LT_{50} was 1.6 days at the highest inoculum concentration,3.9 x 106 (Fig. 5).

4. DISCUSSION

Successful culture of entomopathogenic fungal conidia on cheap and readily available substrates is an important initial requirement in biopesticide production. The fungus, *M.anisopliae* Net 275.86DC colonized and formed conidia on all the cereal substrates differentially at ambient temperature. The growth speed of a fungus determines the rate of colonization of substrate and could potentially reduce contamination by

other saprobes. Conidia yield on millet was significantly the highest, while the local rice produced the lowest. Earlier studies showed significant differences in quantities of conidia produced from different substrates [19]. High quality conidia of *M. anisopliae* have been produced in solid substrate fermentation (SSF) systems using basmati rice [20,21]. In Nigeria, basmati rice is expensive and not readily available. Therefore, this study evaluated the potentials of a readily available local rice and the performance was poor in terms of its ability to support conidiation. However, millet is cheaper than maize, rice and wheat in Nigeria and could easily be used as an alternative substrate for the mass production of conidia.

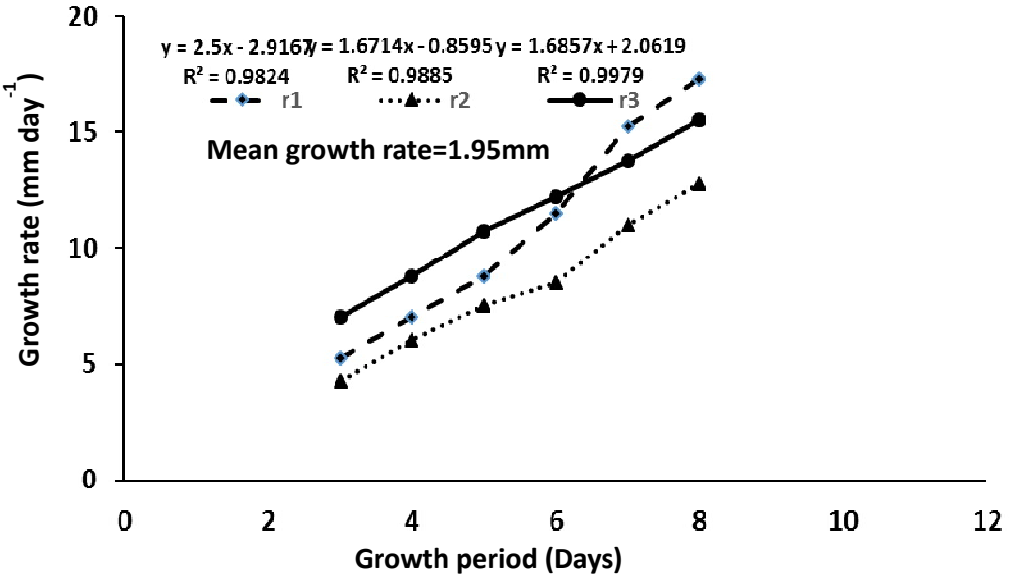

Fig. 1. Growth rate of *M.anisopliae* (Net 275.86DC) at ambient temperature. r1, r2 and r3 are growth curves of replicate cultures

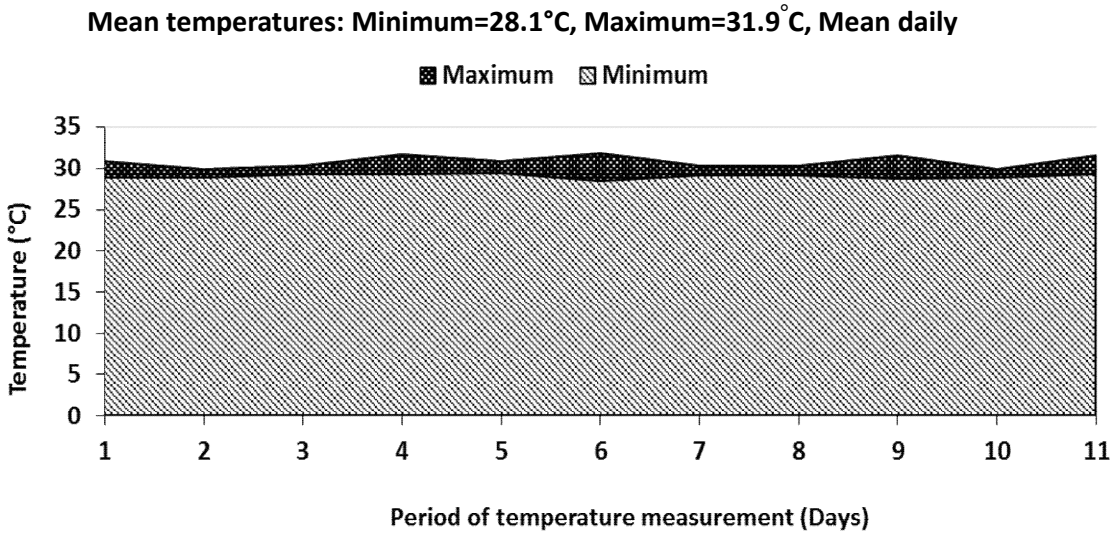

Fig. 2. Variabilities in environmental temperature during the growth of *M. anisopliae*

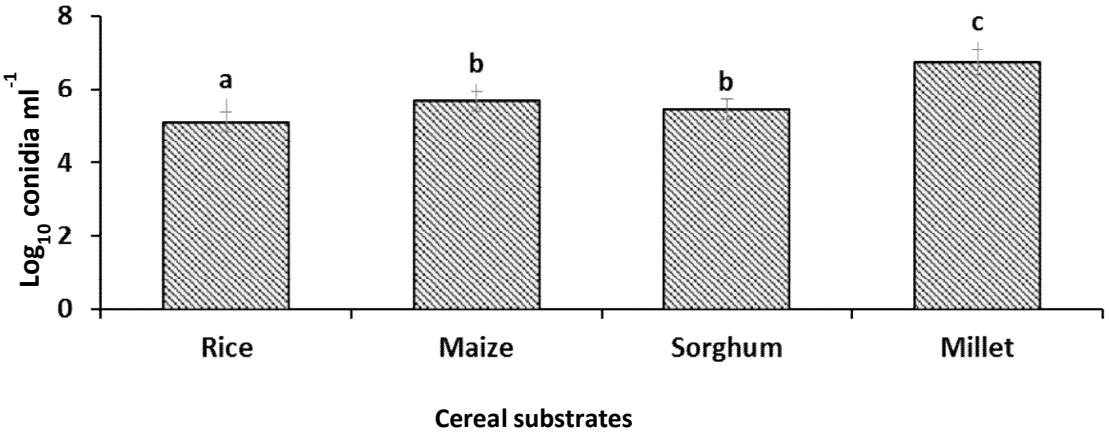

Fig. 3. Yield of *M. anisopliae* conidia on four tropical cereal substrates

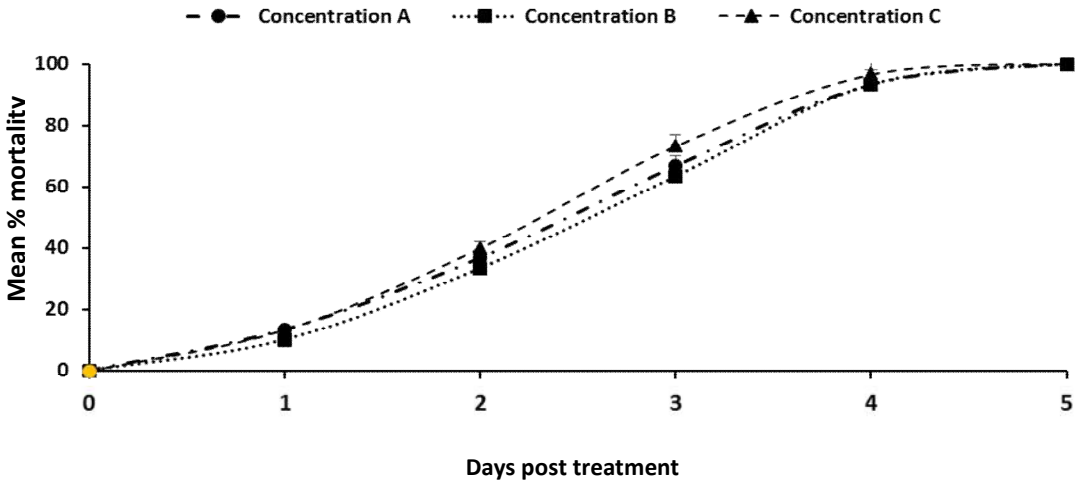

Fig. 4. Mortality kinetics of *C. maculatus* inoculated with different concentrations of *M. anisopliae* conidia

Concentrations: A = 1.0×10^5 conidia mL^{-1}, B = 1.0×10^6 conidia mL^{-1}, C = 3.9×10^6 conidia mL^{-1}

Temperature, water activity (a_w) [18] and the nutrient composition of substrate [22] as well as the length of fermentation and aeration [23] are known to affect conidiation. In this study, it was observed that the structural integrity of the millet was superior to that of rice after autoclaving. It may be possible that the millet permitted better air circulation and gaseous exchange compared to rice and this might have favoured higher conidia yield among other factors. Solid porous matrix with large surface area per unit volume, in the range of 10^3 to 10^6 m^2 per cm^3 are considered suitable for microbial growth. The mechanical properties of the matrix should stand compression and consist of small granular particles which do not tend to stick together [24]. Kinetic evolution of CO_2 and air circulation is an important consideration in SSF [25], however Arzumanov et al. [26] demonstrated that forced aeration is not crucial for sporulation of *M. anisopliae*, as no increase in conidial yield was achieved with increase in aeration. .The conidia were harvested wet using RO water containing the wetting agent, Tween 80 and separated by vacuum filtration through spore filtration cloth, and yield was reported in terms of concentration (Conidia ml^{-1}). This method represents the most realistic method of determination of yield of conidia from solid substrates. The alternative dry harvesting method; by the use of sieve to separate conidia from the growth substrate, where yield is

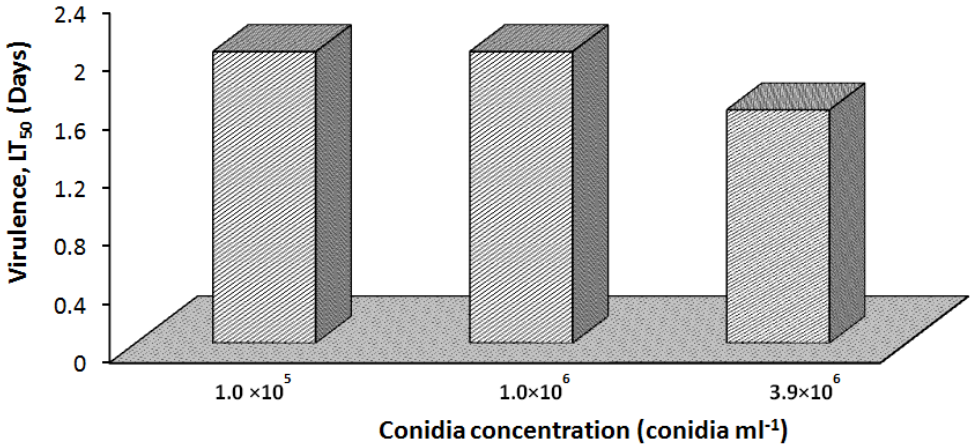

Fig. 5. Virulence of *M. anisopliae* conidia against *C. maculatus* at different concentrations

reported as the dry weight of recovered conidia gram^{-1} substrate [27], can be misleading. Tiny starch granules could pass through the sieve along with the conidia, especially when the substrate is colloidal, and the estimate of the yield could favour a starchy substrate such as rice. The use of the wetting agent has been reported to enhance recovery of conidia from solid substrates compared to the use of water alone [28].

Wet conidia were tested against *C. maculatus* and 100% mortality was recorded within five days. The ability of this fungus to grow, sporulate and infect under the ambient conditions suggest it can be useful in the management of *C. maculatus*, provided that it can be developed into an appropriate formulation without significantly losing its virulence. The onset of mortality was barely 24 hours after the insects were exposed to the infective conidia. Probably the conidia were already primed by the water used for harvesting and ready for germination. This probably favoured the speed of infection process. Development of the conidia into a dry formulation, as may be required in storage environments without loss of infectivity and virulence could be challenging. Different drying methods and drying temperature significantly affect germination and virulence of conidia of entomopathogenic fungi [29]. The equilibrium relative humidity (ERH) boundaries in favour of fungal based biocontrol agents (BCAs) [30] may not be obtainable in a dried legume (cowpea) storage environment.

This study simulated the mode of application which can be applied under storage systems, by applying the inoculum to the pods before the introduction of *C. maculatus* into the Petri-dish system. Mode of application of fungal inoculum could affect the actual load of inocula that gets to the target pests. Achievement of 100% mortality *in vitro* is promising, but this may not translate to the same level of bioefficacy in the field where other biotic abiotic interactions; temperature and relative humidity, may be limiting factors [30]. Field bioefficacy trials may be necessary in future studies to assess the effects of other biotic and abiotic interactions in relation to virulence against *C. maculatus.*

5. CONCLUSIONS

Studies on production of entomopathogenic BCAs using cheap substrates and easily transferable solid substrate fermentation (SSF) technology appear to be a good direction towards alternative entomological storage pest management. However, there is the need for a complimentary study on development of appropriate formulations which could preserve virulence, extend the storage life of propagules and be useful within storage environments.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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