Evaluation of Ethiopian Isolates of Entomopathogenic Fungi as Potential Biological Control Agent of the Desert Locust

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Abstract

Ten native fungal isolates were collected from the different regions of Ethiopia. Six of the isolates were identified as *Beauveria bassiana*, one as *Metarhizium anisopliae*, one as *Paecilomyces* sp. and one as a new *Beauveria* sp. Eight of the tested fungal isolates showed pathogenecity to fifth instar *Schistocerca gregaria*. *B. bassiana* isolate FF and *M. anisopliae* isolate EE were more virulent compared to the rest. Thus two isolates were further assayed in peanut (*Arachis hypogaea)* and noug (*Guizotia abyssinica)* oil formulations, and compared with a known entomopathogen, *M. anisopliae* isolate ICIPE 30. The results indicated that there were strong dose-response patterns. Peanut oil formulation enhanced infectivity of both FF and EE. Infectivity of EE was significantly ($P \leq 0.05$) reduced in noug oil formulation. Comparison of virulence revealed that isolates FF and EE were more pathogenic than ICIPE 30. FF in peanut oil formulation had the lowest LC_{50} value of 2.02×10^5 conidia/ml followed by EE, 2.93×10^5 and ICIPE 30 with 4.98×10^5 conidia/ml. The highest mortality was achieved by FF at 1×10^8 conidia/ml, resulting in 50% in 4.2 days and 100% mortality in seven days. The median lethal time of the fourth, fifth larval instar and adult FF treated insects were 3.5, 4.1 and 4.8 days, respectively, indicating that the fourth larval instar is more susceptible. FF and EE grew at a temperature range of 24°C and 37°C, with peaks at 24°C and 28°C for FF and 28°C for EE. Malt extract agar favored growth and sporulation of FF, whereas EE grew and sporulated faster on sabouraud dextrose agar. Germination of both isolates was significantly ($P \leq 0.05$) higher in peanut oil formulation than in noug oil.

Introduction

The desert locust, *Schistocerca gregaria,* is one of the most serious pests of agriculture in the Sahel (Geddes 1990). During plague years, desert locust causes damage in humid zones of Africa and Asia (Brader 1988). Devastation of crops by locusts has been recorded since biblical times, and the cost of control has been very high (Brader 1988).

Until the mid-1980s, locust control had depended largely on application of persistent chemical pesticides, such dieldrin, which is not environmentally friendly. Because of the long activity of the spray residue, Dieldrin could be used as barrier treatment (Brader 1988, Prior & Street 1997). However, it is now apparent that there are widespread ecological consequences such as soil and ground water contamination, impacts on the food chain and potential health concerns. As a result, persistent chemical insecticides are now prohibited in most countries and replaced by less persistent pesticides such as fenitrothion and Malathion (Brader 1988, Lomer et al. 1992). These chemicals can only be used as contact insecticides, and are therefore much less effective than barrier treatment and often require repeated applications within a season (Brader 1988).

Biological control of insect pests with microbial agents is an appealing prospect,

since it would reduce inputs of chemical pesticides into the environment. Entomopathogenic fungi, particularly *Metarhizium* and *Beauveria* in the Class Deuteromycetes, are attractive as biopesticides for use in integrated pest management (IPM), as they combine host specificity with proven safety (Bateman et al. 1993).

Often, locusts are indigenous to the areas of infestation indicating that their natural enemies co-exist with them. Therefore, this study was carried out to find native pathogenic isolates of fungi and to evaluate their effectiveness in killing *S. gregaria*.

Materials and Methods

Rearing of *S. gregaria*

Eggs of *S. gregaria* were obtained from the Desert Locust Control Organization for East Africa (DLCO-EA) Addis Ababa and the colony was maintained in the Biology Department, Addis Ababa University. The target insects were reared according to the method described by Gillepsie et al. (1998). The eggs were incubated in wooden box at 31 ± 1°C for about 12 -14 days. Every other day, a few drops of water were added to the egg tubes to maintain the moisture 70-80% relative humidity.

When the eggs hatched, the first instar larvae, were transferred into metallic cages (38 x 38 x 52 length x width x height). A 60 watt light bulb was used to maintain an inside temperature of 32 - 37°C. The photo phase was kept on for 12 hours, and the humidity of the cages ranged between 35 and 65%. The insects were fed cabbage leaves, barley and wheat seedlings supplemented with wheat bran.

Collection of pathogens

Roadside surveys were made in 1997/8 to different locations in the regions of Tigrai and Wollo of Amhara Regional States recognized as locust breeding areas. Dead insects with external sporulation were collected in sterile glass vials and brought to the laboratory. Spores were directly transferred from the external surface of the cadaver using a fine sterile needle onto sabouraud dextrose agar media containing 0.5g/l of chloramphinicol. The cultures were incubated at 28°C in the dark for seven days.

Preparation of oil formulation

The conidia of each fungal isolate were harvested 15 days after inoculation by flooding/pipeting 10 ml of pure peanut oil or extra refined noug oil onto the culture. The suspension was then sieved through cheesecloth to remove the hyphal fragments and agitated in test tube shaker for 8 minutes to avoid clumping of spores. The conidial concentration of each isolate was adjusted to 5×10^7 conidia/ml using haemocytometer for the initial screening (Cecil & Lomer 1996).

Insect bioassay

For each new isolate, 20 fifth instar *S. gregaria* nymphs in duplicates were used in the assay. Each insect was kept in 400 ml plastic beaker covered with fine mosquito net and inoculated with 2 µl of 5×10^7 conidia/ml (Cecil & Lomer 1996) under the pronotum using a micropipette. Twenty control insects in duplicates were also treated with 2 µl of oil. For comparison, *Metarhizium anisopliae* isolate ICIPE 30 supplied by the International Center for Insect Physiology and Ecology (ICIPE), Nairobi was formulated in peanut oil and used in the assay. Treated insects were maintained under controlled conditions of 28°C and 39% RH and a photoperiod of 12h. Insect mortality was recorded daily for 21 days beginning from the second day of inoculation as described by Bateman (1994). Cadavers were incubated at 100% RH to confirm fungal infection.

Further bioassays were conducted on two

most virulent native isolates by comparing them with ICIPE 30. Two oil formulations; peanut (*Arachis hypogaea*) and noug (*Guizotia abyssinica*) were used in the assay. Serial dilutions of each oil formulation were made to obtain four different conidial suspensions ($1x10^5$, $1x10^6$, and 1×10^7, 1×10^8 conidia/ml). Twenty insects per replicate were used for each treatment. Each treatment was replicated trice.

The response of different instars of *S. gregaria* to the most virulent isolate was examined by applying a uniform 2 µl of $1x10^8$ conidia/ml formulated in peanut oil. For each instar twenty insects were used in two replicates. Nymphs of each instar were also treated with blank peanut oil and used as a control.

Growth rate and spore yield

The growth media, Oatmeal agar (OA), malt extract agar (MEA) and sabouraud dextrose agar (SDA) were compared for their effect on the growth rates of the fungal isolates. Agar discs of 9 mm in diameter of each isolate were transferred from 15 days old SDA cultures on fresh media of the three different agars. The cultures were incubated for ten days at 28°C in the dark and colony diameter was measured on the tenth day of incubation. The spores from each culture medium were harvested by flooding 10 ml of 0.01% Tween 20 onto the plates. The spore suspension was then filtered through cheesecloth and diluted (1:10) in sterile water. The suspension was vortexed for eight minutes to avoid clumping of the spores and the spore density when adjusted to $1x10^8$ conidia/ml.

Effect of temperature on spore germination

The germination behavior of two most potent isolates was studied at different temperature ranges. A spore suspension that contained about 5×10^5 spores/ml was prepared in sterile water. Four agar discs of 1 mm^2 and about 2 mm thick were placed on a sterile glass slide. Then a drop of water containing suspended spores of the respective isolate was placed on each agar disc. The slides were incubated in the dark at 4, 24, 28, 37 and 45°C and percent germination was determined after 24 hours. Germination was considered when a germ tube grew to the length equal to the minimum diameter of the spore.

The Relative potency of each was calculated using the following formula (Feng & Johnson 1990):

$$\text{Activity of tested isolate} = \frac{LC_{50} \text{ of standard ICIPE30 (conidia/ml)}}{LC_{50} \text{ of tested isolate}} \times 100$$

Effect of oil on spore germination

The effect of peanut and noug oil on spore germination was studied using a conidial concentration of 5×10^5 spores/ml. The slide cultures prepared were incubated at 28°C in the dark for 24 hours. The germinating spores were counted and percent germination in the respective oils was determined.

Statistical analysis

The median lethal time was estimated using the MELTIMOR program. Two-way analysis of variance was used to see the interaction between dose and efficacy of the isolates. Duncan's multiple range test was used to separate the means. Finney's probit analysis program was used to estimate the LC_{50} values of the different concentrations (Finney 1971).

Table 1.Origin and order of host insects found from which entomopathogenic fungi were isolated.

Local code	Species	Location	Altitude (m)	Crop	Stage of insect	Host insect Order	Appearance of sporulation
AA	*Paecilomyces sp.*	Korem	2450	Grass	Adult	Coleoptera	White muscardine
BB	*Beauveria (New sp.)*	Gusquam	2050	Grass	Adult	Arachinida	White muscardine
CC	*Beauveria bassiana*	Woldiya	1950	Millet	Adult	Coleoptera	White muscardine
DD	*Beauveria bassiana*	Debremarko	2030	Teff	Adult	Coleoptera	White muscardine
EE	*Metarhizium anisopliae*	Alamata	1500	Grass	Adult	?Crustacean	Green muscardine
FF	*Beauveria bassiana*	Ashengie	2400	Wheat	Adult	Coleoptera	White muscardine
GG	*Beauveria bassiana*	Ashengie	2400	Wheat	Adult	Coleoptera	White muscardine
HH	*Beauveria bassiana*	Ashengie	2450	Wheat	Adult	Coleoptera	White muscardine
II	?	Woldiya	1950	Millet	Adult	Coleoptera	White muscardine
AK	*Beauveria bassiana*	Tikurinchini	2225	Barley	Larvae	Coleoptera	White muscardine

Table 2. Mean percentage mortality on day 11 of fifth instar *S. gregaria* inoculated with various fungal isolates at a dose of $5x10^7$ conidia/ml in peanut oil.

Isolate tested	% mortality (corrected)	Median lethal time ± s.d.
Beauveria bassiana FF	100[a]	6.71±2.32
Metarhizium anisopliae ICIPE 30	90 (89.5)[ab]	6.67±3.44
M. anisopliae EE	90(88.5) [ab]	8.00±2.37
B. bassiana GG	85 (83.3)[bc]	8.60±3.46
B. bassiana AK	80(76.5)[bc]	8.00±4.04
B. bassiana HH	75 (72.2)[bc]	8.40±3.04
Paecilomyces sp. AA	60 (55.6)[d]	9.00±6.36
B. bassiana CC	40 (33.3)[e]	13.5±4.77
B.bassiana, DD	38 (31.4)[e]	11.6+4.58

Values in columns followed by the same letter are not significantly different using Duncan's multiple range test [P<0.001].

Results

Collection of pathogens

Most of the fungal isolates were found on beetles (Coleoptera) and discovered as infected cadavers (Table 1). All the spores recovered from the cadavers were viable, and a pure culture of each isolate was prepared. The fungal isolates were identified by Dr. Richard Humber from Cornell University, New York. Among the *Beauveria* isolates, BB was identified as a new species.

Insect bioassay

Among ten isolates indicated in table 1, eight were selected on the basis of spore production. Percentage cumulative mortality and the median lethal time (see Bateman 1994) for each isolate against the fifth instar *S. gregaria* are presented in table 2. There were highly ($P \leq 0.001$) significant differences between the isolates with respect to mortality (table 2). Depending on the preliminary bioassay, the isolates could be categorized as low (CC & DD), intermediate (GG, AK, HH & AA), moderately high (EE & ICIPE 30) and highly virulent (FF).

Isolates FF, ICIPE 30 and EE caused high mortality (90-100%) in 11 days after inoculation. FF treated insects began to die three days after inoculation and took peanut oil showed low (15%) mortality throughout the experiment. However, a few times, failure to successfully molt was resulted. The experiments lead to the

Table 3. Mean percentage cumulative mortality of fifth instar *S.gregaria* 8 days after inoculation with 10^5-10^8 conidia/ml of the EE and FF isolates.

Isolate	Cumulative (%) mortality			
	10^5	10^6	10^7	10^8
FF, peanut oil	40 (36.8)^{fg}	75 (73.7)^{cd}	80 (78.9)^{b}	100^{a}
FF, noug oil	35^{g}	60^{e}	60^{e}	95^{a}
EE, peanut oil	40 (36.8)^{fg}	60 (57.9)^{e}	85 (84.2)^{bc}	95 (94.7)^{a}
EE, noug oil	5^{h}	10^{h}	40^{fg}	60^{e}
ICIPE30 peanut oil	45 (42.1)^{f}	45 (42.1)^{f}	70 (68.4)^{d}	95 (94.7)^{a}
Peanut oil (control)	0	4	3	4

Means associated with the same letter are not significantly different as determined by DMRT {Duncan's multiple range test (P≤0.05)}. Cumulative % mortality data were corrected using Abbot's formula.

6.7 and 11 days to achieve 50% and 100% cumulative mortality, respectively. The isolates *M. anisopliae,* ICIPE 30 and EE, each resulted in 90% cumulative mortality in 11 days after inoculation. The isolate ICIPE 30 had a shorter lethal time than isolate EE although both caused 90% mortality.

The isolates of *B. bassiana*, GG, AK and HH showed moderately high virulence resulting in a corrected cumulative mortality of 83.3%, 80% and 72.2%, and median lethal time of 8.6, 8.0 and 8.4 days, respectively. Isolate AA (*Paecilomyces* Sp.) and CC (*Beauveria* Sp.) appeared to have intermediate virulence with corrected cumulative mortality of 55.6% and 33.3%, respectively at 11 days after inoculation.

Mortality caused by these two isolates rose gradually resulting in 75% and 80% by day 21. The isolate DD may be considered as a weak pathogen on the ground that the corrected cumulative mortality recorded at day 11, 15 and 21 were 0%, 22.2% and 47.1%, respectively. Cumulative mortality increased for all isolates with a longer incubation time. Death was quicker with more virulent isolates. Controls treated with blank

identification and selection of two native isolates i.e. EE & FF and these were compared with exotic ICIPE 30 isolate. The efficacy of isolates *M. anisopliae* EE and *B. bassiana* FF was investigated in peanut and noug oil formulations against fifth instar larvae of *S. gregaria*.

Peanut oil based formulation of EE caused 36.8-94.7% mortality at all concentrations tested eight days after application (Table 3).

The noug oil-based formulation reduce efficacy of EE at all doses and at lower doses ($1x10^5$ and 10^6 conidia/ml) 50% mortality was not achieved even until 21 days after inoculation (Table 4). The cumulative mortality at eight days after inoculation ranged 5-60% for all doses examined (Table 3). As the duration increased, however, the cumulative mortality gradually rose. Particularly, at high dose ($1x10^8$ conidia/ml), 100% mortality was achieved in 14 days after inoculation, the median lethal time being 7.3 days (Table 4). Mortality in the control was high (20%) 14 days after treatment. The estimated time in days to cause 50% mortality with the various concentrations is indicated in table 4.

Table 4. LT_{50} in days of three isolates at concentration from 10^5 to 10^8 conidia/ml against *S. gregaria*.

Isolate	$LT_{50} \pm$ s.d.			
	10^5	10^6	10^7	10^8
FF, peanut oil	8.63±5.91	7.17±2.18	7.00±1.75	4.2±1.22
FF, noug oil	8.75±3.73	7.6±1.49	7.5±3.01	5.0±1.17
EE, peanut oil	8.83±3.77	7.39±4.08	7.23±1.96	5.00±1.79
EE, noug oil	-	-	8.33±2.89	7.33±2.46
ICIPE30, peanut	9.5±4.47	8.75±2.14	7.21±2.79	5.5±1.72

There was significant ($P \leq 0.001$) difference between various treatments of the isolate FF, formulated in peanut oil, and low mortality of the controls (Table 3). FF in peanut oil took a median lethal time of 4.2 days at 10^8 conidia/ml (Table 4).

There was no significant ($P \leq 0.05$) difference in the efficacy of noug and peanut oil formulation of FF. The median lethal time taken was five days (Table 4). However, treatment of noug oil without conidia also resulted in higher (45%) mortalities 13 days after inoculation than peanut oil. The median lethal time for the doses $1x10^5$, 10^6 and 10^7 ranged between 8.75 and 7.5 days.

Among the tested isolates, FF in peanut oil formulation had the lowest LC_{50} values ($2.02x10^5$ conidia/ml) (Table 5). The isolates EE and ICIPE 30 in peanut oil and FF in noug oil formulation showed less virulence to *S. gregaria* as compared to peanut oil formulation of FF. EE in noug oil formulation showed lower virulence than in peanut, yielding LC_{50} of $3.66x10^7$ conidia/ml (Table 5).Among the different stages of *S. gregaria* tested with $1x10^8$ conidia/ml, the fourth instar larvae were highly susceptible to infection by the indigenous isolate FF. Locust mortality was 100% for the fourth instar larvae on day five, it took seven days for the fifth instar larvae and nine days for the adults. The treatment of fourth instar nymphs with peanut oil alone showed 25% mortality on day four. In all the treatments, there was a significantly ($P \leq 0.001$) higher mortality in treated insects than in the controls. The median lethal time when the fourth and fifth instar larvae and adults of *S. gregaria* were treated was 3.47±0.83, 4.14±1.27 and 4.83±1.72 days, respectively.

Table 5. Probit analysis of mortality data of EE (*Metarhizium* spp.), FF and ICIPE 30.

Isolate	Upper	LC_{50}	Lower	X^{2a}	Relative potency[b]
FF, peanut oil	$6.39x10^5$	$2.02x10^5$	$2.08x10^4$	1.7289	246.5
EE, peanut oil	$9.39x10^5$	$2.93x10^5$	$3.32x10^4$	0.1513	169.9
ICIPE30, peanut oil	$1.84x10^6$	$4.98x10^5$	$4.28x10^4$	2.7805	100
FF, noug oil	$2.20x10^6$	$6.13x10^5$	$6.52x10^4$	3.0460	81.24
EE, noug oil	$2.85x10^8$	$3.66x10^7$	$1.20x10^7$	0.6280	1.36

Analysis was based on data taken eight days after inoculation, [a]$P \leq 0.05$, df=2

Effect of media on growth and sporulation

Colonies of isolates EE and FF showed similar colony growth rates when incubated on oatmeal agar (Fig. 1). On malt extract agar, EE grew to a diameter of 3.2 cm in 10 days as compared to 2.35 cm for FF.

Effect of temperature on spore germination

Isolate EE and isolate FF germinated at temperature ranges of 24 - 37°C (Fig. 2). Both isolates failed to germinate at 4°C and 45°C. The peaks in germination of FF occurred between 24°C (90.8%) and 28°C (92%), which was significantly ($P \leq 0.05$) higher than at 37°C (65.4%). Isolate EE attained peak germination at 28°C (40.3%) and was significantly ($P < 0.05$) more at these temperatures than at 24°C (27.9%) and 37°C (30.4%).

Effect of oil type on spore germination

Isolates EE and FF had different responses to the oil diluents (Fig. 2). Noug oil did not significantly affect the germination of EE isolate. Isolate FF germinated significantly (94.8%) more in peanut than in noug oil (61.2%).

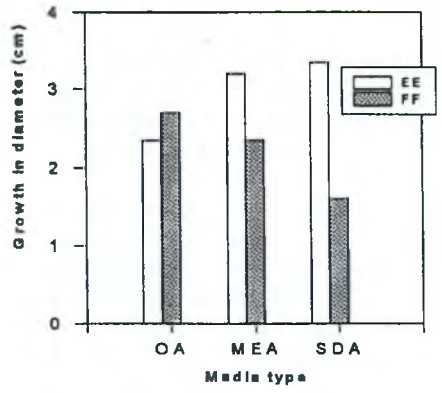

Fig. 1. Effect of media on growth rate

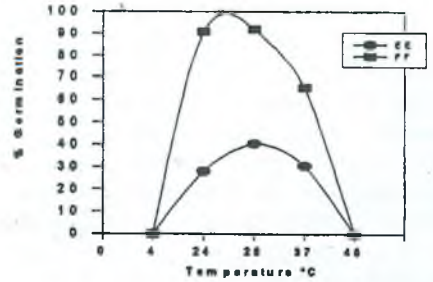

Fig. 2. Effect of temperature on spore germination

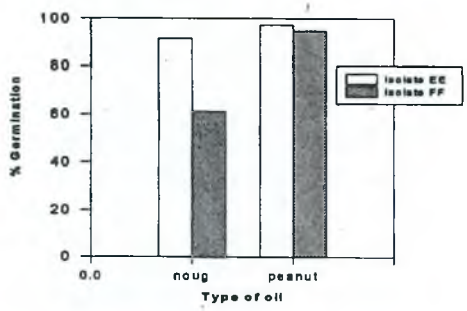

Fig. 3. Effect of oil type on spore germination

Discussion

Although it is true that experiment of this nature could be carried out using either the fourth instar nymphs of *S. grgaria*, the fifth instars were selected during the present assay considering their sufficiently larger sizes than the fourth instar nymphs and suitability for controlled droplet application under the pronotum. Furthermore, the fifth instars are highly voracious in that they can cause economic yield loss even before developing into flying adults. Therefore, this stage could be an appropriate target for application of mycopesticides. In addition, the fifth instars undergo molting to become adults. An advantage of this was taken to see if moulting could affect the progress of infection. The LUBILOSA project (Cecil & Lomer 1996) had mainly focused on adult *S.gregaria* for their bioassays so that the present results could serve as compliment to the program through augmenting their previous findings on adults although the isolates used were different from those used in the present study.

From the collected 10 native fungal isolates, two (BB and II) showed weak sporulation and hence their efficacy was not evaluated. The remaining eight native isolates in a preliminary screening at a dose of $5x10^7$ conidia/ml revealed a continuous gradation of

entomopathogenic effect ranging between 100% mortality within 11 days to no mycosis within the same period in accordance with that described by Prior et al (1997).

The Coleopteran-origin isolate FF in peanut oil suspension was the most virulent with an LC_{50} value of $2.02x10^5$conidia/ml which is equivalent to 406 conidia/insect. Prior et al (1997) have already argued that virulent isolates may originate from a taxonomic group unrelated to the target pest.

The two native isolates EE and FF have superior performance over ICIPE 30 (Table 5). A virulent fungus is considered to have evolved biochemical, morphological, and trophic mechanisms to attach and adhere to the cuticle, to solubilize, penetrate, and utilize the cuticle, and to overcome the resistance mechanisms in the haemocoel (Bidochka et al. 1997). Therefore, further studies are required to determine which of the factors for virulence attribute to the high efficacy of FF and EE compared to the ICIPE 30.

Few infected fifth instar locusts molted and died as adults. This might indicate that once the fungi penetrate the locust's cuticle, moulting has no effect on mycosis. Among the different life stages tested, fourth instar larvae were highly susceptible to infection, in particular FF than the fifth instar or adult stages. This reason might be that at younger stages locusts have soft cuticle, less weight and weaker resistance than the higher instars, all of which could enhance susceptibility to mycosis.

Conidia in peanut oil yielded a significantly higher germination rate and a better efficacy than when formulated in noug oil. Probit analysis of the mortality data indicated that peanut oil formulation of FF and EE resulted in lower LC_{50} and LT_{50} values when compared to noug oil (Tables 4 & 5). Controls treated with blank noug oil showed relatively higher mortality 12 days after treatment. Noug oil is known to contain a high amount (54-73%) of linoleic acid (Dagne & Jonsson 1994) that may have lethal effect on insects although no published data are available. The germination rate of FF was particularly retarded in noug oil diluents. Although no significant difference in cumulative mortality was found in peanut and noug oil formulations of FF, delays in mortality of experimental insects were observed (Tables 3 & 4). The efficacy of EE in noug oil suspension was significantly lower compared to peanut oil formulation. For noug oil to achieve similar mortality in peanut oil about five days delay were noted. The germ tubes, appressoria and penetration structures were formed in the presence of an oil film. Accordingly, even though the germination rate was sufficiently high, suspension in noug oil seemed to impede infection for a reason that was not clear. The experiment was carried out at 28°C and about 39% RH, which indicated that the tested isolates, when formulated in oil suspensions, could infect *S. gregaria* and cause mycoses in dry conditions.

The germination of isolates EE and FF showed that they are capable of germinating in a wide range of temperatures (24-37°C). Germination of isolate EE was lower in water than in oil. High germination (97.2%) attained in peanut oil formulation could be due to the lipophilic layer of the conidia that enhanced germination in oil than water. The peak germination rate attained with EE was at 28°C (40.3%) whereas both 24°C (90.8%) and 28°C (92%) provided non-significant peak germination rates for FF. Germination of FF at 37°C was unusually high (65%). Although, the virulence of the isolates was not examined, the germination potential at different temperatures may imply their possible application in the above temperature ranges.

The bioassay results obtained in the laboratory were generated under

conditions for the growth of the pathogen and adverse weather conditions prevail. In addition, further studies should be done to see if there are detrimental effects on non-target organisms. It is therefore, imperative to carry out a follow up experiment that is required in the field to acertain the present laboratory based findings.

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