Growth rate in media and pathogenicity of two species of entomopathogens found in Fiji

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ABSTRACT

Two species of entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae* were collected in the field on larvae of the Black and Yellow Mud Dauber *Sceliphron caementarium* in the Instructional Agricultural Farm Complex of the Fiji National University's College of Agriculture, Fisheries and Forestry. These fungi were cultured using different nutrient media to determine the type best suited for isolation and mass production. It was identified that both species responded well to growth in Egg Glucose Agar while a slower rate of mycelial formation occurred in Potato Dextrose Agar over a 7-day period under a photoperiod of 16 hours light and 8 hours of darkness at a relative humidity of 85%. Pathogenicity tests on *Helicoverpa armigera* larvae indicated that the isolate of *Metarhizium anisopliae* was more virulent than the strain of *Beauveria bassiana* with an LT_{50} time of 4 and 6 days respectively. *B. bassiana* has not previously been collected in the country while significant gaps still remain in the understanding of the potential for use of bio-control agents in agriculture locally. In this paper we attempt to determine the pathogenicity and growth rates of two entomopathogens using different media.

Key words: Entomopathogenic fungi, Pathogenicity, *Beauveria bassiana*, *Metarhizium anisopliae*

INTRODUCTION

Entomopathogenic fungi have played a uniquely important role in the history of microbial control of insects. Historical evidence indicates that entomopathogenic fungi were the first to be recognized as disease causing microorganisms in insects. Agostino Bassi de Lodi reported a disease in silkworm caused by a fungus, which was later, identified as *Beauveria bassiana* (Ainsworth, 1956). Elie Metschnikoff began with study of disease of a grain beetle *Anisoplia austriaca* that resulted in the discovery of the fungus *Metarhizium anisopliae* (Ainsworth, 1956). *B. bassiana* has been implicated as the cause of white muscardine disease in immature and adult insects while *M. anisopliae*, the causal agent of green muscardine disease, is reported to infect over 200 species of insects and arthropods ((Bridge *et al*., 1993; Mythili *et a*l., 2010).

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The uses of biocontrol agents, particularly the use of entomopathogens, have garnered significant research interest and scrutiny from the scientific community (Vega *et al.*, 2012). These agents exhibit both sexual and asexual reproduction with the formation of a variety of infective propagules (Vega *et al*., 2012). Fungal entomopathogens mode of action involves attachment of infective propagules to the cuticle followed by germination, chitin penetration and proliferation internally (Boucias & Pendland, 1991). During this process, which may involve the production of secondary metabolites, the internal organs of the insect are eventually degraded. Environmental factors such as ultraviolet light, temperature, and humidity can influence the effectiveness of fungal entomopathogens in the field (Inglis *et al*., 2001).

It is increasingly recognized that the biodiversity in agro-ecosystems deliver significant ecosystem services to agricultural production such as biological control of pests (Meyling & Eilenberg, 2007). Conventional management systems rely almost exclusively on applications of synthetic chemical insecticides; however, certain insects, such as the Colorado potato beetle, rapidly develop resistance to such methods of pest control (Gauthier *et al.,* 1981).

The authors aim to identify the pathogens causing muscardine infections in insects, determine the growth rates on various media and the pathogenicity of the pathogens. The Center for Agriculture and Bioscience International (CABI) provides datasheets for the presence of *M. anisopliae* in Fiji (CABI, 2017). However, a literature search yielded no results for identification of *B. bassiana* nor was there any evidence of a large scale studies into its potential as a control agent for local pests (CABI, 2017).

For a country such as Fiji, it is important to recognize natural means of controlling insects rather than focusing on traditional chemical poisons which harms the environment that, in turn, further stresses the amount of limited agricultural land that we already have. The development of cleaner agricultural insect control methods will reduce the impact on our environment allowing for more sustainable agricultural development with a reduced likelihood of adverse impacts on human health due to the fact that infection with entomopathogenic fungi is rare in humans without a predisposing medical condition (Tucker *et al.* 2004; Darbro & Thomas, 2009). The authors believe this would act as the baseline from which further research can be conducted.

MATERIALS AND METHODS

Preparation of Different Media (PDA and TDA)

PDA was prepared using the same method described by Rinaldi (1982) with the only modification being the use of 200 g of potato cut into small cubes. These were boiled and strained using miracloth prior to addition of dextrose and agar while gently mixing with a glass rod. After preparation, media was loaded into 250 ml conical flasks and sterilized in an autoclave at 121 °C and 15 lbs pressure for 20 minutes.

Two hundred and fifty grams of Tapioca (*Manihot esculenta* L.) was peeled and cut into small 2 cm cubes and placed in a beaker to which 1000 ml of water was added. Tapioca was then left to boil on medium heat for 40 minutes. Once the Tapioca had become soft, the mixture was drained through a sieve cloth. The fluid was transferred to a 2000 ml beaker that was placed on low flame. To this 50 g of dextrose and 20 g of agar was added

while gently stirring with a glass rod to ensure even mixing of constituents. Care was taken to ensure that no globules of dry dextrose or agar remained. The mixture was poured into a beaker and the volume was maintained at 1000 ml by adding water. This was then transferred to two 500 ml conical flasks and plugged using cotton plugs. The media was then autoclaved at 121 °C and 15 lbs pressure for 20 minutes.

Egg Glucose Agar (EGA)

Egg Glucose Agar (EGA) was prepared by adding 2 whole eggs (50 grams each minus weight of shell) to 1000 ml of warm water in a beaker that was being warmed over a flame. The eggs solidified as the water was heated for a further 20 minutes with constant stirring until no more egg was found to be free floating. The mixture was blended to break down large pieces of egg using a Black and Decker Blender and Mixer (BX600G). This mixture was then passed through a sieve cloth to trap any large pieces of egg still remaining. Fifty g of glucose and 20 g of agar was then added to the media in conjunction with gentle stirring. The constituents were mixed evenly together with a glass rod until no solid particles of glucose or agar remained. This was then transferred to two 500 ml conical flasks, plugged with cotton plugs and autoclaved for 20 minutes.

Isolation of the Fungi

Fungal isolates were cultured from mummified *Sceliphron caementarium* cocoons (*Metarhizium anisopliae*) and directly from the internal larvae (*B. bassiana*). Larvae of the Black and Yellow Mud Dauber were collected from the Fiji National University's Agricultural Farm Complex located at Koronivia, Nausori with the site of excavation approximately 100 meters from the King's Highway at grid coordinates 18°02'39.5"S 178°31'57.5"E. Experimentation was done at the Fiji National University's Science Laboratory located at grid coordinates 18°03'02.2"S 178°31'49.2"E. The larvae were found in soil mixed with silt that was dredged from the Lokia river. Larvae were washed from the original nesting sites during heavy rain and carried away by currents before being deposited near the river bank.

Metarhizium anisopliae was allowed to produce conidiophores bearing conidia on the surface of the cadavers by moistening the soil containing the cocoons and leaving for incubation for a period of 7 days at a temperature of 25 ± 1 ^{o}C. A mass of sporulating fungal bodies was found at the dorsal tip of the mummified larvae by the end of the 7th day. These were then removed carefully from the cadavers with the use of two forceps and placed on nutrient media for development of fungal mass. The culture was then incubated at 25 °C for a period of 7 days under 85% relative humidity with a photoperiod of 16 hours of light and 8 hours of darkness.

Larvae of *S. caementarium* infected with *B. bassiana* were removed from their cutaneous cocoons and allowed to develop conidia under moist, consistently dark conditions at a mean temperature of 25 °C. The white mycelial mass on top of the larvae was removed with the help of forceps and a needle. The mycelia were then placed on different media and were incubated at 25 °C with 85% relative humidity for a period of 7 days under 16 hours of light and 8 hours of darkness per day. Humidity of all samples was controlled by placing them in a glass cabinet with a soaked sponge. When the humidity rose above 85% the sponge was removed to allow the humidity to settle back down to acceptable levels before being replaced.

In the case of cadavers where no visual signs of pathogen-induced mortality were seen, an approach described by Kaya and Stock (1997) was used which involved surface sterilization in 70% ethanol (2-3 seconds), followed by rinsing in sterile water and subsequent submersion in 1% sodium hypochlorite for 30 seconds, followed by three washes in sterile water and placed in a petri dish for observation. This was done to confirm the presence or absence of entomopathogenic fungi and separate those insects that had not been killed by fungal infection.

Streptomycin and gentamicin were added to all the media prior to culturing to reduce chances of contamination by bacteria in a method described by Tanprasert and Reed (1997). However, no fungicide was used to ensure that all possible fungal pathogens were isolated. All culturing was done in 100 mm petri plates. Methods used are similar to those described by Boom (1971). Relative humidity had to be controlled as it was considered an important factor in insect infectivity (Ramoska, 1984).

Koch's postulates were conducted on *S. caementarium* larvae to confirm the causal agent of the disease. Mortality of insects and subsequent emergence and development of fruiting bodies on larval cadavers confirmed the presence of the pathogens.

Pathogenicity Testing

Pathogenicity testing was done through infection of batches of 24 larvae of *Helicoverpa armigera* being replicated 5 times. Trials were conducted both in the laboratory and in a meshed cage outside to simulate field conditions. Tests conducted indoors were conducted in two separate fume hoods to prevent cross-contamination with fungal isolates upon sporulation. The numbers of spores produced were estimated using serial dilution of 1ml of liquid culture followed by subsequent culturing and counting of colonies.

Insects exposed to the external environment were enclosed in two separate cages with a wire-gauze mesh which prevented the entry of any large foreign insects. The mesh had hole dimensions of 2 mm x 2 mm allowing for free movement of air. The base of the cage was covered with a 1:2 mixture of sterilized sediment and sand to a depth of 5cm. Insect larvae were placed in 6 x 4 cell grids with a larva in each after inoculation with 10^7 conidia of each of the entomopathogens. Mortality rates were noted at 4 hour intervals and are rounded off to the nearest day.

Statistical Analysis

Data was analysed with the use of R version 3.4.1. Tests were conducted for ANOVA and correlation between rate of mycelial growth and insect mortality.

RESULTS

The relative growths of fungal isolates on the various types of media are represented in Figure 1, Figure 2 and Figure 3. It was determined that spore production was proportional to rate of pathogen mycelial growth. For growth on media, it was found that the highest rate of mycelial mass growth for both entomopathogens was on Egg Glucose Agar and the lowest was on Potato Dextrose Agar.

Best results were obtained when cultures were incubated at a constant temperature of 25 ˚C with changes in temperature causing delayed growth. Any fluctuation to temperatures above 35 ˚C resulted in severely delayed growth of fungal mycelium (Figure 5).

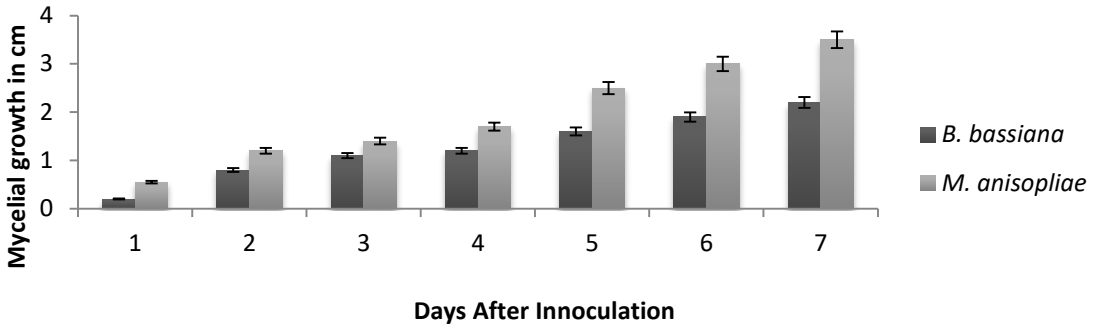

Figure 1. Growth response of *B. bassisana* and *M. anisopliae* to Potato Dextrose Agar. PDA elicited the slowest growth response of all the three types of media tested. While potatoes are cheap and easy to access, the lack of a definite protein source severely limited the proliferation of the entomopathogens. Error bars represent a standard error of mean of ± 5%.

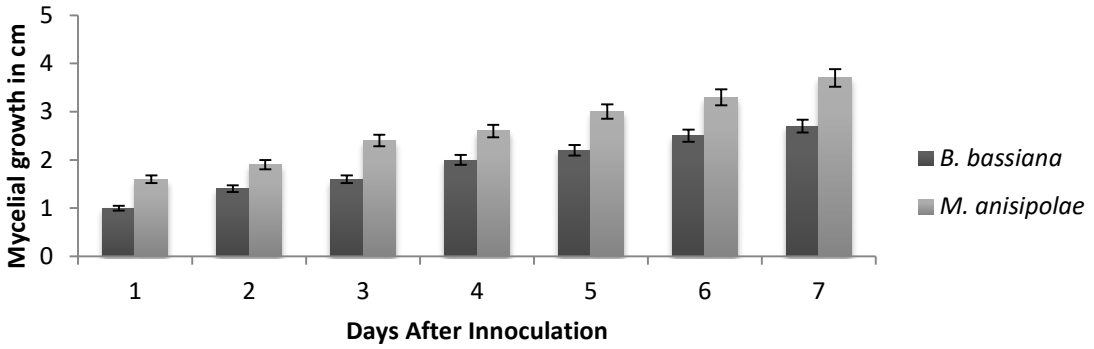

Figure 2. Growth response of *B. bassiana* and *M. anisopliae* to Tapioca Dextrose Agar. Growth response to TDA was slightly better than that achieved with PDA. However, even here the response was not rapid and the lack of a protein source became evident. Supplementation of TDA with a protein source, such as waste meat, has the potential to yield better results. Error bars represent a standard error of mean of ± 5%.

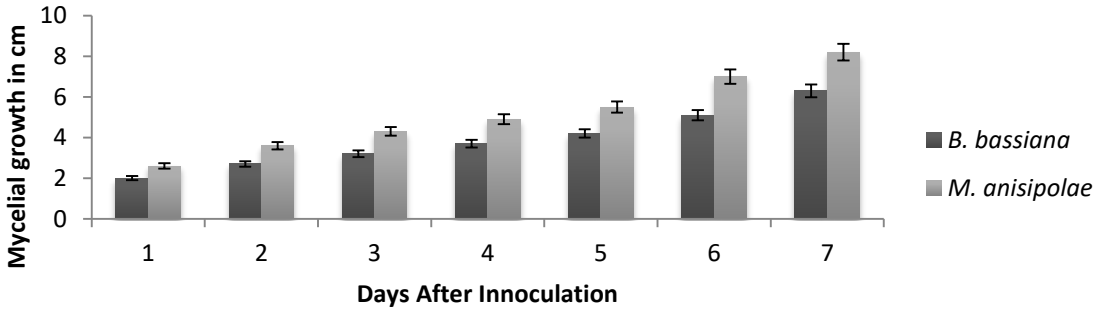

Figure 3. Growth response of B. bassiana and M. anisopliae to Egg Glucose Agar. Rapid proliferation of mycelial mass was seen for both species cultured on this medium. The most likely reason for the rapid response in this case may have been a higher concentration of protein in the media derived from the egg. Error bars represent a standard error of mean of ± 5%.

The infected larvae ceased to feed on third day after inoculation then became slightly bent, hard and mummified on the seventh day after being infected with *B. bassiana*, a phenomenon that has also been noticed by other authors (Jayaramaiah, 1981). In the five replications, 50% of the infected larvae (12) died 5.5, 6.5, 6.8, 5.8 and 5.2 days after with a mean LT_{50} of 5.96 days rounded up to 6 days. This finding is similar to that reported by Inglis and Feniuk *et al.* (1995) who noted mycosis and death of adult female grasshoppers exposed to soil treated with 1 x 10^7 conidia of *B. bassiana* beginning on the 4th day with 100% mycosis and mortality attained by the 9th day. On the eight-day entire larval body was covered with white mycelium. Secondary infection was more prevalent in larvae exposed to external environmental conditions than those left in the laboratory. Mortality rates were consistent in both laboratory and field conditions with no significant variation noted at 5% level of significance.

Mortality after infection with *M. anisopliae* began on the third day with 73% of infected larvae ceasing to feed 16 hours after inoculation with the remaining ceasing to feed by the end of the second day. In all five replications, at least 50% mortality was achieved by 96 hours post-infection. Death was rapid and 100% mortality was achieved by the 5th day in all cases. Insect cadavers were covered with mycelial growth by the end of the 5th day and green spores were present by the 6th day post-infection. Mortality rates were consistent and no significant variation was found at 5% level of significance. Mortality is represented in Figure 4.

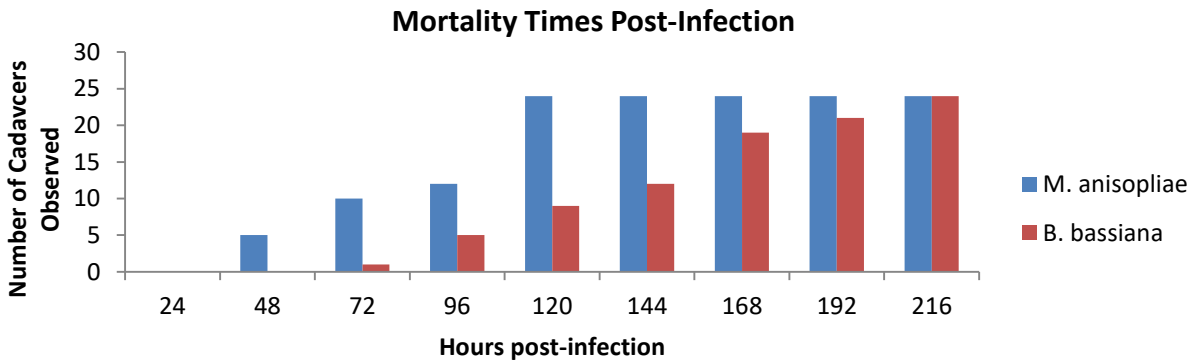

Figure 4. Mortality rates of *H. armigera* post infection. Rapid mortality was observed in larvae infected with *M. anisopliae* 96 hours post-infection. Infection with *B. bassiana* yielded mortality at a much slower rate. However, further testing will be required with multiple hosts to determine actual efficacy.

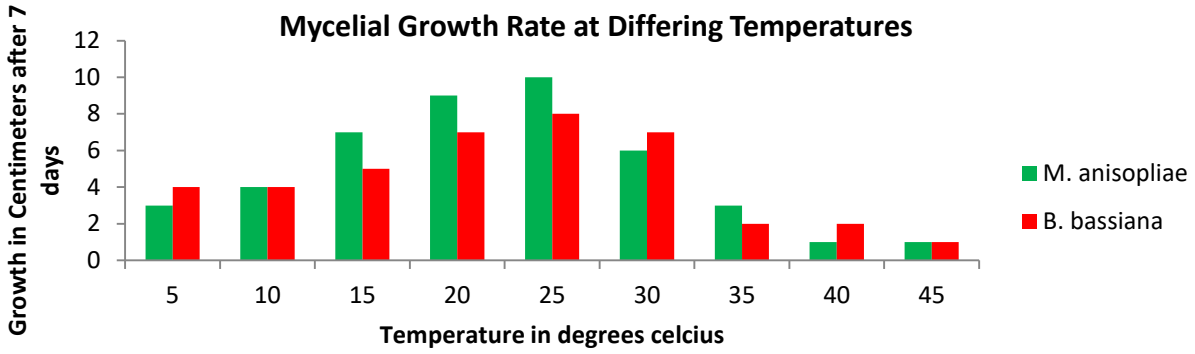

Figure 5. Relative growth rate of *B. bassiana* and *M. anisopliae*. Optimum temperature for rapid proliferation of fungal mycelium was noted to be 25 ◦C. Increasing the temperature led to diminishing growth rate and any increase past 35 ◦C severely slowed down mycelial mass formation.

DISCUSSION

This present finding is consistent with the findings of Hallsworth and Magan (1999) who tested the effect of temperature and water availability on the growth of *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces farinosus.* Fernandes *et al*. (2008), Lingg & Donaldson (1981), and Zimmerman (1982) reported similar findings when testing the thermo sensitivity of entomopathogens and it was reported that abiotic factors are a major problem when employing the use of entomopathogens for the control of crop pests, however, manipulation of the culture medium through the use of salicylic acid can be done to increase thermo tolerance (Rangel and Fernandes, 2012). Furthermore, results suggest that excessive exposure to light would delay the growth of both entomopathogens that is similar to findings of previous research conducted (Chase *et al*., 1986). The isolate of *M. anisopliae* was more virulent than the identified strain of *B. bassiana* based on LT_{50} results obtained.

Moreover, most insect larvae showed signs of secondary infection by bacterium resulting in dry, hardened cadavers when left exposed to the environment.

The rate of growth of the fungi in media and the rate of mortality was found to be showing a strong positive correlation with higher growth rates in media indicating rapid death of infected insects.

Statistical analysis showed significant correlation between mycelial growth in media and subsequent infectivity and mortality of the host. There was a strong positive correlation between rapid mycelial growth and insect mortality as shown by *M. anisopliae.*

Limitations of the study

This study is an introductory experiment into fungal entomopathogens and significant improvement is still possible in design and experimental methodology. Genetic sequencing will allow for better determination of the strains of fungi found which will in turn determine whether these are native microbes found in the soil or whether they have been introduced as a result of foreign trade.

Future studies may also want to consider increasing the scope of the study by including other pests of importance including insects such as the Rhinoceros beetle *Oryctes rhinoceros* (Linnaeus), fruit flies present on Rotuma being *Bactrocera kirki* and *Bactrocera obscura*. Accidental exposure to environmental inoculum will also need to be considered.

CONCLUSION

Results indicate that an increase in the amount of available glucose and protein increases the rate of growth of both *Beauveria bassiana* and *Metarhizium anisopliae*. For successful and rapid mass production of the fungus, the media would require a high amount of protein. Rapid growth in media was also consistent with virulence and this can be used in future trials and control measures designed to biologically control pests in the country and the region.

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