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Evaluation of some native entomopathogenic fungi against pink stem borer (*Sesamia calamistis* Hampson) (Lepidoptera:Noctuidae) in sugarcane

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The present study was carried out to evaluate the indigenous entomopathogenic fungi (EPF), *Metarhizium* and *Beauveria* isolates and to incorporate as potential bio-control agents for the management of the sugarcane pink stem borer (*Sesamia calamistis Hampson*). EPF were isolated from rhizosphere soils of three commercial sugarcane plantations of Ethiopia, indicated from a total of 205 soil samples collected, 10 different fungal isolates were isolated and identified. Out of 10 indigenous EPF obtained, only 4 *Metarhizium* species (i.e. M2E, F3E, W8D and W11D) and 6 *Beauveria* species (i.e. M7A, M9C, M10C, W2D, F10A and F11E) were identified. All the 10 isolated native EPF including with two standard isolates of the Ambo Plant Protection Research Center (MM and PPRC-56) were tested in single dose efficacy experiment (at 1 X 10^8 conidia ml^{-1}) under laboratory conditions. The results indicated that four fungal isolates both from *Beauveria* (M7A and F11A) and *Metarhizium* (M2E and F3E) species produced significantly superior larval mortality over the first four days period. According to 10 days cumulative larval mortality data, M2E, F3E, M7A and F11A were categorized as the most virulent isolates as compared to others. The most virulent isolates were further tested for their potential on *S. calamistis* eggs under laboratory condition and also on pot planted sugarcane plants infested with second to third instars larvae under lath-house condition. Egg susceptibility study indicated that all the isolates were able to kill the eggs as compared to the untreated check. Four days of cumulative results indicated that fungal isolate M2E was significantly superior in affecting egg hatching potential of *S. calamistis* followed by F3E, M7A and F11E under laboratory conditions at 1x 10^8 conidia ml^{-1}. The pot experiment under lath-house condition using the most virulent isolates, namely, F3E, M2E, M7A and F11E were examined at three different conidial concentration levels ($1x10^7$, $1x10^8$ and $1x10^9$ conidia ml^{-1}) including the untreated check. The results showed that isolate F3E at the higher conidial concentration ($1x10^9$ conidia ml^{-1}) produced significantly superior larval mortality after two, four, six and

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eight day's of treatment applications as compared to the others. Similarly, all fungal isolates at their lowest conidial concentration ($1x10^7$ conidia ml^{-1}) level produced significantly inferior larval mortality as compared to other treatments except the untreated check. In general, all fungal isolates showed similar increased trends of control potential as their conidial concentration increased. The results indicated that extremely fruitful prospects of pink stem borer pest management through efficient utilization of biological control of EPF.

Key words: Entomopathogenic fungi, *Beauveria*, *Metarhizium*, Mortality, Virulence, Biocontrol, Pink stem borer, Sesamia calamistis, Sugarc

Introduction

Sugarcane (*Saccharum* spp.) is a perennial crop that is grown as a source of sugar primarily in the tropical and subtropical areas of the world, including several countries in Africa, the Mascarene Islands and Madagascar (Overholt *et al*., 2003). In Ethiopia, sugarcane is grown in some parts even before the commencement of large-scale commercial plantations and establishment of a modern sugar factory at Wonji mainly for local consumption (Aregaw, 2000). The country's annual production of sugar from the sugar estates is about 280,000 tons (Firehun and Tamado, 2006). In Ethiopia, sugar industry plays a great role in the country's economy and sugar and its bi-products are used for local consumption and export. In addition to the aforementioned efforts, intensification of sugarcane cultivation is of a paramount importance. Insect pests are among the major constraints of sugarcane production in the country. Tesfaye and Solomon (2007), about 14 insect pests are reported from sugarcane plantations of Ethiopia. Despite these numerous insect pests, *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae), a pink stem borer is considered to be economically important .insect pest in sugarcane plantations of Ethiopia. In the Ethiopian Sugar Estates, sugarcane borer complex inflicted about 24.86% and 34.34% cane and sugar yield loss, respectively.

There is an increasing interest in the exploitation of Hyphomycetous fungi for the control of invertebrate pests and diseases. Fungal biological control is an exciting and rapidly developing research area with implications for plant productivity, human health and food production. Entomopathogenic fungi (EPF) and several taxa of the other fungi have demonstrated excellent suppression of insect pests in green house conditions (McCoy *et al*., 1988; Ferron *et al*., 1991; Tanada and Kaya, 1993 and Inglis *et al*., 2001). *Beauveria bassiana* has recently been registered against array of green house pests, including stem borers, aphids, thrips, white flies and spider mites (Shah and Goettel, 1999). A recognition of the situation has diverted the attention of scientists to search alternatives to overcome this complex situation, in which biological control is the most desired approach of pest management in that kept

the damage below the economic injury level through least or no contamination of the environment. In the sugarcane plantations of Ethiopia, the presence of naturally occurring biological agents has been reported recently. Yoseph *et al*. (2006) revealed that bacterial (*Bacillus thuringiensis*) and fungal (*Entomophthora spp.* and *Beauveria bassiana*) pathogens were important mortality factors of larvae of all stem borer species on young sugarcane fields at Wonji-Shoa plantation. The results indicated that up to 50 % larvae in young sugarcane (1-3 months old) fields were killed by *B. thuringiensis* with an overall mean percentage mortality of 34.9 % of the stem borer larvae in the estate. However, the potential of these bio-agents is not exploited well. Hence, the present study was carried out to evaluate the indigenous EPF from soils of three commercial sugarcane cultivated fields of Ethiopia and to determined the in vitro efficacy of EPF isolates against S. calamistis larvae and eggs and also to evaluate the selected isolates of EPF for the control of pink stem borer in sugarcane plants under pot culture.

Materials and methods

Survey was carried out in three sugarcane plantation areas viz. Wonji-Showa, Metahara and Finchaa of Ethiopia between October and November, 2010.

Wonji-Shoa Sugar Estate: Wonji-Showa is located in the central part of the East African Rift Valley in Ethiopia at 8^{o} 30' to 8^{o} 35' N longitudes and 39^{o} 20' E latitude and at an altitude of 1540 m. a. s. l. It is situated at about 107 km south east of Addis Ababa, (Fig. 1). The total area under cultivation is about 7050 ha with an average can yield of 155 tons/ha. It receives an average of 831 mm annual rainfall with a bimodal distribution (between mid May- September and February to April). The mean minimum and maximum temperatures are 15^{o}C and 27^{o}C, respectively. In general, soils of Wonji-Showa have been described as a complex of gray cracking clays in the topographic depressions and semiarid brown soils (APECS, 1987).

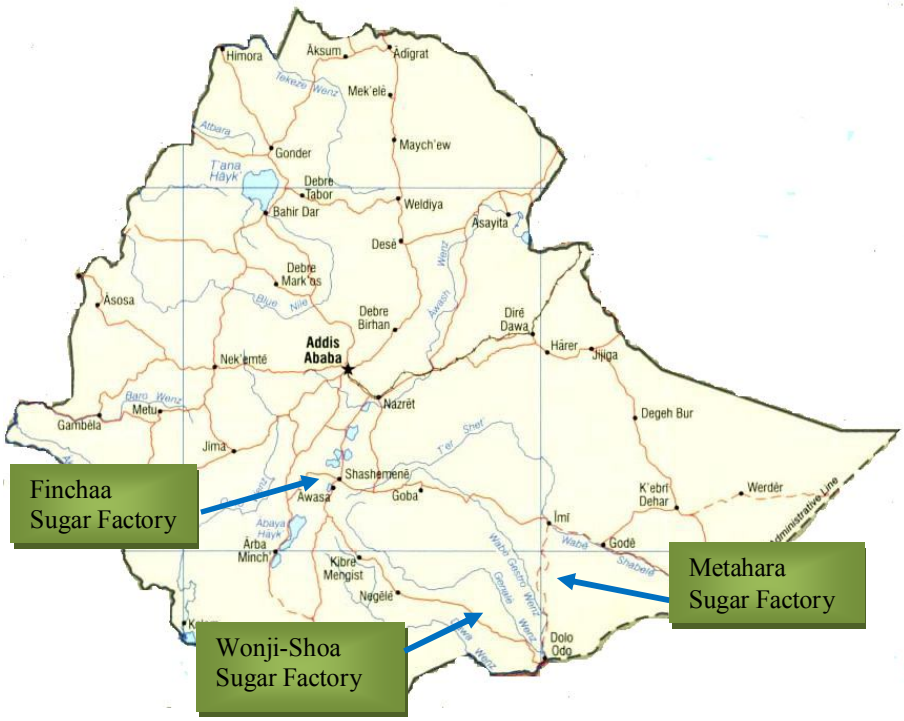


Fig. 1. Location of the governmental sugar factories in Ethiopia (Yoseph *et al.* 2006)

Metahara Sugar Estate, Similarly, Metahara is situated in the central part of the East African Rift Valley at 8°N latitude and 39°52' E longitude and at an altitude of 950 m. a. s. l. The estate is located at about 200 km south east of Addis Ababa, (Fig.1). The total area under cultivation is about 10,000 ha with an average cane yield of 174 tons/ha. It receives an average of 554 mm annual rainfall with a bimodal distribution (between February to April and June to September). The mean minimum and maximum temperatures are 17.4°C and 32.6°C, respectively. Metahara soils were grouped into a complex of vertisol and semiarid brown soils (Girma, 1993).

Finchaa Sugar Estate, the estate is the third plantation established in 1998, and located in the valley of South Western highlands of the country. It is located at a distance of 374 km West of Addis Ababa, between 9° 31' to 10 ° 00' N latitudes and 37 ° 15' to 37 ° 30' E longitude, at an elevation between 1350 – 1650 m. a. s. l., (Fig. 1). The total area under cultivation is about 9500 ha with an average cane yield of 180 tons/ha. It receives an average of 1280 mm annual rainfall with mean minimum and maximum temperature of 14.4 and 30.4°C, respectively. In general soils of Finchaa sugarcane plantation have been described as a Luvisol and Vertisol (Ambachew, 2000).

Experiment I: Survey of native EPF

From Wonji-Shoa, 12 Sugarcane plantation fields were surveyed and 60 soil samples were collected, and from Metahara 11 fields were surveyed and 55 soil samples were collected. Similarly, at Finchaa, 18 fields were surveyed and a total of 90 soil samples were collected. From each field, 5 soil samples were taken at a depth of 5-10 cm by removing the most top surface soil (Tsay *et al*., 2006). A total of 205 soil samples were collected from the three commercial sugar estates. Soil samples were collected at 1 km intervals of the road side of sugarcane fields. The soil samples were collected in plastic bags and transported to Wonji Research Center. The individual soil samples were crushed using mortar and pestle and thoroughly mixed and stored at 4 ^{o} C for further use**.**

Isolation of EPF: Mass rearing of *Galleria mellonella* L. (Lepidoptera: Pyralidae) and used as bait. Adults of *G. mellonella* were collected from Ambo Plant Protection Research Center, Ethiopia. Rearing was performed in plastic boxes incubated in dark inside incubator at 20^{o}C. In a flask, adult moths (in 1:1 female to male ratio) were kept by providing honey and water. In addition, folded tissue paper was placed for oviposition. The paper was removed with the eggs attached and placed in new plastic jar with feed ingredient (Meyling, 2007). The feed composition was 180 g honey, 180 g glycerine and 50 g wheat bran. First honey and glycerine were melted in a cooking pot. After fifteen minutes, it was mixed thoroughly with wheat bran. The feed and egg attached tissue papers were transferred in a bigger jar for rearing inside incubator at 20^{o}C. The feed was changed periodically based on the larval growth stage of *G. mellonella*. Larvae of approximately 2.5-3 cm in length (4 weeks after hatching) and directly used for baiting soil samples (Meyling, 2007).The use of selective media exploits the saprotrophic abilities of hypocrealean EPF. However, to exploit the ability of the fungi to infect host, the insect bait method was used (Zimmermann, 1986).

Before inoculating in the soil, larvae were immersed in boiled water (at 56^{o}C) for 15 seconds and transferred for cooling in a running bath water for 30 seconds in order to prevent webbing. Ten third instar larvae of *G. mellonela* were placed into small glass jars of about 500 ml (on average 300 g soil). Soils were placed on top of the larvae until approximately 2/3 of the containers were filled. The containers were incubated at 22 ^{o} C. Every day, the containers were inverted, so that the larvae continually had a chance to move through the soil, and repeatedly exposed to infective conidia. In the course of the experiment data on mortality was recorded daily for ten days. The dead larvae were collected and submerged into 70% ethanol for one minute and washed in sterile distilled water for three minutes to remove saprophytes and all conidia found on

the outer surface of the larvae body (Odindo, 1994). The disinfected cadavers (dead larvae) were allowed on filter paper to dry for three minutes. This step was added to ensure that mycosis observed on the surface of the cadavers would not be attributed to spores used during the treatment but rather to growth from the interior to the exterior of the insect after colonization of internal organs. Cadavers were held under high humidity on Petri dishes containing damp filter paper to provide sufficient humid conditions to promote fungal outgrowth. Petri dishes were sealed with Para film to maintain greater than 95% RH and were incubated in the dark at 27°C. Larvae were considered mycosis when growth of the fungus was visible on the external surface and those which showed hyphal growth characteristics of the EPF were recorded as infected.

Larvae of *G. mellonela* mortality were checked continuously for ten successive 10 days after inoculating in the soil sample. Fungi samples outgrowing and sporulating on the cadaver were cultured on artificial media, Sabouraud's Dextrose Yeast Agar (SDYA) and pure cultures obtained through successive transfer for identification. Tentative identification was done based on macro and micro growth and morphological characters of fungi (Subramanian, 1971; Lacey, 1997; Sinishaw, 2002). Pure cultures of EPF were sub-cultured on to Sabouraud's dextrose agar with yeast extract (SDAY). These were incubated at 27^{o}C, 75% RH and photoperiod of 12:12h light and dark for ten successive days till there were full culture development and sporulation. The surface of ten-day old cultures were scrapped with a sterile scalpel and suspended in aqueous solution of 0.01% Tween 80. The fungal suspension was vortexed for one minute to break up the conidial chains or clumps and filtered through several layers of sterile cheesecloth to remove mycelia. The dose of conidia in the filtrate was estimated using haemocytometer under a light microscope (40 x magnifications). Conidial suspension ($1x10^7$, $1x10^8$ and $1x10^9$ conidia per ml) was prepared for each isolate and applied on egg and larval growth stages of S. calamistis.

Mass rearing of S. calamistis: For mass rearing of *S. calamistis,* larvae were collected from sugarcane plantation fields and mass reared at Wonji Plant Protection Department laboratory using young sugarcane stalk as a feed (Leul *et al*., 2009). A total of 390 fourth instar larvae of *S. calamistis* of the second generation wasused for in-vitro efficacy experiment and a total of 1040 second to third instar larvae of *S. calamistis* were used for pot experiment. In addition, 200 black head stages of eggs were used for egg susceptibility study.

Experiment- II: In-vitro efficacy of EPF isolates against S. calamistis larvae

In this efficacy study, all the 10 identified native EPF fungal isolates indicated in Table 1 including two standard EPF fungal isolates (MM and

PPRC-56) were screened against larvae of *S. calamistis*. Fourth instar *S. calamistis* larvae were treated in a Petridish. Ten larvae were introduced in each Petridish with filter paper and young fresh chopped cane stems inside. For each isolate an aqueous suspension containing 1.0 x 10^8 conidia ml^{-1} was prepared in 0.01% Tween 80 and applied using micro-pipette. Sterile distilled water with 0.01% Tween 80 was used as free control treatment. The treated insects were incubated at 27 °C and 70 ± 5% RH and maintained for 10 days. The experiment was laid out in a Complete Randomized Design (CRD) with three replications; and a total of 13 treatments were used. In the course of the experiment, data on mortality was recorded daily for ten days. The dead larvae were collected and submerged into 70 % ethanol for three seconds and 5% sodium hypochlorite for two minutes (Odindo, 1994) and washed in sterile distilled water for three minutes to remove saprophytes and all conidia found on the outer surface of cadavers. The disinfected cadavers (dead larvae) were allowed to dry for ten minutes on Watmann No.1 filter paper. Cadavers were held under high humidity on Petridishes containing damp filter paper to provide sufficient humid conditions to promote fungal outgrowth. Petri dishes were sealed with Para film to maintain greater than 95% RH and incubated in the dark at 27°C. A larvae was considered mycosis when growth of the fungus was visible on the external surface and those which showed hyphal growth characteristics of the EPF were recorded as infected.

Mortality data was corrected for the corresponding Control Mortality by the formula:

$$CM(\%) = \frac{(T - C)}{(100 - C)} * 100$$

Where CM is corrected mortality, T is percent mortality in treated insects and C is Percent Mortality in untreated insects (Abott, 1925).

The effect of native fungal isolates on the larval mortality was analyzed using one-way analysis of variance (SAS Institute, 1999). Moreover, LT_{50} value was also determined for all fungal agents using daily records of percent mortality data.

Experiment-III: Efficacy of selected Beauveria and Metarhizium species against eggs of S. calamistis

Four potential fungal isolates, namely F3E, M2E, M7A and F11E were used for this experiment. The isolates were selected based on the observed potential in the screening experiment.For each isolate, aqueous suspension with

0.01% Tween 80 at 1.0 x 10^8 conidial ml^{-1} doses were used for inoculation and for the control group only sterilized distilled water with 0.01% Tween 80 was used. Ten black headed stages (just ready to hatch) of eggs of *S. calamistis* were transferred to a Petridish with young chopped sugarcane seedling. Each was sprayed with half ml of conidial suspension of isolates having a concentration of 1.0 x 10^8conidial ml^{-1}. Each treatment was replicated four times. After spraying, all eggs on Petridishes were incubated at 27^{o}C, 70 ± 5% RH, photoperiod of 12:12h day and night; and examined daily. Data were collected on the number of hatched and un-hatched eggs starting from 24 h after inoculation for seven consecutive days.

Egg mortality due to fungal infection was assessed based upon the distortion or shrinkage in egg shape and non-emergence (Kongchuensin and Takafuji, 2005). Furthermore, confirmation of fungal isolate that caused egg mortality was assured by transferring all the un-hatched egg in moist chambers for 3 days. Then after the fungal outgrowth, it was verified whether eggs death were due to the native fungal isolate infection.

Experiment IV: Evaluation of EPF isolates against S.calamistis in sugarcane under pot culture

This experiment was conducted in the lath-house at Ambo University, Ethiopia on sugarcane (var.B52298) under pot culture.Two double budded sets were planted in each pot (21cm diameter and 19 cm height). The pots were initially filled with composition of black soil, compost and sand at a proportion of 2:1:1 and watered at three days intervals. Urea at a rate of 0.52 g per pot was applied while the crop became one and half months of age. The pots with sugarcane plants were placed in the lath-house at a photoperiod of 12 hrs and mean daily temperature of 28°C on average (range 16-40°C as measured with a thermo hygrograph) and with 30 – 75 % RH.

Four potential EPF isolates, namely F3E, M2E, M7A and F11E were used for this experiment. Conidial suspension of these isolates were prepared, diluted and counted using haemocytometer. Three different doses of each isolate at 1× 10^6, 1× 10^7 and 1× 10^8 ml^{-1} concentrations with 0.01 % Tween 80 including untreated check were used. The control (untreated check) pots were treated with sterilized distilled water having 0.01 % Tween 80. Inoculations of the second to third larval instars of *S. calamistis* were used while the plants became 60 to 70 cm height (2.5 months). After four hours of larval inoculation on pot planted sugarcane, treatments were applied using hand held sprayer early in the morning. Each treated pot plants were placed in the lath-house in a separate cage in order to avoid any damage from natural enemies such as ants, rodents etc. Cage (having 2 m height, 8 m length and 2 m width) was built and

divided into two compartments per test organism of the isolate. The experiment was laid out in a 4 x 4 factorial Randomized Complete Block Design (RCBD) with four replications.Five larvae (2^{nd} to 3^{rd} instars) were inoculated per single shoot. Two vigorous grown shoots per pot were selected and inoculated. A total of 1040 second to third instars larvae were used for this entire experiment.

Inoculation of the larvae was done by slightly detaching the top two leaf sheath and safely transferring the larvae inside the leaf sheath. Data on larval mortality was taken at two days interval from randomly selected shoot per pot. In each sampling, dead and alive larvae count was made destructively for eight days. Those cadavers were transferred to on incubator for further confirmation of the causes of death as indicated by Lacey (1997) and Sinishaw (2002). Percentage of efficacy of the native isolates were determined by means of the Abbott (1925) formula, which was used when the infestation of the pest was homogeneous before treatment application:

% Efficacy = (Cd – Td) X 10/ Cd

Where: Cd: Number of live individuals in the control plots after the treatment. Td: Number of live individuals in the treated plots after the treatment.

Statistical analysis

Data analysis was carried out using SAS software package (SAS Institute, 1999). Before undertaking data analysis to stabilize normality arcsine transformation was made (Gomez and Gomez, 1984). Data presented in the text was the untransformed ones.

Results and discussions

Survey of indigenous EPF in three sugarcane plantations of Ethiopia

A total of 41 fields from the three sugarcane plantations were surveyed and a total of 205 soil samples were collected and identified indigenous EPF isolates through examination of the growth, morphology and microscopic features of conidiogenous cells. Out of 205 soil samples, a total of 10 EPF were isolated and identified. Out of ten different indigenous EPF isolates obtained, only 4 *Metarhizium* species (i.e. M2E, F3E, W8D and W11D) and 6 *Beauveria* species (i.e. M7A, M9C, M10C, W2D, F10A and F11E) were identified (Table 1). and isolates that produced white powdery mass of spores on the external surface of *Galleria* larval cadavers were identified as *Beauveria species* (Fig 2), whereas, those isolates that infected the *Galleria* larvae and produced green

crust like velvet on the walls of the cuticle were identified as *Metarhizium species* (Fig 3).

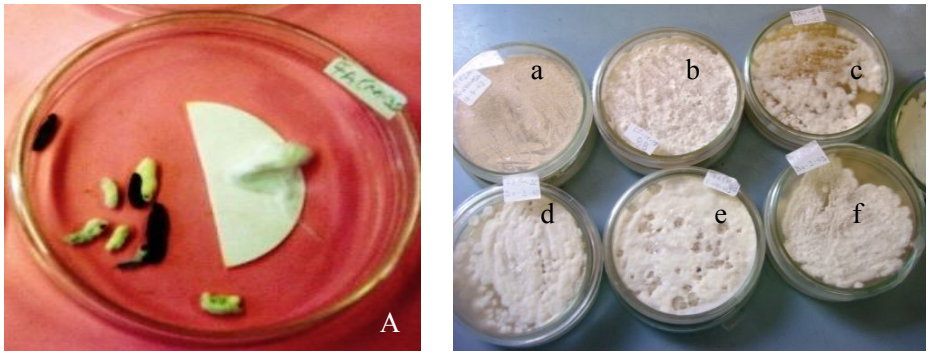


Fig. 2. *Beauveria* species (A) fungal outgrowth on *Galleria* cadaver; six different isolates of *Beauveria* cultured on SDYA media (a) M7A (b) M9C (c) M10C (d) W2D (e) F10A and (f) F11E

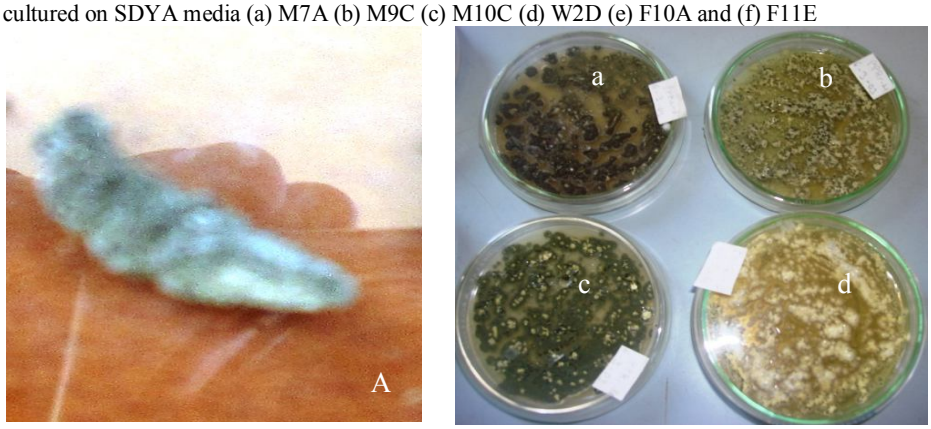


Fig. 3. *Metarhizium* species (A) outgrowth on *Galleria* cadaver; four different isolates of *Metarhizium* cultured on SDYA media (a) M2E (b) F3E (c) W8D and (d) W11D

Table 1. Survey of indigenous EPF in three sugarcane plantations (Metahara, Wonji and Finchaa) of Ethiopia

Sample code	Field name	Geographical location	Altitude (m.a.s.l)	Soil type	Soil pH	Remarks
M2E	O-7	N 08^0 48.182', E 039^0 54.725'	968m	Heavy clay	8.36	M.sps
M7A	M-35	N 08^0 49.904', E 039^0 57.132'	967	Heavy clay	7.77	B.sps
M 9C	H-20	N 08^0 48.718', E 039^0 56.734'	979	Heavy clay	8.37	B.sps
M 10C	H-29	N 08^0 48.292', E 039^0 55.832'	972	Heavy clay	8.35	B.sps
W 2D	33	N 08^0 25.687', E 039^0 14.068'	1550	Heavy clay	8.13	B.sps
W 8D	193	N 08^0 25.146', E 039^0 15.056'	1544	Heavy clay	8.16	M.sps
W 11D	P&L	N 080 27.310',E 0390 13.789'	1546	Heavy clay	7.85	M.sps
F 3E	Forest	N 09^0 49.504', E 039^0 23.726'	1428	Luvisol	6.81	M.sps
F 10A	PS-503	N 09^0 47.818', E 037^0 22.286'	1513	Luvisol	6.61	B.sps
F 11E	Go-127	N 09^0 45.514', E 037^0 23.978'	1503	Luvisol	6.51	B.sps

NB: Sample codes M1A, W1A & F1A implies Metahara, Wonji-Shoa and Finchaa sample number two A. M.spps and B. spps. meaning *Metarhizium species and Beauveria species*

Out of those 10 identified EPF, two *Metarhizium* spp. and one *Beauveria* spp. were found from Wonji-Shoa, one *Metarhizium* spp. and three *Beauveria* spp. from Metahara and one *Metarhizium* spp. and two *Beauveria* spp. were from Finchaa sugarcane plantations (Table 2). *Beauveria* species were the dominant (60%) EPF in sugarcane plantations as compared to *Metarhizium* spp.(40%). Higher proportions of EPF were obtained from Metahara as compared to the other two plantations. These findings are in line with that of Yoseph *et al*. (2006) who indicated that bacterial pathogen, *Bacillus thuringiensis* and fungal pathogens, *Entomophthora spp.* and *Beauveria bassiana* were found at Wonji-Shoa sugarcane plantation fields.

Table 2. Indigenous EPF isolates obtained from three commercial sugarcane plantations

Site	No.of fields surveyed	No.of soils sampled	Fungal isolates obtained	Common fungi spp.
Wonji-Shoa	12	60	2 M.spp., 1 B.spp.	*Metarhizium* species
Metahara	11	55	1 M.spp., 3 B.spp.	*Beauveria* species
Finchaa	18	90	1 M.Spp., 2 B.spp.	*Beauveria* species

NB: B. spp. and M. spp. implies *Beauveria* and *Metarhizium* species respectively.

Screening of indigenous fungal isolates of Beauveria and Metarhizium species against larvae of S. calamistis

Ten indigenous fungal isolates collected from the three sugar plantations and the two standard fungal isolates (MM and PPRC-56) obtained from APPRC, Ambo, Ethiopia were capable of infecting and killing the larval stages of *S. calamistis* under laboratory conditions. Mortality was observed every 24 hours after treatment application for ten consecutive days. Mortality resulted due to the fungal isolates were confirmed based on the visual observation of fungal outgrowth (mycosis) on the surface of larval cadaver as indicated by Sinishaw (2002). All larvae killed by the fungal isolates became tough and rigid upon death. The *Beauveria* isolates F10A, M7A, W10C, M9C, F11E and W2D commenced white sporulation gradually covering the insect body when the cadavers were placed in Petridishes and comparison was made to the respective shape and color of the isolates already preserved on SDAY media (Fig. 4). Similarly, those larval cadavers killed by *Metarhizium* isolates M2E, W11D, F3E and W8D produced green sporulation while placed on Petridishes and comparison was made with the respective isolates cultured on SDAY media (Fig 5). It was observed that there were significant variations in the percentage mortality and the time taken to kill the larvae among the different isolates tested (Tables 3 and 4). Among the fungal isolates, M2E (40%), F3E (76.67%) and M9C (26.67%) incurred highest mortality in the two, four and eight days after treatment application, respectively, as compared to the others isolates (Table 3). Fungal isolates MM and W8D caused highest (30% each) mortalities after six days of treatment application as compared to the other treatments.

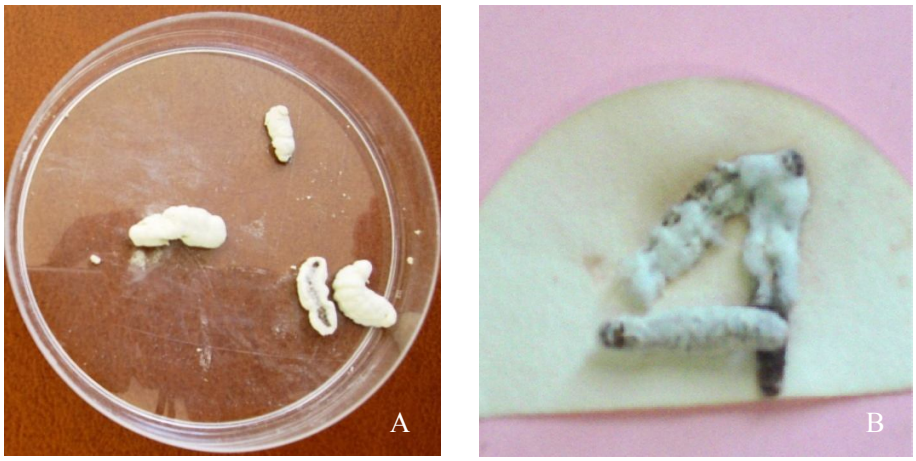


Fig. 4. a and b. S. calamistis larvae killed by native Beauveria isolates (a) M7A and (b) F11E

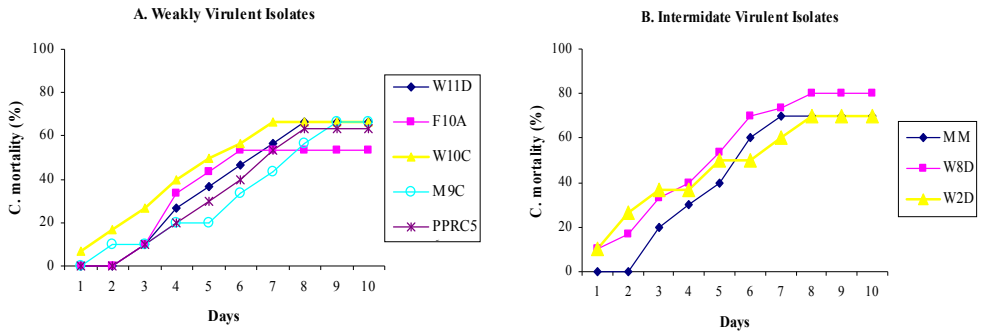
Fig. 5. a and b S. calamistis larvae killed by native Metarhizium isolates (a) M2E and (b) F3E

Table 3. Percentage mortality of the sugarcane stalk borer larvae (S. calamistis) treated with different fungal isolates of Beauveria and Metarhizium species

Fungal Isolates	Mortality* ±SE 2DATAppl.	Mortality* ±SE 4DATAppl.	Mortality* ±SE 6DATAppl.	Mortality* ±SE 8DATAppl.
M2E	40±0.00a	60±0.00b	0±0.00f	0±0.00d
W11D	0±0.00e	26.67±2.21cde	20±0.00bc	20±0.00b
F3E	13.33±2.71cd	76.67±2.21a	10±0.00e	0±0.00d
MM	0±0.30e	30±0.00cd	30±0.00a	10±0.00c
W8D	16.67±2.71c	23.33±2.21de	30±3.66a	10±0.00c
F10A	0±0.00e	33.33±4.22c	20±0.00bc	0±0.00d
M7A	16.67±2.71c	56.67±1.92b	26.67±2.21ab	0±0.00d
W10C	16.67±2.71c	23.33±2.21de	13.33±2.71cd	10±0.00c
M9C	10±0.00d	10±0.00f	16.67±2.71de	26.67±2.21a
F11E	16.67±2.71c	53.33±1.92b	26.67±2.21ab	0±0.00d
PPRC-56	0±0.00e	20±0.00e	20±0.00bc	23.33±2.21ab
W2D	26.67±2.21b	10±0.00f	13.33±2.71de	20±4.27b
Un treated	0±0.00e	0±0.00f	0±0.00f	0±0.00d

*Values followed by the same letter in the same column do not differ significantly (P>0.05) according to DMRT

Depending on the efficacy result, the isolates were categorized as highly (81-100%), intermediately (71-80%) and weakly virulent (60-70%) (Fig.6). These findings are in agreement with that of Thungrabeab *et al*. (2006) who classified the EPF based on the percentage of mortality as highly pathogenic (> 64.49), moderately pathogenic (30.99-64.49) and low pathogenic (<30.99). Similarly, Addiss (2008) also grouped the EPF as most virulent (84.8-98.32), moderately virulent (40-73) and weak virulent (<40) based on percent mortality.



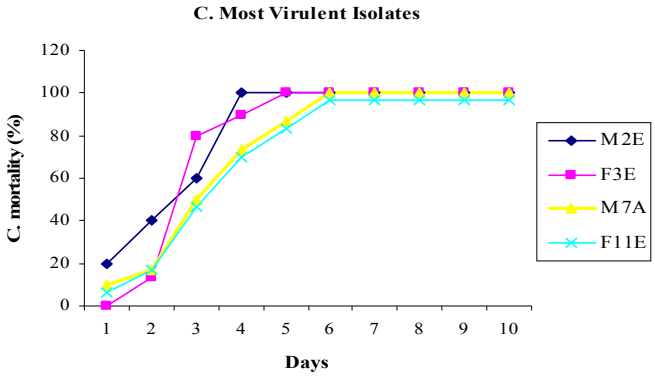


Fig. 6. Percentage cumulative mortality of *S. calamistis* larvae following treatment with different native *Beauveria* and *Metarhizium* isolates, (a) weakly, (b) intermediate and (c) highly virulent isolates

As indicated in Table 3, all the EPF isolates showed reduction in the overall larval population of *S. calamistis* as compared to the untreated check. Four days cumulative larval mortality results indicated that M2E, F3E, M7A and F11E produced above 70% larval population reduction as compared to the other isolates (Table 4). Only two fungal isolates of *Metarhizium* species (M2E (100%) and F3E) showed the highest (100%) virulence potential within the first four days period (Tables 3 and 4) as compared to the others. Similarly, two fungal isolates from *Beauveria species*, (M7A and F11E) caused highest (100% & 96.67%, respectively) mortality over eight days period. Based on, the cumulative mortality over the first eight days it can be seen that all of the isolates of *Metarhizium* and *Beauveria* species gave more than 50% control of the larval population of *S. calamistis* (Tables 3 and 4). The highest virulence was recorded from fungal isolates M2E, F3E, M7A and F11E.Though all the isolates induced mortality, the single dose time mortality experiment indicated significant differences in LT_{50} values among isolates (Table 4). The mortality rates of the isolates were broadly correlated with LT_{50}. The isolates which had the least LT_{50} values showed the highest mortality rates and vice versa. The LT_{50} values ranged from 1.00 to 11.19 days. The most, intermediate and weakly virulent isolates had LT_{50} values ranging from 1.00 to 4.9, 5.4 to 8.21 and greater than 11.00 days, respectively. This was clearly shown that in addition to mortality, LT_{50} values can effectively measure the virulence of the isolates. The activity of the immune system of the host and the fungal response are likely to be important factors determining virulence (Moorehouse *et al*., 1993). The development of fungal pathogen within hosts can be influenced not only by immune reaction of the host but also indirectly by the hosts diet (Tadele, 2003).

The difference in the ability of an isolate to overcome the immune system may be reflected in the increased insect survival following treatment as well as the higher LT_{50}. Moorehouse *et al*. (1993) demonstrated that LT_{50} was correlated with spore dose and it is, therefore, only the differences observed in LT_{50} values in isolates tested here could reflect genetic and physiological differences between isolates or factors such as toxins or characteristics of the insect host.

Table 4. Percentage mortality and LT_{50} of *S. calamistis* larvae after treatment with isolates of indigenous *Beauveria* and *Metarhizium* species at the rate of 1x 10^8 conidia ml^{-1}

Fungal isolates	Cumulative Mortality (±SE) (4 days)	Cumulative Mortality (±SE) (8 days)	LT_{50} (days)	95% Fiducial limits	Intercept ± S.E	X^2	P-value
M2E	100±0.00a	100±0.00a	3.60±1.57	2.50 – 4.35	3.48±1.10	15.41	0.0001
W11D	26.67±2.21fg	66.67±2.00bc	8.212±0.44	3.01-16.11	-0.22±0.37	2.35	0.0001
F3E	100±0.00a	100±0.00a	1.00±0.49	1.29-10.99	0.003±0.4529	1.69	0.0001
MM	30±0.01ef	70±0.00bc	11.19±0.46	5.30-11.00	-0.54±0.42	1.23	0.0001
W8D	40±0.00d	80±4.27b	5.42±0.63	3.28-10.10	1.85±0.49	15.77	0.0001
F10A	33.33±4.22def	53.33±3.84c	4.22±0.80	2.89-9.00	2.11±0.44	32.54	0.0001
M7A	73.34±2.21b	100±0.00a	3.23±0.97	2.21-4.36	2.15±0.57	25.33	0.0001
W10C	40±3.40d	63.33±4.22bc	7.92±0.59	5.29-13.99	2.11±0.44	20.02	0.0001
M9C	20±0.00g	63.34±3.40c	6.62±0.99	4.50-10.71	-2.59±0.82	10.05	0.0015
F11E	70±0.00c	96.67±5.80a	4.92±0.52	5.29-13.99	2.11±0.44	20.01	0.0001
PPRC-56	20±0.00g	63.33±2.00c	7.10±0.45	4.15 – 8.22	-0.11±0.43	1.16	0.0023
W2D	36.67±2.00de	70±7.69bc	5.39±43.00	3.21 – 9.72	38.47±24.12	0.03	0.0002
Control	0±0.00h	0±0.00d	-	-	-	-	-

Values followed by the same letter with in the same column do not differ significantly from each other (P>0.05) according to DMRT

Efficacy of selected Beauveria and Metarhizium species against eggs of S. calamistis

Untreated eggs of *S. calamistis* (at black headed stage) started to hatch within a day and hatching rate increased through time and completed within 4-5 days. The fungal isolates caused different levels of mortality on eggs of *S. calamistis* (Table 5). There were significant differences among treatments and the least mortality (10%) was observed in the untreated control. Isolate M2E resulted in the highest egg mortality (71.92%) followed by F3E (51%), M7A (42.24%) and F11E (26.75%) within four days after treatment application. This study also revealed that hatch rates between fungal isolate treatments and the untreated control were too small following one day after treatment application and the hatch rate showed significant variation afterwards. The highest hatch

rate (90%) was recorded in the untreated control. From day four on, hatch rates at each of the fungal isolate did not change in the following two days. Thus, observations on fourth day were used to indicate mortalities of eggs. The observed hatch rates both in the treated eggs and in the untreated control were considered to determine the level of egg mortality. Those un-hatched eggs were considered as dead ones. Un-hatched eggs due to fungal isolates were confirmed through careful observation using hand lens (40X magnification power) on the egg shape and morphology. Those which looked sunken, distorted and shrunk were considered as dead. Moreover, fungal outgrowth observed on the un-hatched eggs after keeping in moist chambers at 25±3^{o}C for about 3-5 days. The fungal outgrowths were identified as *Beauveria* and *Metarhizium* species based on their characteristic color when the wizened eggs were individually placed in SDYA. Marannino *et al*. (2006) reported a significant reduction in egg hatch for the buprestid *Capnodis tenebrionis* treated with *B. bassiana*. Silva *et al*., (2006) reported higher mortality for *B. bassiana* treated eggs of the tenebrionid *Alphitobius diaperinus* than for other stages of the insect; and similarly, Chikwenhere and Vestergaard (2001) reported that infected eggs of the weevils *Neochetina bruchi* showed highest mortality. Wekesa *et al*. (2006) reported the susceptiblity of various developmental stages of *Tetranchus evansi to M. anisopliae* and *B. bassiana* under laboratory condition. The fungi reduced fecundity and egg fertility. Females treated with the fungi laid fewer eggs than the untreated control. Gouli *et al*. (2005) also described EPF, *B. bassiana* and *M. anisopliae* often able to infect several development stages including eggs. Samuels *et al*. (2002) reported that *M. anisopliae* was highly virulent to eggs of the chinch bug, *Blissus antillus*. Al-Degahri (2008) observed that the EPF, *B. bassiana* had lethal effect against eggs, and young and old nymphs of white fly. Similarly Kaaya *et al*. (2002) reported both *B. bassiana* and *M. anisopliae* induced reduction in engorgement weights, fecundity and egg hatchability (94-100%) on adult tropical African Bont Tick (*Amblyomma variegatum*) which feeds on bloods of animals.

Furthermore, *B. bassiana* reduced egg hatchability to 0%, while 11% of the infected females failed to lay eggs. In agreement with the above reports, in this study, the native EPF (*Metarhizium* and *Beauveria species*) not only were virulent to the larvae of *S. calamistis* but also caused mortality on their egg stage.

Table 5. Percentage of un-hatched four days eggs after application of fungal isolates

Isolates	Cumulative % egg un-hatched (after 4 days)
F3E	51.00±3.19b
M2E	71.92±3.38a
M7A	42.24±2.14b
F 11E	26.75±2.87c
Untreated control	10.00±2.50d

Values followed by the same letter with in the same column do not differ from each other significantly (P>0.05) according to DMRT

EPF isolates against S.calamistis in sugarcane under pot culture

This study conducted using the four most virulent native EPF isolates, namely, F3E, M2E, M7A and F11E at three different level of concentration 1 x 10 ^{7}, 1x10^{8} and 1x 10^{9} conidia ml^{-1} including untreated check indicated that these was a significant differences in mortality among isolates inoculated at different concentrations on two, four, six and eight days after treatment application (Table 6).The least mortality (10%) was recorded in the untreated control even eight days after treatment application. This low record of mortality in the control was a natural death as it was observed that there was no growth of any one of the above four fungus when kept in moist chambers at 25±3^{o}C for about 3-6 days. All the tested native EPF isolates caused significantly higher larval mortality as their concentration and duration increased (Table 6).

Table 6. Percentage cumulative mortality of larval stage of *S. calamistis* treated with different conidial concentrations of indigenous *Beauveria* and *Metaharizium* species

Conc. (ml-l)	Mortality (mean)							
	After 2 days treatment application				After 4 days treatment application			
	F3E	**M2E**	**M7A**	**F11E**	**F3E**	**M2E**	**M7A**	**F11E**
Control	10.00h	10.00h	10.00h	10.00h	9.00h	10.00h	9.25h	6.25h
1 x 10^{7}	48.88c	37.78efg	36.58fg	32.08g	54.17de	46.00ef	41.67f	32.50g
1 x 10^{8}	61.38b	47.22cd	44.44cde	40.00def	63.50c	62.50cd	61.11cd	45.25ef
1 x 10^{9}	75.50a	66.00b	60.00b	59.75b	82.22a	72.75b	73.25b	73.14b
Conc. (ml-1)	**Mortality (mean)**							
	After 6 days treatment application				After 8 days treatment application			
	F3E	**M2E**	**M7A**	**F11E**	**F3E**	**M2E**	**M7A**	**F11E**
Control	8.00h	10.00h	7.00h	7.50h	10.00g	10.00g	10.00g	10.00g
1 x 10^{7}	53.75def	52.50def	48.89efg	38.19g	54.31ef	64.50de	48.75f	47.11f
1 x 10^{8}	75.83bc	64.17cd	61.11de	45.75fg	86.67b	75.83cd	73.75cd	66.67de
1 x 10^{9}	93.00a	81.00b	75.25bc	80.56b	100.00a	80.00bc	84.17bc	84.17bc

* Values followed by the same letter with in the same column do not differ from each other significantly (P>0.05) according to DMRT

The native EPF isolate F3E (i.e. 75.50%, 82.22%, 93.00% and 100%) produced significantly superior mortality at the higher concentration (1 x 10^9 conidia ml^{-1}) in two, four , six and eight days after treatment applications, respectively, as compared to others. Similarly, the fungal isolates M2E, M7A and F11E were produced significant mortality followed F3E at their higher concentration level after two, four, six and eight day's treatment applications. Almost all of the tested native fungal isolates produced lowest larval mortality in their respective lower concentration levels as compared to the others levels. On average the indigenous fungal isolates F3E, M2E, M7A and F11E produced more than 87.09% larval population reduction over the first eight day's period. The results of the pot experiment using the most virulent isolates, F3E, M2E, M7A and F11E at different conidial concentration levels of $1x10^7$, $1x10^8$ and $1x10^9$ conidia ml^{-1} showed that F3E isolate at the highest conidial concentration produced significantly superior larval mortality after two, four, six and eight days of treatment application. Similarly, all fungal isolates at the lowest conidial concentration produced significantly lower larval mortality than the other treatments except the untreated check. All of the fungal isolates showed increased efficacy as the conidial concentration increased.

Conclusions

In the present study, the indigenous EPF isolates identified from the commercial sugarcane fields showed the potential for the management of *S. calamistis* in an eco-friendly and sustainable manner and it can also be used as an alternative for the synthetic pesticides as used in the commercial sugarcane plantations of Ethiopia. Moreover, it can avoid any pesticide resistance development and create a competitive market advantage by reducing the associated problems of agrochemicals at large. This is the first work of its kind to be conducted in commercial sugarcane plantations of Ethiopia. The results indicated extremely fruitful prospects of insect pest management through efficient utilization of biological control at large and EPF in particular. Future research works in techniques for mass production, appropriate formulation to keep the quality, large scale application are needed .and also variation in virulence among the identified few EPF isolates clearly indicated larger collection and screening in the future research activity.

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