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Research article

Phenotypic, molecular, and virulence characterization of entomopathogenic fungi, *Beauveria bassiana* (Balsam) Vuillemin, and *Metarhizium anisopliae* (Metschn.) Sorokin from soil samples of Ethiopia for the development of mycoinsecticide

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ABSTRACT

Fungal entomopathogens are the most effective biocontrol agents against insect pests in the natural ecosystem. This study was conducted for phenotypic, molecular, and virulence characterization of locally isolated entomopathogenic fungi from soil samples of six localities in Ethiopia. Entomopathogenic fungi were isolated from 120 soil samples with the *galleria* baiting method. A total of 65 (54.2%) entomopathogenic fungal isolates belongs to *Beauveria* spp and *Metarhizium* spp were identified based on cultural and morphological features. All isolates were pre-screened based on germination, vegetative growth rate, and spore production as fungal *in-vitro* virulence determinates. Isolate AAUKB-11 displayed the peak germination of 99.67% and isolate AAUMFB-77 achieved the highest radial growth rate of 3.43 mm day^{-1} with the highest sporulation 4.60 × 10^8spores/ml. The phylogenetic analysis of ITS-rDNA confirmed that 7 isolates were identified as *B. bassiana* and 5 isolates were categorized into *M. anisopliae.* Selected *B. bassiana* and *M. anisopliae* strains were evaluated for their pathogenicity efficiency against *G. mellonella* larvae and caused 86.67%–100% mortality. The mortality rates of *G. mellonella* larvae peaked at 100% with 4(33.33%) isolates from *B. bassiana* and 2(16.67%) isolates from *M. anisopliae* after 10 days of treatments. The high virulent isolate, *B. bassiana* AAUMB-29 displayed the least LT_{50} value of 2.36 days followed by isolate *B. bassiana* AAUMFB-77 with LT_{50} of 2.53 days. Future studies should be needed to focus on the evaluation of high virulent isolates against other potential insect pests to assess their vigorous role as favorable biological control agents.

1. Introduction

Entomopathogenic fungi (EPF) are cosmopolitan natural enemies of arthropod pests and they are effective for the regulation of numerous insect pests in natural ecosystems in an eco-friendly manner. The endophytic and epiphytic characteristics of EPF induce plant resistance to insect pests (Klieber and Reineke, 2016; Ramakuwela et al., 2020) and microbial disease-causing agents by increasing plant defense responses (Moonjely et al., 2016). Consequently, they have attracted attention as microbial insecticides to control insect pests because of their high virulence, broad host range and can be isolated from wide ranges of soil habitats particularly cultivated and forest soils existence in a wide range of habitats (Gürlek et al., 2018; Mishra et al., 2015).

The *Beauveria, Metarhizium,* and *Paecilomyces* are predominant genera of entomopathogenic fungi widely used as biocontrol agents throughout the world. Among these, the white muscardine fungus *B. bassiana* and the green muscardine fungus *M. anisopliae*, are the most pronounced fungal entomopathogens for the control of sucking and chewing agricultural insect pests and play a vital role in the integrated pest management strategies (Malekan et al., 2015). The *B. bassiana* is reported to infect 707 species of insect hosts (Imoulan et al., 2016) whereas *Metarhizium anisoplae* is infecting over 200 species of insect pests (Jitendra et al., 2012).

The commercial products of *M. anisoplae* and *B. bassiana* are in use for bio-control of diverse insect pests in agriculture for a long time. However, they are non-adaptable to different agro-ecological conditions and their continuous application in an ecosystem encounters reduced efficacy on

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target insect pests (Dhar et al., 2019). This can be overcome with isolation and identification of potential local isolates for pest management. The indigenous isolates of EPF from different localities or hosts deliver control programs with available and specific controlling methods against indigenous pests because they are more adapted to a range of environmental conditions (Zayed, 2003). Different studies also showed that indigenous isolates of fungal entomopathogen are effective against various agricultural pests under local conditions (Clifton et al., 2019; Hernandez-Trejo et al., 2019).

Isolation of Entomopathogenic fungi is frequently based on insect bait method using *Galleria mellonella* larvae from soils (Mantzoukas et al., 2020b) and insect cadavers (Meng et al., 2017), of which insect bait using *G. mellonella* is a very sensitive detection method. Besides *in vitro* characterization of entomopathogenic fungi through germination, radial growth and sporulation parameters are important in defining the virulence of fungal isolate (Islam et al., 2014). Furthermore, identification of EPF based on molecular technique is a prerequisite for distinguishing species more accurately for the successful control of insect pests. Internal transcribed spacer ITS1-5.8S-ITS4 region of ribosomal DNA (rDNA-ITS) is the most widely used for detection and identification of various *Beauveria* spp (Belay et al., 2017) and *Metarhizium* spp (Islam et al., 2014).

In Ethiopia, several studies have revealed the virulence efficacy of Ethiopian indigenous isolates of entomopathogenic fungi, *Beauveria* and *Metarhizium* spp. against diverse insect pests such as *Tuta absoluta* (Tadele and Emana, 2017), spotted spider Mites (Negash et al., 2017), and *Helicoverpa armigera* (Fite et al., 2020). These studies showed that the local entomopathogenic fungi were more effective than the reference exotic isolates. Hence, biological control of insect pests by using local isolates of fungal entomopathogens may be a promising choice regarding environmental suitability with pest species in comparison to exogenous fungal isolates (Imoulan et al., 2011; Lee et al., 2015). This necessitates isolation, identification, and pathogenicity screening of indigenous entomopathogenic fungal bio-control agents is crucial to identify novel virulent isolates for effective insect pest management under greenhouse and field conditions. Thus, this study aimed at isolation, phenotypic characterization, molecular identification, and pathogenicity screening of *B. bassiana* and *M. anisopliae* for effective control of insect pests of tomato grown from different parts of the Rift Valley.

2. Material and methods

2.1. Description of soil sample collection sites

Soil samples were collected from various tomato-growing farmlands (Debre Zeit, Koka, and Ziway) in Central Rift Valley and forests (Menagesha and Entoto) (Table 1). Debre Zeit is situated at 8° 45′26.10″ N latitude, 39°00′46.42″ E longitude with an altitude of 1879 m.a.s.l. The area has to mean annual maximum and minimum temperatures of 28 °C and 10.2 °C, with sub-humid climate type and means rainfall of about 47mm and relative humidity of about 51% during the off-season, respectively. Koka is located at 8° 25′07.25″ N latitude, 39°02′29.25″ E longitude, and altitude 1605 masl. The area is characterized by mean minimum and maximum temperature of 12.14 °C and 27.39 °C, respectively (ESDA, 2010). The Meki town is located at central rift valley at 8° 01'to 8° 25′N Latitude and 38° 32′to 39°04′E Longitude. It has an altitude range from 1600 to 2000 m.a.s.l. The mean annual temperature and rainfall are 22–28 °C and 700–800 mm, respectively (CSA, 2011). Ziway is situated at 7°58′25.39″ N latitude, 38° 43″21.15″ E longitude with an altitude of 1645 m.a.s.l. Entoto forest located between latitudes 9°04′N - 9°06′N and longitudes 38°44′E - 38°49′E and average altitudes of 2850 m a.s.l which ranges between 2, 600 and 3,100 m a.s.l (EWNHS, 2001). The Menagesha national forest is with geographical coordinates of 9°02′08.946″ N 38°34′57.507″ E and with an average altitude of 2696.

2.2. Soil samples collection techniques

A total of 120 soil samples were collected from 6 localities (20 samples from each site) of tomato rhizosphere and forest soils. The soil sampling strategy was adapted from (Tuininga et al., 2014). Approximately 1.5 kg soil was collected from randomly selected five points of each sampling site at 4m apart in transect using The samples soil core borer to a depth of 20 cm. All five sub-samples from each site were homogenized thoroughly and 1 kg of total weight was collected in alcohol sterilized ziplock polyethylene bags. They were transported to the applied microbiology laboratory, Addis Ababa University, Ethiopia. The soils were mechanically crushed and sieved using a 500 μm aperture sieve and stored at 4 °C for further processing.

2.3. Rearing of insects

2.3.1. Rearing of Galleria mellonella

Larvae of *Galleria mellonella* (Lepidoptera, Pyralidae) were reared at the Ambo plant protection agricultural research center according to the method described (Meyling, 2007). Adult moths were kept in 500ml flasks containing folded tissue paper to facilitate their mating and egg-laying potential. Eggs were laid on folded tissue paper and each tissue paper was transferred from the flask into rearing plastic containers containing 80g honey, 50g wheat bran, and 180g glycerol for hatching third to fourth instar larvae, and incubated at 20 °C for four weeks under darkness.

2.4. Isolation of entomopathogenic fungi from soil

The entomopathogenic fungi (EPF) were isolated from the soil samples with the Galleria bait method following the protocol of (Meyling and Eilenberg, 2006). *Galleria mellonella* larvae were given heat shock by immersing in 56 °C water before baiting and cooled by immersing in sterile cold water for 30 s and placed on dry sterile tissue paper in the dark for 3–5 h. About 1 kg each soil sample was moisturized with sterile

Table 1. Soil sample collection sites, habitat, and geographical location.

No	Sample Sites	No of the soil samples	Collection zone	Habitat	Altitude	Latitude and Longitude
1	Debrezeit	20	East Shoa	Cultivated land	1879	8°45′26.10″N 39°00′46.42″E
2	Koka	20	East Shoa	Cultivated land	1605	8° 25′07.25″ N 39° 02′29.25″ E
3	Meki	20	East Shoa	Cultivated land	1636	8°10′N 38°50′E
4	Ziway	20	East Shoa	Cultivated land	1645	7° 58′25.38°39″N 38° 43′21.15″E
5	Menagesha	20	West Shoa	Forest	2696	9°02′08.946″N 38°34′57.507″E
6	Entoto	20	North Addis Ababa	Forest	2850	9°05′00″N 38°47′00E

water and then filled into a glass container with a screw cap leaving some space on the top to inoculate Galleria larvae. Ten (10) *G. mellonella* larvae were inoculated into each glass container and incubated at 25 ± 5 °C in the dark. To ensure permanent soil contact, all containers were inverted every other day. Dead larvae were removed every 3 days from the glass containers for ten days. The moisture level was maintained by moistening with sterile water each time following the inspection of the dead larvae. The dead larvae were surface sterilized with 1% sodium hypochlorite for 2–3 min, rinsed in sterile distilled water for several times. The cadavers (dead larvae) were placed on a plate over layered with Whatman filter paper No.1 (Macherey-Nagel, Duren, Germany) moistened with sterile water and incubated at 25 ± 2 °C in the dark. The sporulated fungi on dead larvae were subcultured on potato dextrose agar (PDA; Merck Ltd., Darmstadt, Germany) to isolate pure cultures and preserve on PDA slants at 4 °C.

2.5. *Cultural characterization*

Entomopathogenic fungal isolates were culturally examined based on their colony characteristics such as colony color, shape, texture, and growth pattern on PDA.

2.6. *Morphological characterization*

2.6.1. *Slide culture method*

Microscopic features of fungal isolates were characterized by slide culture techniques (Larone, 1995). A bent glass rod was placed into a sterile Petri dish containing a piece of filter paper, and a sterile glass slide was put on the glass rod. A 1-by-1-cm block potato dextrose agar (PDA) cut with a sterile scalpel was then transferred to the glass slide. The 15 days old fungal culture was inoculated with the help of a sterile needle on the four sides of the agar block. Then a coverslip was placed over the block and pressed to ensure adherence. Approximately 2ml of sterile distilled water was added to the bottom of the Petri dish and incubated at 25 °C for 2–5 days. After fungal growth, the coverslip was removed with the help of forceps and placed on a drop of lactophenol cotton blue on another clean glass slide and then observed under a microscope with 400 x magnification.

2.7. *Preparation of entomopathogenic fungi suspension*

The spore suspensions of all isolates were prepared according to the procedures described by (Erper et al., 2016). The isolates were grown on potato dextrose agar (PDA) for 14–20 days at 25 °C. The fungal spores were scraped using a sterile spatula and transferred into 10 ml of sterile distilled water having 0.1% Triton X-100 solution. Suspensions were filtered with layers of cheesecloth to remove the mycelium and vortexed to homogenize the inoculum. Spore concentrations of the filtrates were determined using Hemocytometer at 400 x magnification.

2.8. *Pre-screening of entomopathogenic fungal isolates*

2.8.1. *Screening for spore germination*

Isolates were screened for germination according to the conidial viability assessment (Yeo et al., 2003). A conidial suspension (200 μl) of each isolate at (1×10^6 -spores ml^{-1}) was sprayed onto PDA medium and incubated at 25 °C for 24 h. Each plate was treated with 1 ml formaldehyde (0.5%) 24h post-inoculation to halt germination. Then one drop of lactophenol blue and a sterile coverslip was put on each Petri-dish in triplicates to count approximately 300 germinated and non-germinated conidia to estimate the percentage of germination under the light microscope (400 x magnifications). Conidia were regarded as germinated, when they produced a germ tube, at least half of the conidial length. The percentage of germination for each isolate was calculated using the following formula: (Vega et al., 2008).

$$\% \text{ of spore germination} = \frac{\text{Number of spores germinated}}{\text{Total spore count}} \text{ x } 100$$

2.8.2. *Screening for vegetative growth rate and spore production*

Vegetative growth and sporulation of isolates were assessed following the protocol of Bugeme et al. (2008). One ml suspension 1×10^8 spores/ml of each isolate was spread on PDA plates. The plates were then sealed with Parafilm and incubated at 25 °C under complete darkness for 72 h. Then, 5 mm mycelial plugs were cut from the cultures and transferred onto the centers of freshly prepared PDA plates. The plates were then sealed with Parafilm and incubated at 25 °C for 15 days under complete darkness in triplicate. The mycelial growth rate (mm day^{-1}) was calculated using simple linear regression (Fargues et al., 1997). Spore production was examined after 15 days of growth from which 5mm agar discs were randomly taken and transferred in 10 ml of 0.02% Tween 80 solution and vortexed for five minutes to make spore suspension. The number of spores for each isolate was counted using a compound microscope with a Neuberger Hemocytometer at 400 x magnification.

2.9. *Molecular identification of EPF*

2.9.1. *DNA extraction and purification*

The genomic DNA was extracted from entomopathogenic fungal isolates following the protocol of (Chi et al., 2009). Selected pathogenic isolates were inoculated onto potato dextrose agar (PDA) and incubated at 25 °C for 4 days. Then 500 mg fungal propagules were transferred into 2 ml of sterile Eppendorf tube containing 500μl DNA extraction buffer (Tris-HCl, 100mM; KCl, 1M; EDTA, 10mM) and crushed with sterile plastic pestle fitted with an electronic instrument black and decker portable electronic drill for 2–4s. The mycelia suspensions were vortex and centrifuged at 12,000g for 10 min. Each supernatant was transferred to a new Eppendorf tube containing 300μl of 2-propanol, mixed, centrifuged at 13000g for 20 min. After having suspended the supernatant, each pellet was washed with 0.5ml of 70% ethanol, dried at room temperature, and resuspended in TE buffer (10 mMTris-HCl, 1mM EDTA, and pH 8.0). Finally, the extracted DNA was stored at -20 °C for further use.

2.9.2. *PCR amplification and sequencing*

The internal transcribed spacer (ITS) regions of rDNA of the fungal isolates were amplified using universal Primers ITS1 (5′- TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′- TCCTCCGCTTATTGATATGC-3′) as described by (White et al., 1990), generating estimated 500bp products. The PCR amplification was carried out in a total volume of 30μl. The mixture was 14.5μl of water, 4μl of the buffer, 1μMgCl2, 3μl of dNTP, 1μl *Taq* polymerase, 2.5μl of each primer, and 3μl of genomic DNA. The PCR thermocycler settings were initial denature at 94 °C for 4min followed by 35 cycles denaturation at 94 °C for the 30s, annealing at 59 °C for 30s, extension at 72 °C for 1 min, and a final extension at 72^{o}Cfor 1min. The PCR product was resolved by 1.5% agarose gel electrophoresis run with TBE buffer (Tris-Boric EDTA) at 100V. The gel was stained with Ethidium bromide and visualized with UV-light for band confirmation. The amplified PCR products (50μl) were purified with NucleoSpin ® Gel and PCR cleanup system. The sequencing was done at Macrogen Inc. (Geumchengu, Seoul, Korea). Sequenced data were deposited into the GenBank database. Molecular sequences were edited with BioEdit 7.1.9 (Hall, 1999) and multiple sequence alignment carried out by using CLUSTALX2 (Thompson et al., 1994). All sequences were compared with already published sequences of known fungi from the NCBI GenBank Database. Phylogenetic and molecular evolutionary analysis was constructed by using Molecular Evolutionary Genetics Analysis (MEGA-X-10.1.8) software (Tamura et al., 2007). Finally, a phylogenetic tree was done based on the neighbor-joining method (Saitou and Nei, 1987).

2.10. Pathogenicity test of selected isolates against G. mellonella

Based on screening for viability, vegetative growth, and sporulation, isolates were selected for pathogenicity tests against *G. mellonella* larvae to select the virulent isolates (Ibrahim et al., 2016). The spore suspension was prepared from well-sporulated culture and adjusted to 1 × 10^8 spores/ml as before. Ten larvae of third to fourth *G. mellonella* were immersed into 10ml conidial suspension for 10–30s in a sterile beaker against the control that was immersed in 10ml sterile distilled water. Each treatment was replicated three times. The larvae were then transferred into plastic Petri-dishes containing filter paper and incubated at 25 °C. The number of dead larvae was recorded daily for the following 10 days. Mortality data were corrected for the corresponding control mortality by using the formula:

$$\%CM = \frac{(\%T - \%C)}{(100 - \%C)}x100$$

Where; CM is corrected mortality, C is mortality in the untreated larvae, and T is mortality in the treated larva (Abbott, 1925). Dead larvae were surface sterilized by briefly immersing in 70% of alcohol for 3 min and rinsed with sterile distilled water three times. Finally, the larvae were transferred to a sterile Petri-dish containing wet filter paper, sealed with parafilm, and incubated at room temperature to stimulate conidia germination.

3. Data analysis

The spore germination and spore production data were analyzed using analysis of variance (One-way ANOVA) with statistical significance of $p < 0.05$, followed by a post hoc Tukey test. The mean percentage of corrected mortality data were arcsine transformed to stabilize the efficacy of analysis of variance (Gomez and Gomez, 1984) and subjected to the ANOVA procedure of SPSS version 20. Means were separated using Tukey's Honestly Significant Difference (HSD) at $P < 0.05$ for screening experiments against *G. mellonella*. The LT_{50}, values were determined with probit analysis (IBM SPSS statics 20).

4. Results

In this particular study, a total of 65 (54.2%) entomopathogenic fungal isolates belonging to *Beauveria* and *Metarhizium* genera were

Table 2. Morphological and cultural characteristics of *Beaveria* isolates.

No	Isolates	Colony color		Colony shaped	Colony texture	Elevation	Shapes of spore
		Front side	Reverse side				
1	AAUDB-1	White	White	Round	Powder	Raised	globose
2	AAUEB-3	White	White	Round	cottony	Raised	globose
3	AAUEB-53	White	Yellowish white	Round	Powder	Raised	Sub-globose
4	AAUEB-8	White	Yellowish white	Round	Powder	Raised	globose
5	AAUEB-13	White	White	Round	Powder	Flat	globose
6	AAUEB-89	White	Yellowish white	Round	Powder	Raised	Sub-globose
7	AAUMB-20	Yellowish white	White	Round	Cottony	Raised	globose
8	AAUMB-19	White	yellow	Round	Cottony	Raised	globose
9	AAUMFB-10	White	White	Round	Powder	Flat	globose
10	AAUMFB-19	White	White	Round	Powdery	Raised	globose
11	AAUMB-25	White	White	Round	Powder	Flat	globose
12	AAUMFB-15	White	White	Round	Cottony	Raised	globose
13	AAUMB-21	Yellowish white	Yellow	Round	Cottony	Raised	Sub-globose
14	AAUMB-26	White	Yellowish White	Round	Cottony	Raised	globose
15	AAUMB-29	White	Yellow	Round	Cottony	Raised	globose
16	AAUMFB-82	White	White	Round	Powder	Raised	Sub-globose
17	AAUZB-3	White	Yellowish White	Round	Powder	Flat	Sub-globose
18	AAUDB-6	White	white	Round	Powder	flat	globose
19	AAUKB-11	White	White	Round	Powder	Flat	globose
20	AAUMFB-16	White	Yellowish White	Round	Cottony	Raised	globose
21	AAUDB-39	White	White	Round	Powder	Raised	globose
22	AAUZB-9	White	White	Round	Powder	Raised	globose
23	AAUEB-11	White	Yellowish White	Round	Cottony	Raised	globose
24	AAUMFB-79	White	Yellowish white	Round	Powder	Raised	globose
25	AAUDB-46	White	White	Round	Cottony	Raised	Sub-globose
26	AAUMFB-3	White	White	Round	Powder	Raised	globose
27	AAUMFB-77	White	Yellowish White	Round	Powder	Raised	globose
28	AAUEB-59	White	Yellowish White	Round	Cottony	Raised	globose
29	AAUMB-76	White	Yellowish White	Round	Powder	Raised	globose
30	AAUEB-46	White	White	Round	Powder	Raised	globose
31	AAUDB-14	White	Yellowish White	Round	Cottony	Raised	Sub-globose
32	AAUMFB-5	White	White	Round	Powdery	Raised	globose
33	AAUMFB-12	Whit	White	Round	Cottony	Raised	globose
34	AAUMFB-33	White	White	Round	Powdery	Raised	globose
35	AAUZB-14	White	White	Round	Cottony	Raised	Sub-globose
36	AAUMFB-11	White	Yellowish White	Round	Powdery	Flat	globose

identified based on morphological and cultural characteristics from 120 soil samples. The macroscopic (cultural) characters of *Beauveria* and *Metarhizium* isolates were confirmed by their colony color, shape, texture, and growth pattern. The *Beauveria* isolates showed dispersed growth patterns, white to yellowish-white colony color, smooth powdery to cottony texture, raise to flat elevation, and round shape (Table 2). Regarding *Metarhizium* isolates, colonies had greenish colony color at the top side whereas the reverses of colonies were brownish, white, and yellowish-white. Besides, *Metarhizium* isolates indicated round colony shape, thin to thick adpressed texture, and flat to slightly raised elevation (Table 3).

In the morphological studies, isolates were observed under a light microscope using 400x magnifications with slide culture techniques to express their microscopic features. Microscopic observation of *Beauveria* isolates revealed the globose to subglobose conidia with hyaline hyphae and zig-zag extension of the rachis (Figure 1) whereas *Metarhizium* isolates were characterized with ellipsoid, and cylindrical spore shapes (Figure 2).

The distribution and occurrence of indigenous entomopathogenic fungi, *Beauveria,* and *Metarhizium* obtained from six soil sample sites were displayed (Table 4). Out of the total 65 (54.17%) entomopathogenic fungal isolates, *Beauveria* species were slightly higher in the number of 30% compared to 24.17% that represented *Metarhizium* species. The highest number of isolates 20 (100%) were obtained from Menagesha forest of which 12 (60%) and 8 (40%) were identified as *Beauveria* and *Metarhizium* isolate respectively. Entoto forest site harbored the next higher entomopathogens (85%) of which 40% and 45% of the isolates belonged to *Beauveria* and *Metarhizium* isolates respectively. In contrast, the lowest number of isolate 1 (5%) *Beauveria* and without any *Metarhizium* isolate was recorded from Koka farmland. The distributions of isolates in the case of habitat ecology were 92.5% from forest soil and 35% from farmland soil (Figure 3). The entomopathogenic *Beauveria* was dominant in all samples except the menagesha forest site indicating variation in the distribution of *Beauveria* and *Metarhizium* isolates in agricultural and forest soils.

4.1. Pre-screening of entomopathogenic fungal isolates

4.1.1. Screening for spore germination

All *Beauveria* and *Metarhizium* species were primarily screened for spore viability (germination). Their mean percentage spore germination was between 43.33% and 99.67% upon 24 h of incubation of which twenty-six isolates (40%) with spore germination >85% were selected and further screened for growth and sporulation efficacy from which 57.69% of the isolates belonged to *Beauveria* (Table 4). They showed variation in their conidia germination ($F = 5.04$; $df = 25, 52$; $P < 0.001$). Isolate AAUKB-11 displayed the higher germination (99.67%) followed by isolate AAUMFB-77 (99.00 %) and AAUMB-21 (98.83%).

4.1.2. Screening for vegetative growth and spore production

The vegetative growth and spore production of *Metarhizium* and *Beauveria* isolates were examined as the basis for *in vitro* virulence screening method. Statistical analysis indicated significant differences among isolates in both growth rate day^{-1} ($F = 30.42$; $df = 25, 52$; $P <$

Table 3. Morphological and cultural characteristics of *Metarhizium* isolates.

No	Isolates	Colony color		Colony shaped	Colony texture	Elevation	Shapes of spore
		Front side	Reverse side				
1	AAUDM-45	Light green	Orange	Round	Thin Addpressed	Flat	Ellipsoid
2	AAUEM-11	Dark Green	Brown	Round	Thick Adpressed	Flat	Cylindrical
3	AAUEM-13	Dark green	Brown	Round	Addpressed	Flat	Cylindrical
4	AAUEM-3	Light green	Yellowish white	Round	Thick Addpressed	Flat	Ellipsoid
5	AAUEM-30	Dark green	Brown	Round	Thick Addpressed	Flat	Cylindrical
6	AAUEM-2	Dark green	Orange	Round	Thick Addpressed	Flat	Ellipsoid
7	AAUEM-58	Dark green	Brown	Round	Thin Adpressed	Flat	Ellipsoid
8	AAUEM-1	Green	Brown	Round	Thick Adpressed	Slightly raised	Cylindrical
9	AAUMM-30	Green	Brown	Round	Cottony	Slightly raised	Cylindrical
10	AAUMM-20	Green	Orange	Round	Thin Adpressed	Flat	Ellipsoid
11	AAUMM-50	Green	Orange	Round	Thin Adpressed	Flat	Ellipsoid
12	AAUMM-11	Green	Brown	Round	Thick Addpressed	Flat	Cylindrical
13	AAUMFM-12	Green	Brow	Round	Thin Adpressed	Flat	Ellipsoid
14	AAUMM-10	Dark green	Brown	Round	Thin Addpressed	Flat	Cylindrical
15	AAUMM-23	Green	Orange	Round	Thick Adpressed	Flat	Ellipsoid
16	AAUEM-8	Green	Brown	Round	Thick Adpressed	Flat	Ellipsoid
17	AAUZM-60	Dark green	Brown	Round	Thick Adpressed	Flat	Cylindrical
18	AAUKM-45	Dark Green	Brown	Round	Cottony	Slightly raised	cylindrical
19	AAUMFM-13	Green	Brown	Round	Cottony	Flat	Ellipsoid
20	AAUMFM-78	Green	Brown	Round	Thin Adpressed	Slightly raised	Cylindrical
21	AAUZM-18	Dark green	Brown	Round	Thick Adpressed	Flat	Cylindrical
22	AAUMFM-9	Dark green	Brown	Round	Thick Adpressed	Slightly raised	Ellipsoid
23	AAUMFM-48	Yellowish white	Yellowish white	Round	Cottony	Slightly raised	Ellipsoid
24	AAUDM-43	Green	Brown	Round	Thick Adpressed	Slightly raised	Cylindrical
25	AAUMFM-6	Dark green	Brown	Round	Thin Adpressed	Flat	Cylindrical
26	AAUMFM-21	Green	White	Round	Cottony	Slightly raised	Ellipsoid
27	AAUZM-7	Green	Orange	Round	Thick Adpressed	Flat	Ellipsoid
28	AAUZM-53	Green	Brown	Round	Thin Adressed	Flat	Cylindrical
29	AAUMFM-28	Dark green	Brown	Round	Addpressed	Flat	Ellipsoid

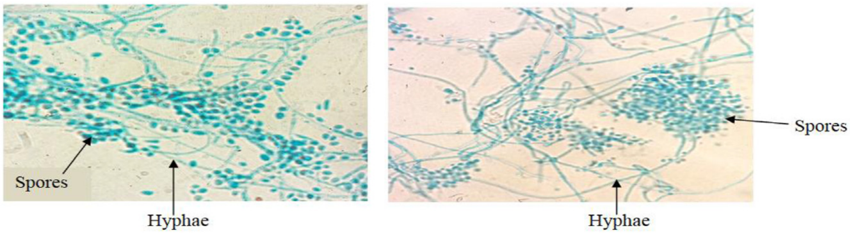

Figure 1. Spores and hyphal structures of *Beauveria* isolates observed with compound microscope (400 X magnification).

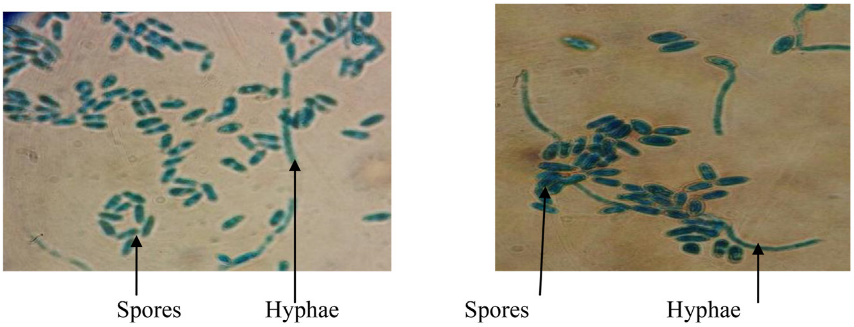

Figure 2. Spores and hyphal structures of *Metarhizium* isolates observed with compound microscope (400 X magnification).

Table 4. Distribution of the indigenous entomopathogenic fungi, *Beauveria* and *Metarhizium* obtained from the different sampling sites.

Sites	*Beauveria*		*Metarhizium*		Total	
	No	%	No	%	No	%
Debrezeit	5	25	2	10	7	35
Koka	1	5	-	-	1	5%
Zeway	3	15	4	20	7	35%
Meki	7	35	6	30	13	65%
Menagesha	12	60	8	40	20	100%
Entoto	8	40	9	45	17	85%

0.001) ranging from 0.89 to 3.43 mm day^{-1} and conidial yield ($F = 36.37$; df = 25, 52; $P < 0.001$) ranging from 0.03×10^8 to 4.60×10^8 spores/ml (Table 5). The highest spore production (4.60×10^8 spores/ml) and radial growth (3.43 mm day^{-1}) were achieved by isolate AAUMFB-77 whereas the lowest sporulation (0.03×10^8spores/ml) and radial growth (0.89 mm day^{-1}) attained with isolate AAUDM-45 and AAUEM-3 respectively (Table 5). Generally, isolate AAUKB-11 displayed a higher germination percentage (99.67%), and isolate AAUMFB-77 showed a higher radial growth rate (3.43 mm day^{-1}) with higher sporulation of 4.60×10^8spores/ml.

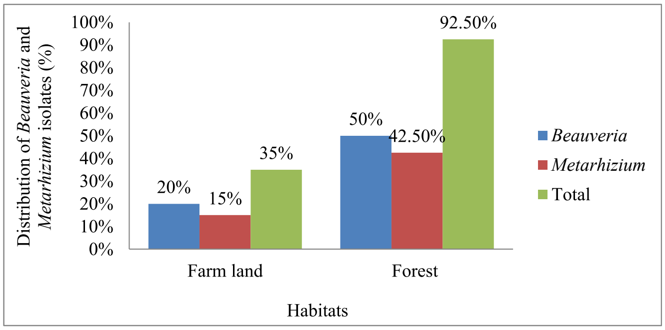

Figure 3. Distribution of entomopathogenic fungi, *Beauveria* and *Metarhizium* from the two ecological habitats.

4.2. Molecular characterization

The isolates of fungal entomopathogens were also genetically characterized by using rDNA-ITS regions. Consequently, the PCR amplification of the ITS region of rDNA resulted in a single product that produced approximately 545bp fragment size for all isolates of *B. bassiana* (Figure 4) and *M. anisopliae* (Figure 5).

The sequences of ITS1-5.8S-ITS4 rDNA region of all indigenous entomopathogenic fungal isolates showed 99–100% sequence similarity with *B. bassiana* and *M. anisopliae* deposited in NCBI/Genebank. The phylogenetic tree verified that all isolates of *B. bassiana* were strongly supported clade with a bootstrap value of 90%, which additionally partitioned into 2 distinct sub-clades (Figure 6). Sub-clade I contained isolate AAUMFB-5 and AAUMFB-77 associated with the sample from Gene bank (AY532045), Sub-clade II includes isolate AAUMB-29, AAUMB-20, AAUEB-59, AAUMB-21, and AAUKB-11 together with Gene bank sample of MG670098. The phylogenetic tree clustered five *Metarhiziumin* isolates into *Metarhizium anisopliae* with bootstrap support of 98% that grouped into 2 sub-clades (Figure 7). Sub-clade I contained isolates AAUMF-6, AAUEM-30, AAUZM-60, and AAUDM-43 analogous to Gene bank sample of KU647722 whereas Sub-clade II encompassed

Table 5. List of *Beauveria* and *Metarhizium* isolate from Ethiopia and their percent germination, growth rate and spore production.

No	Isolate code	Genera	Host of isolates	localities	%Germination (Mean ± SE)	Growth rate (mm Day^{-1})	Sporulation ($\times 10^8$ spores/ml)
1	AAUDB-1	*Beauveria spp*	Farm soil	Debre Ziet	86.00 ± 0.76^{ab}	0.90 ± 0.11^{a}	0.60 ± 0.10^{abc}
2	AAUDM-45	*Metarhizium spp*	Farm soil	Debre Ziet	$88.17 \pm 2.96^{a\text{-}d}$	1.13 ± 0.08^{ab}	0.03 ± 0.01^{a}
3	AAUEB-3	*Beauveria spp*	Forest soil	Entoto	87.17 ± 2.19^{abc}	2.03 ± 0.07^{efg}	0.83 ± 0.03^{abcd}
4	AAUEM-3	*Metarhizium spp*	Forest soil	Entoto	$89.67 \pm 2.33^{a\text{-}f}$	0.89 ± 0.05^{a}	1.60 ± 0.15^{cdefg}
5	AAUEM-30	*Metarhizium spp*	Forest soil	Entoto	$97.67 \pm 1.45^{c\text{-}f}$	2.20 ± 0.06^{eh}	1.67 ± 0.09^{cdefg}
6	AAUEB-53	*Beauveria spp*	Forest soil	Entoto	$91.33 \pm 2.02^{a\text{-}f}$	2.73 ± 0.14^{hi}	1.47 ± 0.24^{cdefg}
7	AAUEB-89	*Beauveria spp*	Forest soil	Entoto	98.67 ± 1.33^{def}	2.13 ± 0.09^{eh}	0.07 ± 0.01^{ab}
8	AAUMB-20	*Beauveria spp*	Farm soil	Meki	$97.33 \pm 1.20^{c\text{-}f}$	2.23 ± 0.23^{eh}	3.23 ± 0.12^{j}
9	AAUMM-20	*Metarhizium spp*	Farm soil	Meki	$96.83 \pm 0.92^{b\text{-}f}$	1.93 ± 0.12^{idg}	1.13 ± 0.08^{cdef}
10	AAUMB-21	*Beauveria* spp	Farm soil	Meki	98.83 ± 1.16^{ef}	$2.00 \pm 0.10^{d\text{-}g}$	1.73 ± 0.29^{defghj}
11	AAUMM-10	*Metarhizium* spp	Farm soil	Meki	$92.00 \pm 2.64^{a\text{-}f}$	$1.67 \pm 0.03^{b\text{-}e}$	1.40 ± 0.06^{cdefg}
12	AAUMB-29	*Beauveria spp*	Farm soil	Meki	$98.17 \pm 1.83^{c\text{-}f}$	3.00 ± 0.17^{ij}	4.33 ± 0.32^{k}
13	AAUMFB-82	*Beauveria spp*	Forest soil	Menagesha	$88.83 \pm 0.88^{a\text{-}f}$	$1.97 \pm 0.13^{d\text{-}g}$	0.07 ± 0.01^{ab}
14	AAUZB-3	*Beauveria spp*	Farm soil	Ziway	$87.67 \pm 2.84^{a\text{-}d}$	2.50 ± 0.17^{ghi}	1.23 ± 0.08^{defg}
15	AAUMFB-5	*Beauveria spp*	Forest soil	Menagesha	$96.67 \pm 2.40^{b\text{-}f}$	$1.90 \pm 0.11^{c\text{-}g}$	1.90 ± 0.06^{defghi}
16	AAUKB-11	*Beauveria spp*	Farm soil	Koka	99.67 ± 0.33^{f}	2.47 ± 0.12^{ghi}	1.77 ± 0.06^{defghi}
17	AAUZM-60	*Metarhizium spp*	Farm soil	Ziway	$92.33 \pm 3.33^{a\text{-}f}$	2.30 ± 0.01^{fgh}	2.17 ± 0.15^{efghij}
18	AAUKM-45	*Metarhizium spp*	Farm soil	Koka	$94.17 \pm 2.42^{a\text{-}f}$	$1.40 \pm 0.05^{a\text{-}d}$	0.90 ± 0.12^{abcd}
19	AAUZM-18	*Metarhizium spp*	Farm soil	Ziway	$95.50 \pm 3.54^{a\text{-}f}$	1.80 ± 0.05^{cdef}	3.00 ± 0.58^{j}
20	AAUMFB-77	*Beauveria spp*	Forest soil	Menagesha	99.00 ± 0.57^{ef}	3.43 ± 0.88^{j}	4.60 ± 0.23^{k}
21	AAUEB-59	*Beauveria spp*	Forest soil	Entoto	$93.67 \pm 1.85^{a\text{-}f}$	2.33 ± 0.15^{fgh}	2.76 ± 0.23^{ij}
22	AAUMB-76	*Beauveria spp*	Farm soil	Meki	$95.00 \pm 2.51^{a\text{-}f}$	1.90 ± 0.10^{cdg}	1.30 ± 0.31^{defg}
23	AAUEB-46	*Beauveria spp*	Forest soil	Entoto	98.33 ± 1.20^{def}	1.30 ± 0.12^{abc}	$3.13 \pm 0.03j$
24	AAUDM-43	*Metarhizium spp*	Farm soil	Debre Ziet	$95.67 \pm 1.45^{a\text{-}f}$	2.40 ± 0.06^{fi}	2.83 ± 0.07^{ij}
25	AAUMFM-6	*Metarhizium spp*	Forest soil	Menagesha	$96.50 \pm 1.75^{b\text{-}f}$	1.83 ± 0.09^{cdef}	$2.47 \pm 0.03h^{ij}$
26	AAUZM-7	*Metarhizium* spp	Farm soil	Ziway	85.43 ± 1.37^{a}	0.90 ± 0.15^{a}	1.83 ± 0.24^{dhi}

Means followed by the same letter within a column are not significantly different according to Tukey's Studentized Range (HSD) test, at $\alpha = 0.05$. Isolates of *Beauveria* and *Metarhizium* with germination >85%, growth rate >1.4mm day^{-1}, and sporulation $>1.6 \times 10^8$ spores/ml were selected for molecular identification.

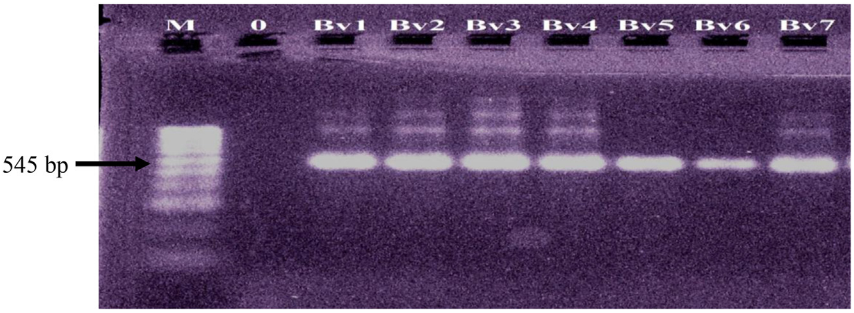

Figure 4. Amplified PCR products using ITS1 and ITS4 primers from seven selected isolates of *B. bassiana*. M = 1000bp ladder, 0 = blank, Bv1 = AAUMB-20, Bv2 = AAUMFB-77, Bv3 = AAUMB-29, Bv4 = AAUMFB-5, Bv5 = AAUEB-59, Bv6 = AAUMB-21, Bv7 = AAUKB-11. The full gel image and blot indicated in supplementary file (see Fig S1).

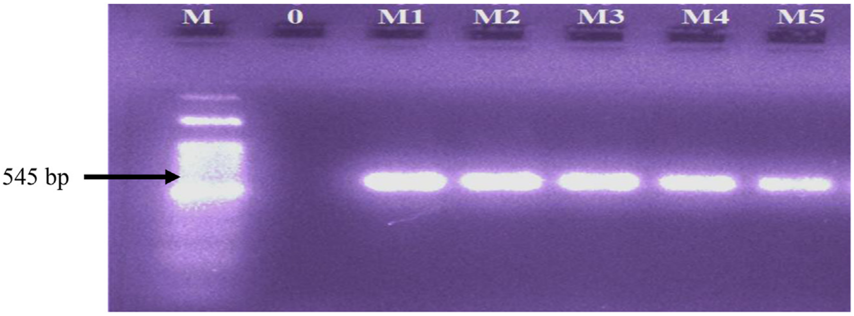

Figure 5. Amplified PCR products by using ITS1 and ITS4 primers from five selected isolates of *M. anisopliae*. M = 1000bp ladder, 0 = blank, M = AAUMF-6, M2 = AAUEM-30, M3 = AAUZM-60, M4 = AAUDM-43, M5 = AAUZM-18. The full gel image and blot indicated in supplementary file (see Fig S2).

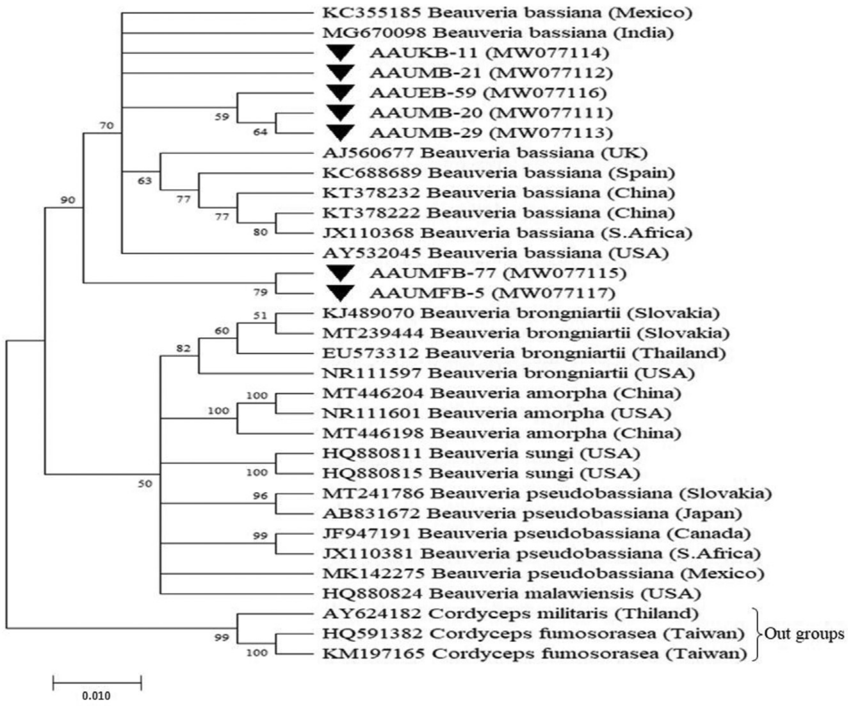

Figure 6. Phylogenetic analysis based on ITS-5.8S-ITS4 rDNA sequences of 7 *Beauveria bassiana* isolates from Ethiopia and other related sequences deposited in GeneBank of NCBI. Phylogenetic tree constructed based on the neighbor-joining method using MEGA-X-10.1.8 software. Bootstrap value > 50% are showed and isolate *Cordyceps militaris* and *Cordyceps fumosorasea* were displayed as out-group taxa.

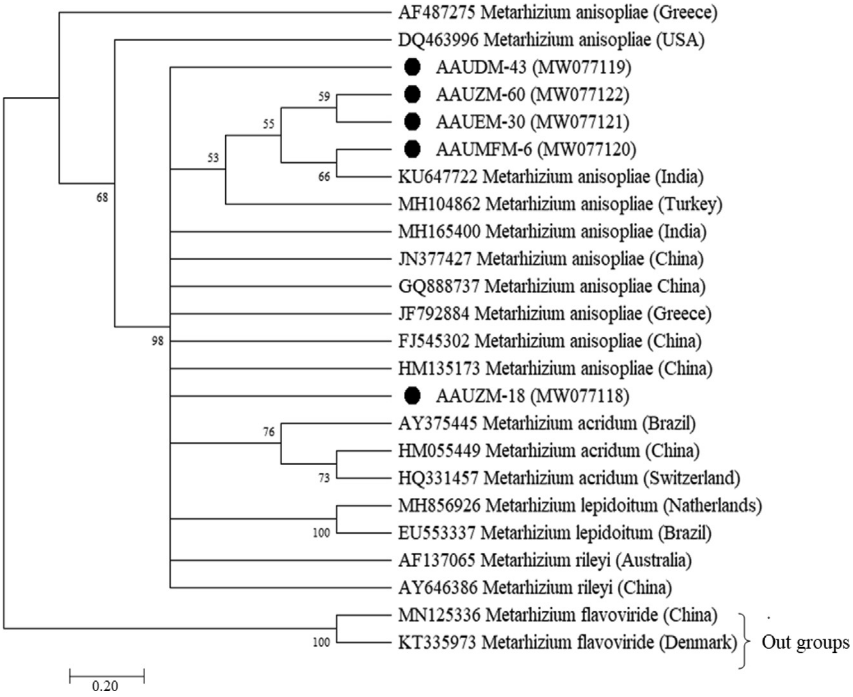

Figure 7. Phylogenetic analysis of ITS – rDNA region sequences of 5 *Metarhizium anisopliae* isolates from Ethiopia compared to other related sequences derived from GeneBank. Phylogenetic tree constructed using the neighbor-joining method with MEGA-X- 10.1.8 software. Bootstrap value > 50% are indicated and isolate *Metarhizium flavoviride* was displayed as out-group taxa.

Table 6. Mean percentage mortality on *Galleria mellonella*, germination and median lethal time (LT $_{50}$) of Entomopathogenic fungi, *B.bassiana* and *M.anisopliae.*

Isolate code	Species	Mortality (%) (Mean ± SE)	LT$_{50}$ (days) (95%CL)	X^2	intercept	*P*-value	Virulence level
AAUMB-20	*B.bassiana*	93.33 ± 6.67^{bc}	4.22 (3.13–5.40)	1.33	-1.61	$P < 0.001$	HV
AAUMB-21	*B.bassiana*	96.67 ± 3.33^{bc}	3.83 (2.53–5.18)	1.72	-1.18	$P < 0.001$	HV
AAUMB-29	*B.bassiana*	100.00 ± 0.00^{c}	2.36 (1.68–2.98)	3.87	-1.25	$P < 0.001$	HV
AAUMFB-5	*B.bassiana*	100.00 ± 0.00^{c}	4.10 (3.31–4.86)	4.07	-2.56	$P < 0.001$	HV
AAUKB-11	*B.bassiana*	100.00 ± 0.00^{c}	3.69 (2.66–4.75)	6.93	-1.42	$P < 0.001$	HV
AAUMFB-77	*B.bassiana*	100.00 ± 0.00^{c}	2.53 (1.37–3.51)	2.60	-0.79	$P < 0.001$	HV
AAUEB-59	*B.bassiana*	96.67 ± 2.45^{bc}	3.48 (2.31–4.64)	2.54	-1.17	$P < 0.001$	HV
AAUDM-43	*M.anisopliae*	100.00 ± 0.00^{c}	3.48 (2.39–4.53)	1.04	-1.29	$P < 0.001$	HV
AAUMFM-6	*M.anisopliae*	100.00 ± 0.00^{c}	2.96 (1.98–3.86)	2.83	-1.15	$P < 0.001$	HV
AAUZM-60	*M.anisopliae*	90.00 ± 5.73^{bc}	4.55 (3.71–5.34)	0.74	-2.77	$P < 0.001$	HV
AAUZM-18	*M.anisopliae*	86.67 ± 3.33^{bc}	5.01 (3.98–6.13)	0.84	-2.31	$P < 0.001$	MV
AAUEM-30	*M.anisopliae*	90.00 ± 5.48^{bc}	3.98 (2.84–5.11)	0.79	-1.46	$P < 0.001$	HV

Mean with Different letters in a column indicates the significant difference at Tukey's HSD test, $P < 0.05$ SE = standard error, CL = confidence limit, MV = moderate virulent, HV = high virulent.

Figure 8. The growth of *B. bassiana* (A) and *M. anisopliae* isolates (B) on *G. mellonella* larvae.

only isolate AAUZM-18 corresponding to HM135173(Gene bank sample).

4.3. Pathogenicity assessment of selected entomopathogenic fungal isolates against G. mellonella

Selected *B. bassiana* and *M. anisopliae* were evaluated for their pathogenicity efficiency against *G. mellonella* larvae. Isolates of both *B. bassiana* and *M. anisopliae* caused 86.67%–100% mortality on *G. mellonella* with a concentration of 1×10^8spores/ml (Table 6). The white muscardine fungus *B. bassiana* and green *muscardine* fungus *M. anisopliae* showed growth on the surface of *G. mellonella* larvae (Figure 8).

All isolates of *B. bassiana* and *M. anisopliae* showed >86% larval mortality which indicated that these isolates are virulent against *G. mellonella*. Among isolates, 4 (-33.33%) from *B. bassiana* isolates (AAUMB-29, AAUMFB-5, AAUKB-11, and AAUMFB-77) and 2 (16.67%) isolates from *M. anisopliae* (AAUDM-43 and AAUMFM-6) caused higher mortality of 100%. The mean percent mortality of *G. mellonella* larvae with *B. bassiana* and *M. anisopliae* strains were significantly varied (df = 16, 34; F = 6.61; $P < 0.001$). The median lethal time (LT$_{50}$), the time required 50% of death of *G. mellonella* larvae by isolates of *B. bassiana* and *M. anisopliae* differed among isolates with the range between 2.36-5.01 days (Table 6). Among 12 verified isolates, 11 (91.67%) of highly virulent and 1 (8.33%) of moderately virulent. The high virulent isolate,

Table 7. The *in-vitro* and *in-vivo* virulence efficacy of selected entomopathogenic fungi *B. bassiana* and *M. anisopliae* from Ethiopia.

Isolate code	Species	Host	Accession number	G (%)	GR (mm day^{-1})	SP(x10^8 spores/ml)	M (%)	LT$_{50}$ (hrs)	AR
AAUMB-20	*Beauveria bassiana*	Soil	MW077111	97.33	2.23	3.23	93.33	4.22	9
AAUMB-21	*Beauveria bassiana*	Soil	MW077112	98.83	2.00	1.73	96.67	3.83	8
AAUMB-29	*Beauveria bassiana*	Soil	MW077113	98.17	3.00	4.33	100.00	2.36	2
AAUKB-11	*Beauveria bassiana*	Soil	MW077114	99.67	2.47	1.40	100.00	3.69	4
AAUMFB-77	*Beauveria bassiana*	Soil	MW077115	99.00	3.43	4.60	100.00	2.53	1
AAUEB-59	*Beauveria bassiana*	Soil	MW077116	93.67	2.33	2.76	96.67	3.48	6
AAUMFB-5	*Beauveria bassiana*	Soil	MW077117	96.67	1.90	1.90	100.00	4.10	7
AAUZM-18	*Metarhizium anisopliae*	Soil	MW077118	95.50	1.80	3.00	86.67	5.01	12
AAUDM-43	*Metarhizium anisopliae*	Soil	MW077119	95.67	2.40	2.83	100.00	3.48	3
AAUMFM-6	*Metarhizium anisopliae*	Soil	MW077120	95.50	1.83	2.74	100.00	2.96	5
AAUEM-30	*Metarhizium anisopliae*	Soil	MW077121	91.33	2.73	1.47	90.00	3.98	10
AAUZM-60	*Metarhizium anisopliae*	Soil	MW077122	92.33	2.30	2.17	90.00	4.55	11

G = germination, GR = growth rate, SP = sporulation, M = mortality against *G. melonella*, LT$_{50}$ = median lethal time, AR = average rank.

AAUMB-29 displayed the least LT_{50} value of 2.36 days followed by isolate AAUMFB-77 with LT_{50} of 2.53 days (Table 6).

Generally, the virulence effectiveness of *B. bassiana* and *M. anisopliae* isolates were studied based on *in-vitro* (spore germination, vegetative growth, and spore production) and *in-vivo* (bioassay evaluation against *G. mellonella*) bio insecticide assessment. The result of the *in-vitro* and *in-vivo* virulence test presented that isolate AAUMFB-77 (*B. bassiana)* and AAUMB-29 (*B. bassiana*) as the most virulent, followed by AAUDM-43 (*M. anisopliae)* and AAUKB-11 (*B. bassiana*) were showed high virulence level compared to other tested entomopathogenic fungal isolates (Table 7). In contrast, isolate AAUZM-18 followed by AAUZM-60 from *M. anisopliae* displayed the lowest virulence level.

5. Discussion

Environmentally sound insect pest management strategies critically require the continuous isolation and identification of proper bio-control agents from farmland and forest soil based on the insect bait method (Mantzoukas et al., 2020b). Accordingly, a total of 65 (54.2%) entomopathogenic fungal isolates belongs to *Beauveria* spp and *Metarhizium* spp were identified based on morphological and cultural characteristics using *galleria* baiting method. Another study (Masoudi et al., 2018) preliminarily identified 22 (36.7%) of *Metarhizium* and *Beauveria* spp based on their morphological and cultural features by using a semi-selective agar medium. The number of detected entomopathogenic fungi from soil samples can be affected by the methods of isolation as described by (Keyser et al., 2015) who stated that from the total of 132 *Metarhizium* spp, 14 isolates were attained by selective media whereas 118 isolates were obtained using insect baiting methods from the same sample sites. The macroscopic characters of *Beauveria* isolates showed white colony color, powdery to cottony texture, and round shape (Kirubhadharsini et al., 2017). Concerning *Metarhizium* isolates, colonies had greenish color and round shape (Sapna Bai et al., 2015). Microscopic observation of *Beauveria* isolates revealed the globose to subglobose conidia with hyaline hyphae whereas *Metarhizium* isolates were characterized with ellipsoid, and cylindrical spore shapes (Dangi and Lim, 2018; Moslim and Kamarudin, 2014).

This study also detected the distribution and occurrence of native isolates of *Beauveria* and *Metarhizium* obtained from six soil sample sites. From the total 65 (54.17%) entomopathogenic fungal isolates, *Beauveria* species were slightly higher which designated 36 (30%) compared to 29 (24.17%) that represented *Metarhizium* species. The entomopathogenic *Beauveria* was dominant in all samples except the menagesha forest site indicating variation in the distribution of *Beauveria* and *Metarhizium* isolates in agricultural and forest soils. A study conducted by Wakil et al. (2013) stated the greater abundance and distribution of *Beauveria* spp. 10 (5.95%) followed by *Metarhizium* sp 5 (2.98%) from 168 soil samples collected from different cultivated and uncultivated lands. Moreover, Sharma et al. (2018) also identified slightly higher 26.55% *Beauveria bassiana* among various entomopathogenic fungi isolated from 183 soil samples using the insect bait method. The variation in the occurrence of entomopathogenic fungi from sample sites could be due to the influence of biotic and abiotic factors including availability of insect hosts, temperature, humidity, UV-radiation, soil type, and organic matter. In general, the distributions of isolates in terms of their habitats were 92.5% from the forest and 35% from farmland soils. Likewise, Yilma et al. (2019) found a greater abundance of 64.58% *Beauveria* and *Metarhizium* isolates in forest soils compared to 28.08% isolates detected from farmland soils. In the present study, the collection site influenced the occurrence and distribution of isolates of fungal entomopathogens. The forest sites comprised a higher number of isolates than farmlands this might be due to diverse insect hosts, undisturbed soil, shading area that protect fungi from UV-radiation and free from any chemical pesticide application in forest habitats.

Entomopathogenic fungal isolates of *Beauveria* and *Metarhizium* species were primarily screened based on spore germination, vegetative growth, and sporulation as suited parameters for studying the *in vitro* virulence effectiveness of entomopathogenic fungal isolates. The mean percentage spore germination of 26 (40%) isolates of *Beauveria* and *Metarhizium* was ranged from 85.43% to 99.67% within 24 hrs. Isolate AAUKB-11 displayed the higher germination (99.67%) followed by isolate AAUMFB-77 (99.00 %). This is similar to 76.33–95.75% conidial viability reported from *Beauveria* isolates (Belay et al., 2017), 89.30–99.00% conidia viability for 22 isolates of *B. bassiana* and *M. anisopliae* (Mkiga et al., 2020), and the conidia viability ranged from 85.3 to 99% for 10 *Beauveria* and *Metarhizium* isolates (Habtegebriel et al., 2016).

The highest spore production (4.60×10^8 spores/ml) and radial growth (3.43 mm day^{-1}) were achieved by isolate AAUMFB-77 whereas the lowest sporulation (0.03×10^8spores/ml) and radial growth (0.89 mm day^{-1}) attained with isolate AAUDM-45 and AAUEM-3 accordingly. By the same token (Schemmer et al., 2016), screened *B. bassiana* and *M. anisopliae* for radial growth ranged between 5.17 to 9.83mm^2/day with conidial production of $9.08–31.87 \times 10^5$ conidia/mm with significant variability among isolates. The variability in spore production and radial growth rates of entomopathogenic fungi might be influenced by the geographical origin of isolates and fungal species. Generally, the present study recognized superior germination percentage (99.67%) rated by isolate AAUKB-11 whereas the radial growth rate (3.43 mm day^{-1}) and sporulation (4.60×10^8sporse/ml) attained with isolate AAUMFB-77. In entomopathogenic fungi, fast conidia germination, radial growth (Dotaona et al., 2015), and high sporulation rates (Mar et al., 2012) are pathogenicity determinates that positively correlated with fungal virulence. Therefore, screening of entomopathogenic fungi by germination, radial growth, and sporulation parameters could be paramount important to determining high virulent isolates.

A DNA-based molecular characterization is a powerful tool for the accurate identification of entomopathogenic fungi. Regarding this, isolates were genetically characterized using rDNA-ITS regions. Consequently, the PCR amplification of the ITS region of rDNA resulted in a single product that produced approximately 545bp fragment size for all isolates of *B. bassiana* and *M. anisopliae*. This was similar to the 500bp fragment size demonstrated from ITS-rDNA regions of isolates amplified with ITS1-ITS4 primers for 8 *Beauveria bassiana* and *Metarhizium anisopliae* isolates (Mora et al., 2016) and 560bp fragment size for 8 isolates of *Beauveria bassiana* (Belay et al., 2017). The amplification and sequencing of the ITS-rDNA region of entomopathogenic fungi have considerably facilitated the detection of fungal isolates particularly *B. bassiana* and *M. anisopliae* (García et al., 2018). The molecular phylogenetic tree constructed from ITS-rDNA sequence of isolates with the neighbor-joining method by using MEGA-X-10.1.8 software at 1000 bootstraps replication (Sayed et al., 2018). The phylogenetic tree verified that isolates of *B. bassiana* were strongly supported clade with a bootstrap value of 90%, which partitioned into sub-clade I contained isolate AAUMFB-5 and AAUMFB-77 associated with the sample from Gene bank (AY532045) and sub-clade II includes isolate AAUMB-29, AAUMB-20, AAUEB-59, AAUMB-21, and AAUKB-11 together with Gene bank sample of MG670098. The five isolates of *Metarhiziumin* clustered into *M. anisopliae* with bootstrap support of 98% that grouped into Sub-clade I contained isolates AAUMF-6, AAUEM-30, AAUZM-60, and AAUDM-43 analogous to Gene bank sample of KU647722 whereas sub-clade II encompassed only isolate AAUZM-18 corresponding to HM135173.

Selected *B. bassiana* and *M. anisopliae* were evaluated for their pathogenicity efficiency against *G. mellonella* larvae. Isolates of both *B. bassiana* and *M. anisopliae* caused 86.67%–100% mortality on *G. mellonella*. Interestingly, all isolates of *B. bassiana* and *M. anisopliae* showed >86% larval mortality which indicated that these isolates are virulent against *G. mellonella*. The mortality rates of *G. mellonella* larvae peaked at 100% with 4(33.33%) *B. bassiana* and 2(16.67%) *M. anisopliae* isolates after 10 days of treatments. These high virulent isolates showed comparable efficiency with *B. bassiana* and *M. anisopliae* that caused

100% and 98.4% mortality of *G. mellonella* larvae respectively at 10 days post-application with the concentration of 1 × 10^8 spores/ml in Egypt (Saleh et al., 2016). As well, this study presented more effective indigenous isolates against *G. mellonella* compared to other native isolates in Ethiopia (Habtegebriel et al., 2016) recorded high mortality of 71.3% by *Beauveria* spp. and 75% with *Metarhizium* spp at 1 × 10^8 spores/ml of 10 days post-application. The effectiveness of infection by entomopathogenic fungi to a specific insect host might be due to the particular genetic assemblage of virulence factors encompassing a pathotype that adapted to singular or broad host (Valero-Jiménez et al., 2016) and their ability to out-compete the host defense mechanisms (Boston et al., 2020).

The median lethal time (LT_{50}) of isolates of *B. bassiana* and *M. anisopliae* differed among isolates with the range between 2.36 -5.01 days. The high virulent isolate, AAUMB-29 displayed the least LT_{50} value of 2.36 days followed by isolate AAUMFB-77 with LT_{50} of 2.53 days. The present study directed that the highest mortality rate was recorded at the lowest lethal time with isolates of *B. bassiana* AAUMB-29 and *B. bassiana* AAUMFB-77. Correspondingly (Ibrahim et al., 2016), reported that two isolates of *B. bassiana* triggered high mortality of *G. mellonella* larvae within the shortest time of 2.2 and 2.3 days. It has been suggested that isolates with the high mortality rate in the shortest time were found to be promising in terms of time taken as a management strategy against *G. mellonella* larvae. (Kaur and Padmaja (2008) categorized that the entomopathogenic fungal isolates with LT_{50} value < 5 day -high virulent, between 5-6 days-moderate virulent and >6 days -less virulent.

The study of *in-vitro* (spore germination, vegetative growth, and spore production) and *in-vivo* (bioassay evaluation against *G. mellonella*) virulence efficacy of isolates demonstrated that isolate AAUMFB-77 (*B. bassiana)* and AAUMB-29 (*B. bassiana*) followed by AAUDM-43 (*M. anisopliae*) showed high virulence level compared to other tested fungal isolates. In contrast, isolate AAUZM-18 (*M. anisopliae*) followed by AAUZM-60 (*M. anisopliae*) were displayed the lowest virulence effect. The screening of high virulence isolates of entomopathogens may be a vigorous parameter in detecting effective biocontrol agents for the development of biopesticides. The virulence and pathogenicity characters are vital properties of entomopathogenic fungi, *B. bassiana,* and *M. anisopliae* strains for sustainable insect pest management (Mantzoukas et al., 2020a). Hence, this study provides encouraging data for the development of potential biopesticides from entomopathogenic fungi.

6. Conclusion and recommendation

The study shows the morphological, cultural, and bio-insecticidal variation of entomopathogenic fungi (*Beauveria and Metarhizium* spp) isolated from soil samples in Ethiopia. The *in-vitro* virulence evaluation of isolates showed that *Beauveria bassiana* AAUKB-11 displayed the highest germination of 99.67% and *Beauveria bassiana* AAUMFB-77 produced the highest radial growth rate of 3.43 mm day^{-1} with the highest number of spores 4.60 × 10^8spores/ml. Phylogenetic analysis confirmed that 7 isolates of AAUMB-20, AAUMFB-77, AAUMB-29, AAUMFB-5, AAUEB-59, AAUMB-21 and AAUKB-11 were identified as *B. bassiana* and 5 isolates coded as AAUMF-6, AAUEM-30, AAUZM-60, AAUDM-43 and AAUZM-18 were *Metarhizium anisopliae.* Prospective isolates of AAUMB-29, AAUMFB-5, AAUKB-11, and AAUMFB-77 from *B. bassiana* and isolates AAUDM-43 and AAUMFM-6 from *Metarhizium anisopliae* caused higher mortality with corrected mortality of 100%. The *B. bassiana* isolate of AAUMB-29 and AAUMFB-77 exhibited the highest virulence on *G. mellonella* with the lowest LT_{50} values of 2.36 and 2.53 days, respectively. Future studies needed to focus on the evaluation of high virulent isolates against other potential insect pests to assess their vigorous role as a promising biological control agent against different insect pests.

Declarations

Author contribution statement

Amha Gebremariam: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Yonas Chekol: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Fassil Assefa: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data associated with this study has been deposited at NCBI GenBank Database under the accession numbers MW077111, MW077112, MW077113, MW077114, MW077115, MW077120, MW077117, MW077116, MW077121, MW077118, MW077122 and MW077119.

Competing interest statement

The authors declare no conflict of interest.

Additional information

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