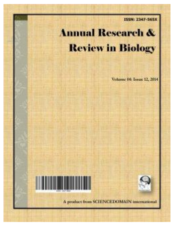

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Characterization of Growth and Virulence of Five Nigerian Isolates of Entomopathogenic Fungi Using *Galleria mellonella* Larvae for Pathogenicity Testing

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Authors' contributions

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

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ABSTRACT

Entomopathogenic fungi (EF) are naturally occurring insect population regulators, with several species that are exploited as biocontrol agents against insect pests. Five EF consisting of two strains of *Isaria farinosa*, (IF-I and IF-II) and one strain each of *Metarhizium anisopliae*, *Beauveria bassiana* and *Entomophaga* sp. (Sensu lato) were isolated from soil using *Tribolium castaneum* (Coleoptera: Tenebrionidae) (Herbst) larvae as bait. The isolates were cultured on standard Potato Dextrose Agar (PDA) (Sigma-Aldrich, UK) and identified based on phenotypic appearance and micro-morphology. Growth rates (mm day^{-1}), number of conidia per cm^2 colony area after incubation for 14 days at ambient temperature (25±2°C), viability of conidia (% germination), based on 24-hours incubation period and virulence of the infective conidia against *Galleria mellonella* were evaluated. The data on growth was subjected to analysis of Variance (ANOVA) procedure and means were separated using Tukeys Honestly Significant Difference (P=0.05). The number of conidia produced by *Entomophaga* sp was ($7.0×10^5$ conidia cm ^{2} per colony area), while the isolate

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of *M. anisopliae* produced (5.2×10^4 conidia cm^2 per colony area). The number of conidia produced by the two isolates of *I. farinosa*, IF-I and IF-II and *B. bassiana* were 9.4×10^4, 7.2×10^4 and 2.1×10^5 conidia cm^2 per colony area respectively. Eighty percent of *Entomophaga* sp conidia germinated after incubating for 24 hours at 25°C while 100% germination occurred in the other fungal isolates. There were statistically significant variabilities in the rates of growth of the EF isolates F(4,2.064) =12.97, P=0.001. The *B. bassiana* isolate had the fastest growth rate, with statistically significant value of 3.3 mm day^{-1}. The rates of growth of the two *I. farinosa* isolates: IF-I, IF-II and *M. anisopliae* were comparable, being 1.53, 1.4 and 1.28 mm day^{-1} respectively, without statistically significant difference. The growth rate of *Entomophaga* sp was 2.0 mm day^{-1}, which was significantly higher than the growth rates of *I. farinosa* and *M. anisopliae*. The mean percentage mortality values of, *G. mellonella* larvae treated with 1×10^8 conidia ml^{-1} of the infective conidia of *I. farinosa*, IF-I, IF-II, *M. anisopliae*, after five days were 70, 60, 60% respectively while *Entomophaga* sp and *B. bassiana* caused 50% mortality. The results suggest that the five isolates examined can potentially be developed into experimental formulations and tested against important horticultural pests in future studies.

Keywords: Conidiation; growth rate; virulence; isolation; mortality.

1. INTRODUCTION

Entomopathogenic fungi are natural pest population regulators, freely occurring in soils. Some important members of the species, especially the anamorphs of Hypocreales: *Beauveria bassiana, Isaria farinosa* and *Metarhizium anisopliae,* have been isolated from dead insects [1], soil [2], tree barks [3] and many have been developed into biopesticides.

Increased development of resistance to chemical pesticides by insects and environmental toxicity problems associated with intensive use of chemicals in Agriculture have promoted interests in search for alternative, environment-friendly pesticides that can be exploited as stand-alone pest control agents or as a component of integrated pest management (IPM) system [4]. Sustainable Integrated Pest Management (IPM) system using fungal biocontrol agents as a component, alongside existing conventional methods under tropical climate is promising [5]. However, success of entomopathogenic fungal biopesticides under field conditions in the tropics depends largely on ability of the propagules to exploit the relatively short, favourable windows of temperature and relative humidity available for growth and infectivity [6,7]. It is expected that entomopathogenic fungal biopesticides developed from non-tropical bioactive propagules may not be perfectly adaptable for use under tropical agroecological conditions, where temperatures and relative humidity often fall outside the critical limits for growth and infection. Borisade and Magan [8] reported the critical upper and lower temperature and water activity (a_w) boundaries for nineteen strains of entomopathogenic fungi, including *B. bassiana*, *I. farinosa, M. anisopliae* and *I. fumosorosea* isolated from tropical and temperate environments and demonstrated that species from tropical environments showed better adaptability to marginal temperatures and a_w and variabilities in the rates of growth, sporulation and virulence of some of the species were temperature and relative humidity (RH)-dependent.

As far as we know, eco-physiology of Nigerian isolates of entomopathogenic fungal species and characterization of growth, sporulation and pathogenicity have not been reported. However, previous studies on the biocontrol efficacy of experimental formulation of *M. anisopliae* (*Ma* 275.86DC), non-indigenous but tropical isolate, showed promising results under ambient temperature and relative humidity when tested against the banana weevil, *Cosmopolites sordidus* in laboratory [9] and the tomato whitefly, *Bemisia tabaci* under field conditions.

The aim of this study was to isolate entomopathogenic fungal species from Nigerian forest soil using the rust red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae) larvae for baiting, characterize growth, sporulation and evaluate virulence of the isolates using *Galleria mellonella* larvae for pathogenicity testing.

2. MATERIALS AND METHODS

2.1 Source of *Galleria mellonella* and *Tribolium castaneum* Larvae

Galleria mellonella and *T. castaneum* were obtained from the Department of Crop and Horticulture, Plant Protection Unit, Faculty of

Agricultural Sciences, Ekiti State University Ado-Ekiti, Nigeria. The *G. mellonella* larvae were reared on diet containing rice bran, wheat grit, rice grit, bee wax and honey while *T. castaneum* larvae were reared on wheat flour.

2.2 Isolation and Identification of Entomopathogenic Fungi

Soil samples were collected from 1-5 cm top soil of the experimental garden, Department of Plant Biology, Federal University of Technology Minna. The soil was spread evenly, air-dried for two weeks. Thereafter the soil was homogenized and weighed into five disposable plastic Petridishes (60 g per Petridish). The soil in each Petridish was sprayed with 10 ml sterile distilled water to moisten the surface. Ten larvae (second instar larvae) of *T. castaneum* were introduced into each Petridish and the lid was replaced. The set-up was incubated at ambient temperature (25 ± 2°C) in the dark and dead larvae were checked daily. Dead larvae were removed, rinsed in sterile distilled water containing 0.02% Polyoxyethylene sorbitan monooleate (Tween-80®) as surfactant. The rinsed larvae were placed on sterile filter paper to absorb water droplets and thereafter surface sterilized by dipping in 0.02% sodium hypochlorite solution for 2-3 seconds, rinsed in three changes of sterile distilled water and air dried on filter paper for ten minutes. The larvae samples were arranged on filter paper at 2 cm apart and further surface-disinfected by spraying with 70% ethanol. The surface-sterilized larvae were placed singly on freshly prepared Potato Dextrose agar (PDA) media (Sigma-Aldrich, UK) containing 0.01% chloramphenicol, inside 9 cm disposable Petridishes. The Petridishes were sealed with Parafilm and incubated in the dark at ambient temperature for 2-3 days. Fungal out-growths from the larvae were subcultured and pure culture of each isolate was prepared using single-spore isolation technique. The isolates were identified using their morphological characteristics under microscope and phenotypic appearance on MEA with reference to identification guides [10].

2.3 Measurement of Growth Rates

One µl of conidia suspension containing 1.0×10^3 conidia ml^{-1}, prepared from 14 days old culture was inoculated at the centre of sterile PDA plates using Micropipette and replicated three times. 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, radial extension was measured daily along two orthogonal axes marked at the back of the plates for a period of 7-10 days or until three quarter (¾) of the media surface was covered by the mycelia. The radial extension values (mm) were plotted against the incubation period (days) to estimate growth rate, using a linear model [7].

2.4 Evaluation of Sporulation Rates

Five agar plugs were randomly taken from 14 days old culture using 1 cm cork borer. The agar plugs were placed in 20 ml disposable sterile Falcon bottles and 10 ml sterile distilled water containing 0.02% Tween 80® water was added. The Falcon bottles were vortexed for 2-3 minutes to dislodge the conidia into the water. Estimation of the number of conidia in the suspension was done using a Neubauer Haemocytometer and microscope. The number of conidia cm^{-2} colony area was calculated as:

$$\frac{Estimated\ conidia\ number}{Colony\ area}$$

2.5 Determination of Conidia Viability

Viability of conidia was determined by spread plating10µl of conidia suspension containing 1×10^6 conidia ml^{-1} on fresh PDA plates. Three portions on the SDA plates were covered with sterile coverslip, the plates were sealed with Parafilm and incubated at ambient temperature for 24 hours. The coverslip areas were viewed under microscope under x40 Objective and the percentage of conidia showing development of germ tube was estimated for 50 randomly counted conidia per coverslip field. The conidia were scored as viable when the germ tubes were longer than half the size of the conidia.

2.6 Evaluation of Pathogenicity of Conidia

Conidia suspension was prepared from 14 days old culture of each isolate and standardized to 1×10^6 conidial ml^{-1}. *Galleria mellonella* larvae were dipped into the conidia suspension for 1-2 seconds and placed on filter paper inside a modified Petridish (Borisade et al. 2018). Each Petridish contained 10 larvae and replicated three times. The Petridishes were arranged inside a cupboard and equilibrated to 95-98%

humidity by placing two beakers containing of 500 ml distilled water each [7]. Mortality of larvae was recorded daily and the mean percentage values of cumulative mortality were plotted against the incubation period to define the trends of larvae mortality.

2.7 Data Analysis

The data on growth rates of the fungal isolates was checked for compliance with the requirements of Parametric Tests and subjected to Analysis of variance (ANOVA). Where there were significant differences, a Post-hoc test was conducted and means were separated using Tukey's Honestly Significant Difference (HSD), P=0.05% (IBM SPSS) and graphs were plotted using Microsoft Excel 2013.

3. RESULTS

3.1 Entomopathogenic Fungal Isolates

Four morphologically distinct entomopathogenic isolates were obtained and consisted of *Entomophaga* sp, *Beauveria bassiana, Metarhizium anisoplia*e and *Isaria farinosa* (Sensu lato). Considering the phenotypic appearance and colony morphology, the *I. farinosa* were two separate strains and they were assigned names as *I. farinosa*-I (IF-I) and *I. farinosa*-II (IF-II) (Fig. 1)

3.2 Viability of Conidia, Growth and Sporulation Rates

Germinations rates of conidia of the entomopathogenic isolates are shown in Fig. 2. Germination of *B. bassiana*, I. *farinosa*-II and *M. anisopliae* was 100% while the *Entomophaga* sp. had 80% germination after 24 hours of incubation. However, no germination occurred in *I. farinosa*-I after 24 hours of incubation. There were statistically significant variabilities in the rates of growth of the *I. farinosa* isolates F(4, 2.064) =12.97, P=0.001. The *B. bassiana* isolate had the fastest growth rate, with statistically significant value of 3.3 mm day^{-1}. The rates of growth of the two *I. farinosa* isolates: IF-I, IF-II and *M. anisopliae* were comparable, being 1.53, 1.4 and 1.28 mm day^{-1} respectively, without statistically significant difference. The growth rate of *Entomophaga* sp was 2.0 mm day^{-1}, which was significantly higher than the growth rates of *I. farinosa* and *M. anisopliae* (Fig. 3). The number of conidia produced by *Entomophaga* sp was 7.0×10^5 conidia cm-^{2} colony area), while the isolate of *M. anisopliae* produced 5.2×10^4 conidia cm-^{2} colony area. The number of conidia produced by the two isolates of *I. farinosa*, IF-I and IF-II and *B. bassiana* were 9.4×10^4, 7.2×10^4 and 2.1×10^5 conidia cm-^{2} colony area respectively (Fig. 4).

3.3 Pathogenicity of Isolates against *G. mellonella* Larvae

All the isolates of EF infected and caused mortality of *G. mellonella* larvae during a five-day incubation period and the highest percentage mortality was caused by *M. anisopliae*. The mean percentage mortality of, *G. mellonella* larvae treated with 1×10^8conidia ml^{-1} of the infective conidia of *I. farinosa*, IF-I, IF-II, *M. anisopliae*, were 70, 60, 60% respectively while *Entomophaga* sp and *B. bassiana* caused 50% mortality after five days (Fig. 5).

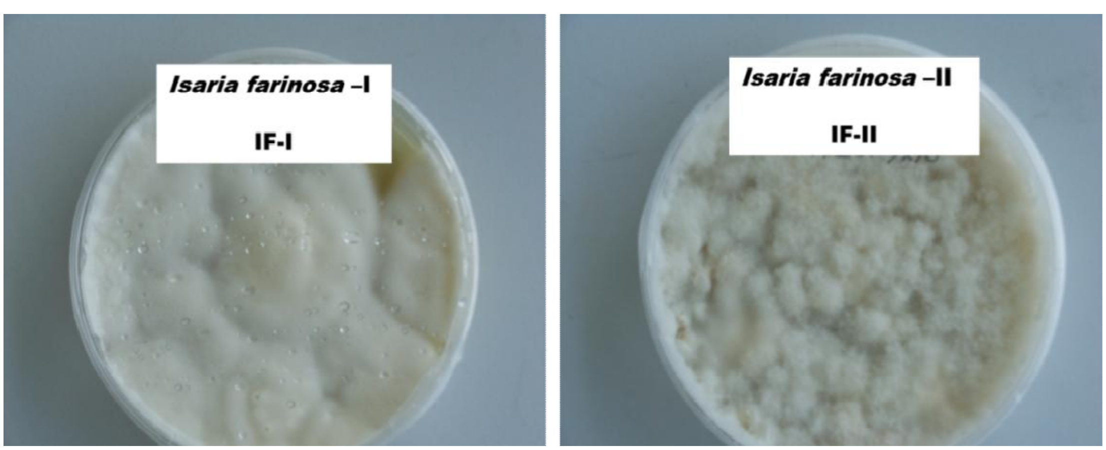

Fig. 1. *Isaria farinosa* isolates showing variabilities in growth morphology on SDA media

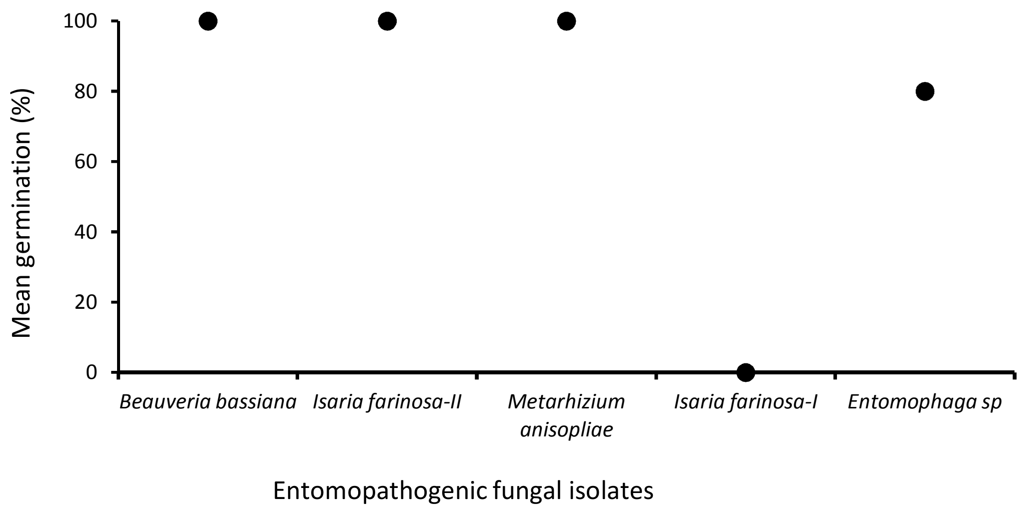

Fig. 2. Germination rates of five entomopathogenic fungal conidia on SDA media after incubation for 24 hours

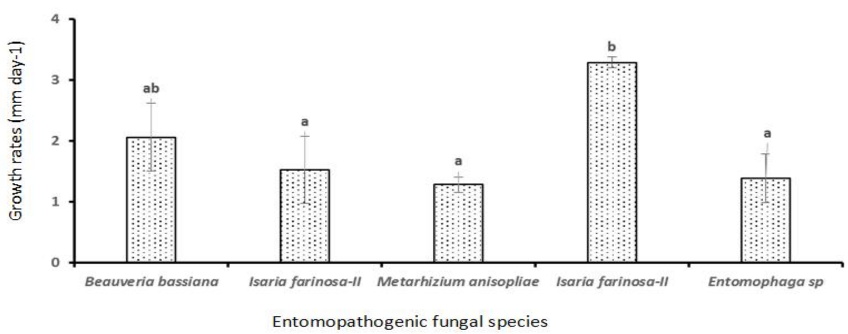

Fig. 3. Growth rates of five entomopathogenic fungi cultured on SDA media and incubated at ambient temperature

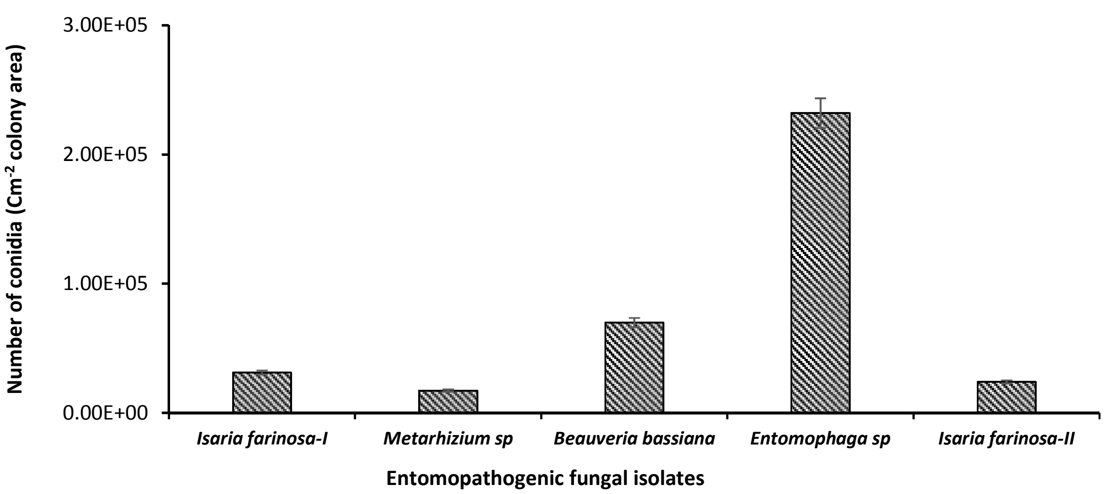

Fig. 4. Conidia production rates of five entomopathogenic isolates on sabouraud dextrose agar after incubation for 14 days

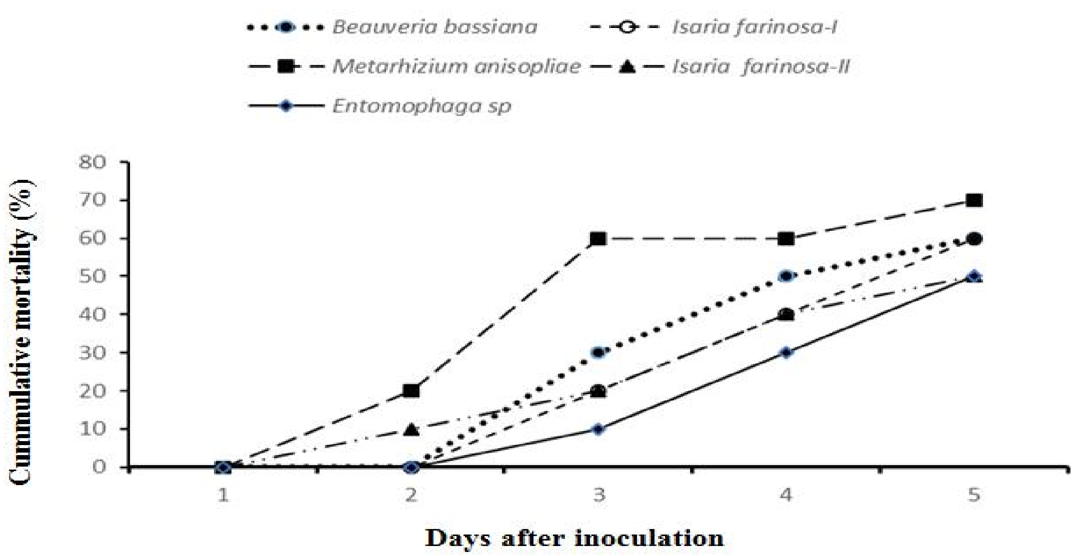

Fig. 5. Cumulative mortality of *Galleria mellonella* inoculated with infective spores of five different entomopathogenic fungi

4. DISCUSSION

Five distinct isolates of entomopathogenic fungi (EF) which comprised of *Entomophaga* sp, *B. bassiana, M. anisopliae* and two strains of *I. farinosa* were isolated from the soil samples, an indication that EF species which can be potentially developed into biopesticides in the future are present and well distributed in the soil. The soil sample from which the fungi were isolated was collected from a single spot, it is expected that greater diversity of isolates would occur if a wider area is profiled. Nussenbaum and Lecuona [11] reported high distribution and occurrence of *B. bassiana* and *I. farinosa* in soil samples collected from different locations. Diversity of EF in soil is attributable to tolerance to a wide range of climatic conditions [12] and other anthropogenic factors, such as land use practices [5].

The conidia of all the isolates except IF-I germinated after incubation for 24 hours at rates that ranged between 70% - 100%. Germination is an important characteristic of EF which necessary for infection and the rate at which it occurs often correlate with virulence [5,13]. Evaluation of germination in this study was based on a 24-hour incubation period, during which IF-I failed to germinate. Yeo et al. [14] and Borisade et al. [5] reported that failure of entomopathogenic fungi to germinate within a 24-hour incubation period does not imply the spores are not viable, as optimal conditions required for germination and growth are well differentiated among species and strains. Lag time is known to vary among EF and those that are same species often show a wide differentiation in abiotic requirements, especially temperature and water activity that permits germination and growth [8]. It is interesting that the isolate (IF-I) which failed to germinate after incubating for 24 hours caused 60% mortality among the host population within five days. However, there is no literature to explain the relationship between the time taken by EF to adjust to its environment prior to germination (Lag time) and eventual virulence, except the contrasting report in Borisade, *et al.* (2016), where the relationship between lag time, infectivity and virulence of EF was studied and it was suggested that lag time need to be considered as an important factor in characterization of virulence of EF that are exploited in biocontrol programs.

The variabilities in virulence in this study is similar to earlier reports by Nussenbaum and Lecuona [11], where mortality rates of 32 to 100% was recorded in boll weevil inoculated with the infective conidia of *B. bassiana* strains. Similarly, Domingues da Silva [15]) recorded 15% to 83% mortality in boll weevil populations infected by *B. bassiana*. Susceptibility of insects to entomopathogenic fungi may be dependent on the type of host, host resistant factors, innate virulence of the EF and interacting abiotic factors [16]. *Galleria* larvae has been adopted in several studies in modeling virulence of EF and results obtained in the laboratory using Galleria-model pathogenicity assay often correlate with results from field trials [17].

The EF isolates sporulated under the prevailing ambient conditions on artificial media. This suggests that these species possess the complementary attribute that is required for their development into biocontrol agents (BCAs). The basic characteristics of EF that determine their suitability for being developed into biopesticides is the ability to produce large numbers of conidia that are environmentally stable and infective [18, 19]. Production of large conidia of EF is often achieved using solid fermentation systems (SF) on artificial media that are readily available and cheap [20,21]. However, the physical and nutritional requirements for conidia production are more stringent than those required for mycelial growth [21]. It is therefore important that eco-physiology of the species be studied to determine the optimal conditions in favour of conidiation.

5. CONCLUSION

The results suggest that the five isolates examined can potentially be developed into experimental formulations and tested against important horticultural pests in future studies. However, there is need for more studies on eco-physiology of the species, especially their temperature boundaries within which they can grow and infect, water activity profiles and relative humidity (RH) relations. Studies are required on development of experimental formulations, which can be further tested to generate data on the host range of the species and evaluation of safety to humans and aquatic species before embarking on field trials.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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