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Morphological, molecular, and pathogenicity characteristics of the native isolates of *Metarhizium anisopliae* against the tomato leafminer, *Tuta absoluta* (Meyrick 1917) (Lepidoptera: Gelechiidae) in Ethiopia

Birhan Aynalem Ayele[1*] ⓘD, Diriba Muleta[1], Juan Venegas[2] and Fassil Assefa[3]

Abstract

The South American tomato leafminer, *Tuta absoluta* (Meyrick 1917) (Lepidoptera: Gelechiidae), is a newly introduced and a major threat of tomato production in Ethiopia. Laboratory bioassay was conducted to evaluate locally isolated *Metarhizium anisopliae* (Metsch.) (Sorokin), against larvae of *T. absoluta*. Twenty-five *Metarhizium* isolates were isolated from different soil types, using the great wax moth, *Galleria mellonella* L. baiting method. From these, 13 isolates were prescreened by biological efficiency index model and tested against 2nd and 3rd larval instars of *T. absoluta* at the concentration of 10^7 spores ml^{-1}. Sterile water plus Tween 80 (0.1% v/v) was used as a control. Greenhouse reared larvae of *T. absoluta* were used as experimental organisms. All tested *Metarhizium* formulations were pathogenic to *T. absoluta* in all conducted bioassays. Three isolates, AAUM78, AAUM39, and AAUM76, were the most effective and scored 88, 90, and 95% and 90, 93.3, and 95.7% mortality against 2nd and 3rd larval instars of *T. absoluta*, respectively. The lowest (48.5 and 50%) mortality rate was recorded by isolate AAUM30 against 2nd and 3rd larval instars, respectively, in 7 days post inoculation. However, all isolates showed significant statistical differences with $F (24, 50) = 6.825$, $p < 0.001$ and $F (23, 15) = 3.97$, $p < 0.001$ of mortality to 2nd and 3rd larval instars, respectively. Molecular analysis categorized these selected isolates under species of *M. anisopliae* and grouped into 2 different groups. AAUM78 and AAUM39 were recorded LT_{50} values of 3.93 and 3.5 days at the lowest (10^4 spore ml^{-1}) concentration and LC_{50} values of 1.2×10^3 and 2.9×10^3 spore ml^{-1}, respectively. Although AAUM78 and AAUM39 were virulent to the target pest, further field evaluation is required to determine their potential efficacy for *T. absoluta* control.

Keywords: *Tuta absoluta*, Entomopathogenic fungi, *M. anisopliae*, Mycoinsecticide, Biocontrol

* Correspondence: berha.bat@gmail.com
[1]Institute of Biotechnology, Addis Ababa University, Addis Ababa, Ethiopia
Full list of author information is available at the end of the article

Background

Fungal entomopathogens (EPFs) are cosmopolitan organisms in different habitats and environmentally suited alternatives for pest control (Niu et al. 2019). Several mycoinsecticides have been formulated from different species of EPFs worldwide (Fang et al. 2014). Among these, the genus *Metarhizium* comprises generalist and specialist species based on their host range (Moonjely and Bidochka 2019). *Metarhizium anisopliae*, *M. brunneum*, and *M. robertsii* are generalists and infect insect pests from several orders, whereas *M. acridum* is a specialist and a specific to order Orthoptera (Wang et al. 2016). However, both groups have similar infection mechanism described as spore adhesion, spore germination, cuticle penetration, and mycelial dissemination through body hemolymph, outgrow over the cadavers of dead insects and produce disseminative green muscardine (conidial masses), which is important for horizontal dispersion to other insects (San Aw and Hue 2017).

Further molecular phylogenetic analysis categorized 4 of the most important species, *M. pingshaense*, *M. anisopliae*, *M. robertsii*, and *M. brunneum*, into monophyletic groups, which were descended from *M. anisopliae* (Mayerhofer et al. 2019). These *Metarhizium* species were abundant in versatile habitats particularly, forest soil (Nishi et al. 2018), organic soil (Clifton et al. 2015), farm soil (Stranne 2014), rhizospheric soil (Nishi and Sato 2019), and insect cadavers (Meng et al. 2017). Usually, a large occurrence of *Metarhizium* was reported from farm and forest soil (Nishi et al. 2018), which is an important attribute to have candidates easily adaptable to farmland ecosystem.

Formulated *Metarhizium* mycoinsecticides showed sounding efficacy against several important arthropod pests (Ndereyimana et al. 2019). Especially, the effectiveness of *Metarhizium* species against invasive (Onsongo et al. 2019), insecticidal resistance (Lovett et al. 2019), and secondary pests (FAO 2017) emerged through time were noteworthy. These characteristics were capturing the attention of many stakeholders to upgrade biopesticide development. However, the virulence level of EPFs depends on the environmental conditions that they were originated and the efficiency was variable in between strains based on the biotic and abiotic situations that they were obtained. Thus, augmentation of locally isolated strains through pathogenicity screening has paramount importance to increase the chance of obtaining effective microbial biopesticides.

Tuta absoluta (Meyrick 1917) (Lepidoptera: Gelechiidae) is the South American tomato leafminer moth, which is invasive, devastating, and a major insect pest of tomato production in the world (Santana et al. 2019). Shiberu and Getu (2018) reported 78% loss of tomato by this pest in Ethiopia. The larvae cause up to 100% tomato production losses if not controlled timely (Desneux et al. 2010) as well as expose plants to secondary infection by facilitating the entry of pathogens (Rwomushana et al. 2019).

Several types of synthetic insecticides have been attempted to control *T. absoluta* worldwide. However, the insect has become less responsive and highly resistant to synthetic insecticides (Roditakis et al. 2015). Hence, searching of ecofriendly alternatives for pest control has been taken as the most significant solution to minimize abovementioned problems. EPFs are parts of the potential alternatives. Attempts have been made for the search for effective EPF species to control *T. absoluta* (Ndereyimana et al. 2019). However, only one strain of *M. anisopliae* has been evaluated against *T. absoluta* in Ethiopia so far (Tadele and Emana 2017). Therefore, in the present study, 25 native *Metarhizium* isolates were recovered from different soil types, indexed for their biological efficiency, characterized by their morphological and molecular features, and evaluated for their pathogenicity against 2nd and 3rd larval instars of *T. absoluta*.

Materials and methods

Sampling and sample collection

Metarhizium species were isolated from 3 soil types: farmland, grazing land, and forest soils. Farm soil and grazing land soil were collected from central rift valley area of Ethiopia, particularly, from East Shewa Zone located at 7.3578° N and 38.4850° E coordinates in central Oromia that the tomato is extensively produced, using an irrigation system, and *T. absoluta* was highly manifested without a history of biopesticide application, whereas the forest soils were collected from Menagesha National Forest with geographic coordinates of 8.9782° N and 38.5585° E that covers the altitude between 2574 to 2948 masl and located 20 km west to the capital city, Addis Ababa, Ethiopia.

Rhizospheric soil were collected from tomato farms by uprooting of aged tomato plants, whereas grazing land and forest soils were taken from undisturbed places. All types of the soil were pooled from 5 to 15 cm depth by avoiding external parts and debris in triplicate diagonals of spots by considering 5 m apart from each spot of each site. Three replicates of the soil samples from each location were composited together and only 2 kg of mixed soils composed into ethanol-sterilized (70%) plotline bag. Totally, 52 soil samples were collected from abovementioned sites and taken into Applied Microbiology Laboratory, Addis Ababa University (AAU) for further work.

Rearing of EPF susceptible insect

The great wax moth (*Galleria mellonella* L.) was used for EPF trapping. The insect was reared at Ambo Plant Protection Research Center (APPRC), using the methods described by Meyling (2007). Briefly, 5 pairs of female and male moths were maintained in lid-capped 500-ml conical flasks containing strip-folded tissue paper infused with honey and kept for a week to facilitate mating and egg laying. After 1 week, the folded tissue paper with lied eggs was carefully transferred into insect rearing plastic container, filled with a mix of wheat bran (100 g), honey (160 g), and glycerol (360 ml) as a food source for emerging larvae. Egg-inoculated rearing containers were incubated at 35 °C for 4 weeks until larvae were attained ages of appropriate instars of interest for EPF baiting.

Rearing of *T. absoluta*

The infected tomato leaves and fruits harboring larvae and pupae of *T. absoluta* collected from the central refit valley area using insect collection box were taken into greenhouse of AAU. The larvae and pupae were transferred into pot-grown tomato plants kept under zipped cages constructed from wooden poles and meshed cotton cloth. Pots with growing tomato plants were replaced in rearing cages once per 3 weeks for insect egg laying and for emerging larval feed. Infected tomato leaves in the rearing cages were inspected continuously for larval development and suitable larval instars were collected and used for EPF screening and pathogenicity bioassay.

EPF isolation

Metarhizium species were isolated from the soil using *G. mellonella* baiting method (Belay et al. 2017). Briefly, 3rd instar larvae of wax moth were shocked for 10 s in heat (65 °C) warmed water to reduce extensive waving in the soil. One kilogram of soil samples moisturized by sterilized water was filled into 1½ l capacity screw capped glass jar. Ten of heat-shocked wax moth larvae were introduced in to jars filled with soil samples separately and incubated at 28 °C for 10 days under complete dark condition. Larval death was inspected every 3 days and moisture content of the soil was adjusted by gentle moistening with sterile water each time following the inspection. The cadavers of dead larvae were carefully removed from the soil, surface sterilized by using sodium hypochlorite (3%), followed by ethanol (70%) for 10 s each, and rinsed 5 times with sterile water. Surface-sterilized larval cadavers were placed on sterile plastic plates lined with moistened tissue paper and incubated at room temperature until outgrown mycelia were sporulated under dark condition. The mycelial sporulation of EPF over the cadavers was checked, spores scraped by incinerated inoculating wire loop, transferred onto potato dextrose agar (PDA) medium, and incubated at 28 °C for 20 days. Isolates were purified by sub-culturing onto fresh PDA medium and pure cultures were maintained on agar slants at 4 °C for further work.

Morphological identification

Morphology of *Metarhizium* isolates were identified following the methods of Fernandes et al. (2010). The growth characteristics of isolates on the culture medium (PDA) was inspected continuously for 20 days and their colony size, mycelial color, colony reverse, and color of conidial mass were visualized. Spore feature characteristics of *Metarhizium* isolates particularly shape and size were examined using wet mount glass slide microscopic techniques, using light microscope (Fish Olympus phase contrast microscope).

Prescreening of isolates

Spore germination potential

Spore viability of isolates was checked through conidial germination test, using standard procedures (Habtegebriel et al. 2016). Fungal spores were collected from the 3-week-old culture by scraping with a sterilized spatula. Collected spores were added into 10 ml of sterile distilled water supplemented with Tween 80 (0.1% v/v) as a surfactant in falcon tube and evenly mixed through vortexing. Spore concentration was adjusted into 1×10^6 conidia ml^{-1} by using improved Neubauer hemocytometer under light microscope. A 100-μl suspension was spread over the fresh PDA and 2 sterilized glass slides were laid over inoculated medium and incubated at 25 °C for 24 h. After 24 h of incubation, over germination progresses of spores were halted by ethanol (70%) dispensing. Then 100 spores of both germinated and non-germinated were counted, following the glass slides, using × 40 magnifying objectives of light microscope and the experiment was repeated three times. Spores with germ tubes become high and then the spores itself were considered as germinated.

Sporulation rate on the agar medium

The sporulation rate of *Metarhizium* isolates was tested through plate culture method by incubating at 25 °C under complete dark condition. The plate cultures were checked daily for sporulation initiation since 4 days of initial inoculation. The sporulation rate of each isolate was recorded for 20 consecutive days and isolates that began to sporulate at the short time were considered as potential for pathogenicity screening procedure, using susceptible host, *G. mellonella*. Each plate served as a replicate and there were three replicates per isolates.

Pathogenicity screening of isolates using G. mellonella

The pathogenicity of isolates was evaluated using 3rd instar of G. mellonella larvae. Spores harvested from 3 weeks of old culture through sterilized spatula scraping were adjusted for conidial concentration at a maximum of 10^8 spores ml^{-1} as explained above and 10 ml of suspension were prepared with sterile water and Tween 80 (0.1% v/v) in sterilized falcon tube. Twenty larvae of G. mellonella were deepened into spore suspensions of each isolates for 15 s and transferred into sterile small jars filled with a mix of wheat bran (25 g), honey (40 g), and glycerol (90 ml) separately. Treated larvae-containing jars were placed at room temperature for 10 days at dark conditions. Dead larvae were collected every 3 days, surface-sterilized, and transferred into sterilized plates lined with moistened tissue paper and incubated at room temperature in complete dark condition. The moisture content of the tissue paper was adjusted using sterile water spray to enhance mycelial outgrowth over the larval cadavers. The other 20 larvae were deepened into sterilized water with Tween 80 (0.1% v/v) as control and incubated at the same condition. Treatments were repeated 3 times to minimize uncertainty (Habtegebriel et al. 2016).

Pathogenicity of isolates against T. absoluta

Pathogenicity of 13 Metarhizium isolates prescreened by the cumulative biological efficiency index model was evaluated against 2nd and 3rd larval instars of T. absoluta (Sabbour and Singer 2014). Briefly, the spore suspensions of isolates were prepared into 1×10^7 conidia ml^{-1} consecration, using sterile water with Tween 80 (0.1% v/v). The tomato leaves were surface-sterilized using ethanol (70%) disinfection for 3 min and rinsed 3 times with sterile distilled water. The tomato leaf petioles were tied by UV sterilized cotton wool to retain water and prevent leaf drying. The surface-sterilized leaves were maintained in sterile plastic plates and sprayed with 3 ml of concentration-adjusted fungal spores and air-dried under the safety cabinet for 3 min. Then 20 of each 2nd and 3rd larval instars of T. absoluta were released separately over the spore-sprayed leaves and incubated at room temperature for 7 days. The other 20 larvae of each instar of T. absoluta were released over surface-sterilized leaves sprayed with Tween 80 (0.1% v/v) plus water and incubated in the same condition as a control. Mortality of larvae was checked daily and dead larvae were surface-sterilized and transferred into other sterile plastic plates containing moisten tissue paper. Plates with larval cadaver were incubated at room temperature in dark conditions until developing the mycosis (Sabbour and Singer 2014). These cultures were maintained for 20 days on the plate to determine spore concentration per larval cadavers from the 3rd instar of larvae by using spore washing and microscopic counting method.

Dose response of isolates to T. absoluta

The concentrations (LC_{50} and LC_{90}) of mortality response and time taken to kill 50% (LT_{50}) for 6 of the most virulent Metarhizium isolates were evaluated (Tefera and Pringle 2004). The stock spore suspensions of each isolate were prepared in sterile distilled water containing Tween 80 (0.1% v/v). Concentrations were down-adjusted to 1×10^4, 1×10^5, 1×10^6, 1×10^7, and 1×10^8 spore ml^{-1} and evaluated for the 3rd instar of larvae, following the same procedure as described for pathogenicity assay. Twenty larvae were used for each concentration and control. Fungal infections of the dead larvae were confirmed after the outgrowth of mycosis on the cadavers. Mortality of larvae was recorded daily for 10 days, but lethal time calculation was performed only for three intermediate concentrations (1×10^4, 1×10^6, and 1×10^8 spore ml^{-1}) and all treatments were replicated 3 times.

Molecular characterization of EPF isolates

DNA extraction

The genomic DNA of the fungi was extracted from 4 days old mycelial culture grown on potato dextrose agar, using a quick and safe fungal DNA extraction method (Chi et al. 2009). Approximately 400 mg of mycelia grown on the PDA was transferred into 1.5 ml Eppendorf tube containing 0.5 ml of DNA extraction buffer (1 M KCl; 100 mM Tris-HCl; 10 mM EDTA) using a sterile toothpick. Soon after mycelia transfer, mycelial tissue was pulverized by using sterile plastic pestle fitted with an electronic instrument Black and Decker portable electronic drill (American manufacturer of power tools, Stanley Black & Decker, Inc.) for 2 to 3 s. Mycelial lysates were centrifuged at 12,000g for 10 min in order to separate cell debris and contaminants from the supernatant. The DNA containing supernatant was carefully transferred into another 1.5 ml Eppendorf tubes containing 0.3 ml of 2-propanol and mixed through tube inverting and centrifuged at 13,000g for 10 min. After discarding the supernatant, pellet in the Eppendorf tube was gently washed with 0.7 ml of ethanol (70%) and allowed for ethanol evaporation at room temperature for 15 min. Then DNA pellet was dissolved by using 100 μl of 20 mM Tris solution through gentle tapping and stored at − 20 °C for PCR use. The DNA amount in the suspension was quantified by transferring 2 μl of aliquots on to nano-drop microplates in duplicate using an instrument, BioTek Synergy$_2$^{TM} Multi-mode Microplate Reader controlled by Gen5^{TM} Dada analysis software, USA. Furthermore, DNA purity was confirmed by

running PCR products under 1% agarose gel electrophoresis.

PCR amplification

The PCR amplification of DNA was performed by using ITS1 and ITS4 primers. Primers ITS1 TCCGTAGGTG AACCTGCGG forward and ITS4 TCCTCCGCTT ATTGATATGC reverse were used to amplify the target regions (White et al. 1990). The PCR amplification reaction was conducted in a total volume of 20 μl. The master mix was prepared from the components of 6.8 μl of water, 4 μl of buffer, 1 μl (2.5 mM μl^{-1}) MgCl$_2$, 1 μl (0.5 mM μl^{-1}) of dNTP, 0.2 μl (1 U μl^{-1}) of GoTaq polymerase, 2.5 μl (2.5 μM μl^{-1}) of each ITS1 (forward) and ITS4 (reverse) primers, and 1 μl (30 μg μl^{-1}) of genomic DNA. PCR thermocycler settings were 4 min initial denaturation at 94 °C followed by 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 56 °C, and 1 min extension at 72 °C. The final extension was performed at 72 °C for 5 min with a storage temperature of 4 °C. The PCR products were assayed by electrophoresis on a 2.5% agarose gel with TBE buffer (Tris; Borate; EDTA) at 100 V for 55 min. Then the gel was stained by shaking within 200 ml of TBE buffer supplemented with 10 μl (v/v) of non-carcinogenic dye "SafeView^{TM} Plus" for 50 min and photographed under UV light using eight-mega pixels canon pc1201 digital camera. The PCR amplicons amplified in a volume of 50 μl was purified by using NucleoSpin® Gel and PCR Clean-upkits (Germany), checked for DNA purity using 2.5% agarose gel electrophoresis, and send to Macrogen Inc. Seoul, Korea for sequencing.

Data analysis

The obtained data for larval mortality were calculated by using Abbott's formula (Abbott 1925). Spore germination, sporulation date, and screening test results were analyzed by using one-way of variance (ANOVA) using SPSS software version 25 statistical programs. Mean separations were calculated using Tukey's HSD test when the value were significant at   = 0.05. The relative sporulation rate (RSR) of the isolates was calculated using the formula $RSR = \frac{Exprimental\ lasting\ date}{PCS\ date\ of\ isolate}$ where PCS, plate culture sporulation, and biological efficacy (BE) index were computed by using the biological indexing formula of Sain et al. (2019) with some modification: BE index (%) = 37 (SG) + 13 (RSR) + 50 (LM) where SG, spore germination and LM, larval mortality in 7 days post inoculation of spores. Lethal concentration (LC$_{50}$ and LC$_{90}$) was analyzed by using Probit analysis software in SPSS version 25. The dose responses of each replicate were checked for estimation of lethal time to kill 50% (LT$_{50}$) of exposed larvae. Analysis of variances and means were separated by using least significant differences (LSD).

Results and discussion

In this study, 25 *Metarhizium* isolates were isolated from the soil by using *G. mellonella* baiting method (Table 1). The total isolates trapped from the collected soil samples by insect baiting was 48%. The preliminary characterization showed that all of these isolates produced typical greenish conidial masses of *Metarhizium* on the culture plate with smooth plate reverses (Fig. 1). Such cultural morphology was an important preface distinguishing attributes to identify the intended EPF from the other relatives (Du et al. 2019). Furthermore, microscopic examination of spore features (spore shape and size) supported partial identification of the isolates. Obtained isolates produced intermediate and small-sized and ellipsoid spores (Fig. 1). Sometimes, spore features of *Metarhizium* strains may vary in color and size (Tangthirasunun et al. 2010) as recognized in this study.

The distribution and occurrence of isolates per soil types were 52% in the farm soil, 36% in the grazing land soil, and the remaining 12% in the forest soil (Fig. 2a). Sometimes, EPF could be abundant in the farm soil (Stranne 2014), especially *Metarhizium* spp. dominated in agricultural habitat (Sánchez-Peña et al. 2011). Remixing of the soil during land plowing could allow better distribution of fungal propagules or contentious pest and entomopathogens interaction through natural dynamics may increase the fungal abundance in the farm soil (Vega et al. 2012). Moreover, as our soil samples collected from irrigation farm, sufficient moisture content of the source may favor spore germination to stay propagules alive.

The biological efficiency (BE) index model was emanated as a promising approach for EPF screening (Sain et al. 2019). The data computed by BE indexing model was prescreened 13 (52%) candidates from 25 isolates considering three main biological aspects, spore viability, sporulation rate, and their pathogenicity potential to hosts. In fact, most of the isolates showed statistically significant differences for each criterion, but these abovementioned isolates scored cumulative BE index values of above 80% and screened as efficient candidates as asterisks (*) (Table 1). The 2 isolates (AAUM40 and AAUM12) showed superior (85.6 and 85.74%) cumulative BE index; however, the other eleven scored between 80.75 and 84.85%, respectively. Several reports emphasized that strains with best spore germination potential (Posada and Vega 2005), fast sporulation rate (Mar et al. 2012), and high virulence to their host (Saleh et al. 2016) are important criteria for selecting promising candidates for agriculturally important pest control.

Table 1 Prescreening and biological efficiency index calculation of isolates based on spore germination, culture sporulation dates, and larval mortality of *Galleria mellonella* at 1×10^8 spore ml^{-1} in 10 days of post inoculation

Isolates	Genera	Source (soil)	SG (%) ± SD	Mean of DPCS ± SD	RSR	LM (%) ± SD	BE index (%)
AAUM46*	*Metarhizium*	Farm	92.66 ± 2.51[ab]	12.83 ± 1.52[ab]	1.559	93.33 ± 11.54[ab]	81.15[b]
AAUM32*	*Metarhizium*	Grazing	89.00 ± 2.64[bc]	14.00 ± 1.32[c]	1.429	100 ± 0.00[a]	83.12[ab]
AAUM30*	*Metarhizium*	Grazing	96.33 ± 1.52[a]	12.33 ± 1.52[ab]	1.622	98.00 ± 2.00[b]	84.85[a]
AAUM01	*Metarhizium*	Farm	94.00 ± 3.00[ab]	14.33 ± 0.57[bc]	1.397	87.66 ± 0.2.08[c]	78.79[bc]
AAUM17	*Metarhizium*	Farm	94.00 ± 2.00[ab]	14.00 ± 2.00[c]	1.429	83.00 ± 6.08[dc]	76.47[c]
AAUM28	*Metarhizium*	Grazing	90.00 ± 3.60[b]	15.33 ± 1.52[cd]	1.305	86.00 ± 1.45[b]	76.47[c]
AAUM22	*Metarhizium*	Farm	92.33 ± 3.51[ab]	16.00 ± 2.00[d]	1.250	86.66 ± 5.77[dc]	77.65[c]
AAUM10	*Metarhizium*	Farm	65.00 ± 48.50[ef]	13.50 ± 2.29[b]	1.481	88.33 ± 11.06[c]	68.41[e]
AAUM39*	*Metarhizium*	Grazing	91.66 ± 2.51[b]	13.50 ± 3.50[ab]	1.481	100 ± 0.00[a]	84.11[a]
AAUM20*	*Metarhizium*	Forest	92.33 ± 2.08[ab]	11.66 ± 1.52[ab]	1.715	98.33 ± 2.88[a]	83.55[ab]
AAUM42	*Metarhizium*	Forest	74.00 ± 11.13[d]	14.00 ± 1.00[c]	1.426	73.33 ± 5.77[f]	64.23[ef]
AAUM14	*Metarhizium*	Farm	69.66 ± 6.65[e]	14.00 ± 2.00[c]	1.429	93.00 ± 6.55[b]	72.46[d]
AAUM40*	*Metarhizium*	Farm	95.66 ± 3.21[a]	12.83 ± 0.76[ab]	1.559	100 ± 0.00[a]	85.60[a]
AAUM43*	*Metarhizium*	Grazing	92.00 ± 3.60[ab]	12.63 ± 2.12[ab]	1.584	93.00 ± 2.00[b]	80.75[b]
AAUM76*	*Metarhizium*	Forest	92.00 ± 6.08[ab]	11.16 ± 0.76[ab]	1.792	96.66 ± 5.77[ab]	82.60[ab]
AAUM12*	*Metarhizium*	Grazing	96.00 ± 3.60[a]	12.00 ± 2.00[ab]	1.667	100 ± 0.00[a]	85.74[a]
AAUM66	*Metarhizium*	Grazing	59.00 ± 8.88[ef]	14.00 ± 1.73[c]	1.429	86.66 ± 11.54[de]	65.35[e]
AAUM21*	*Metarhizium*	Grazing	91.66 ± 2.51[b]	13.50 ± 3.12[b]	1.481	93.33 ± 5.77[b]	80.77[b]
AAUM08	*Metarhizium*	Farm	76.66 ± 3.05[de]	15.33 ± 0.57[d]	1.305	53.33 ± 5.77[ghi]	55.20[fgh]
AAUM78*	*Metarhizium*	Grazing	90.33 ± 2.51[b]	12.00 ± 1.00[ab]	1.667	100 ± 0.00[a]	83.64[a]
AAUM04	*Metarhizium*	Farm	88.00 ± 2.00[cd]	14.66 ± 1.15[c]	1.364	90.00 ± 0.00[b]	77.74[c]
AAUM44	*Metarhizium*	Farm	80.66 ± 6.11[c]	13.33 ± 1.52[b]	1.500	80.00 ± 1.00[de]	70.04[d]
AAUM16	*Metarhizium*	Farm	86.66 ± 3.05[cd]	13.00 ± 1.00[b]	1.538	86.66 ± 11.54[de]	75.59[c]
AAUM80*	*Metarhizium*	Farm	91.66 ± 2.51[ab]	13.66 ± 1.52[b]	1.464	93.33 ± 5.77[b]	80.77[b]
AAUM06*	*Metarhizium*	Farm	91.33 ± 3.05[ab]	13.20 ± 3.12[b]	1.515	100 ± 0.00[a]	83.99[a]

Letters in the columns (a, b, c, d, e, f, g, and h) are ranks of the mean
DPCS date of plate culture sporulation, *SG* spore germination, *LM* larval mortality, *SD* standard deviation, *RSR*, relative sporulation rating, *BE* biological efficiency
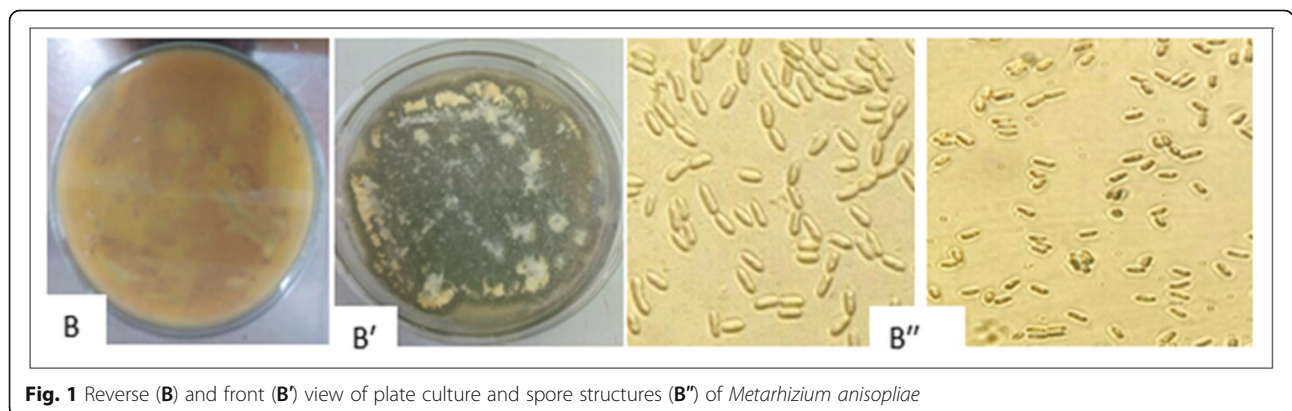*Selected isolates

Fig. 1 Reverse (**B**) and front (**B′**) view of plate culture and spore structures (**B″**) of *Metarhizium anisopliae*

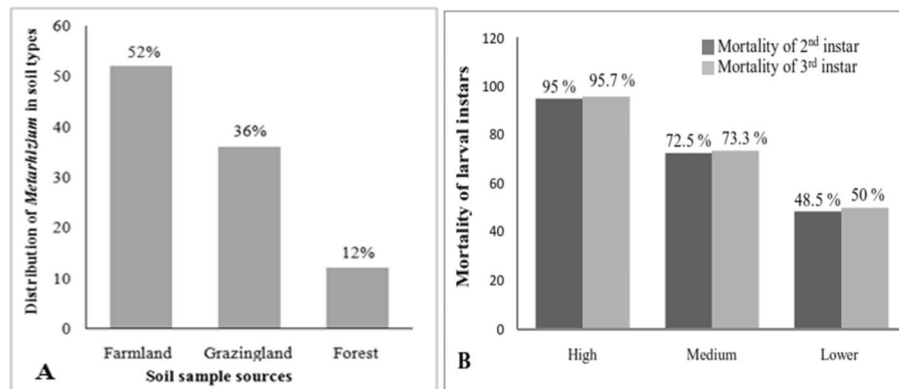**Fig. 2** Distribution of *Metarhizium* in the soil types (**a**) and summary of *Metarhizium* pathogenicity potential against 2nd and 3rd larval instars of *Tuta absoluta* (**b**)

Pathogenicity test revealed that most of these pre-screened isolates were effective against *T. absoluta* with a spore concentration of 10^7 spore ml^{-1} in 7 days of post inoculation (Table 2). Maximum, medium, and low mortality rates caused by *Metarhizium* isolates on both 2nd and 3rd larval instars of *T. absoluta* were almost the same (Fig. 2b). However, mean pathogenicity among isolates showed marked difference with statistical values of F (23, 15) = 3.97, $p < 0.001$ for 3rd instar and F (24, 50) = 6.825, $p < 0.001$ for 2nd instar of larvae. The death of larvae by *Metarhizium* was ensured through green muscardine production on the cadavers.

Among the evaluated isolates, 23% showed markedly high mortality rates (90 to 95.66%), 30.8% scored high mortality (81.66 to 88.33%), and 38.5% caused modest

mortality (73.33 to 76.66%); however, only one isolate scored relatively low (50%) mortality rate on the 3rd larval instar of *T. absoluta* (Table 2). The isolates AAUM78 and AAUM39 exhibited the highest pathogenicity on the 2nd larval instar of *T. absoluta* with mortality rate of 90 and 95%, respectively. However, other isolates showed relatively reduced virulence against 2nd larval instar than 3rd ones. The data clearly indicated that some of the isolates were as effective as the commercially formulated *Metarhizium* (Metatech® WP) that caused 82.8% larval mortality of *T. absoluta* at 10^8 spores ml^{-1} in Rwanda (Ndereyimana et al. 2019), with 87.5% mortality recorded from the treatment of 2.5×10^9 spores ml^{-1} of *M. anisopliae* in Ethiopia (Tadele and Emana 2017).

Table 2 Rescreening of pathogenically potential isolates against second and third larval instars of *Tuta absoluta* at 1×10^7 spore ml^{-1} for 7 days exposure and sporulation potential over cadavers

Screened isolates	Genus	2nd instar LM (%) ± SD	3rd instar LM (%) ± SD	Spore conc. per cadaver ml^{-1}
AAUM30	*Metarhizium*	48.47 ± 2.00[j]	50.00 ± 10.00[h]	8.4×10^5
AAUM43[♦]	*Metarhizium*	86.66 ± 7.63[b]	81.66 ± 2.88[bc]	3.2×10^6
AAUM76[♦]	*Metarhizium*	88.00 ± 10.00[b]	95.66 ± 5.77[a]	3.1×10^7
AAUM06	*Metarhizium*	56.52 ± 3.56[h]	75.00 ± 15.00[c]	9.6×10^5
AAUM32	*Metarhizium*	66.52 ± 3.56[ef]	75.34 ± 39.00[c]	2.4×10^5
AAUM80[♦]	*Metarhizium*	83.33 ± 7.63[bc]	83.33 ± 15.27[b]	7.9×10^6
AAUM12	*Metarhizium*	72.54 ± 6.68[d]	73.33 ± 10.40[cd]	1.5×10^6
AAUM46	*Metarhizium*	69.02 ± 4.82[e]	76.66 ± 14.43[c]	4.8×10^6
AAUM20[♦]	*Metarhizium*	89.00 ± 5.00[b]	86.66 ± 11.54[b]	4.7×10^6
AAUM40	*Metarhizium*	73.33 ± 7.63[d]	88.33 ± 7.63[ab]	4.4×10^6
AAUM78[♦]	*Metarhizium*	90.00 ± 5.00[a]	90.00 ± 10.00[ab]	5.4×10^7
AAUM39[♦]	*Metarhizium*	95.00 ± 8.66[a]	93.33 ± 5.77[a]	1.8×10^7
AAUM21	*Metarhizium*	69.23 ± 2.12[e]	75.00 ± 15.00[c]	4.9×10^5

Letters in the columns (a, b, c, d, e, f, g, and h) are ranks of the mean
LM larval mortality, *SD* standard deviation
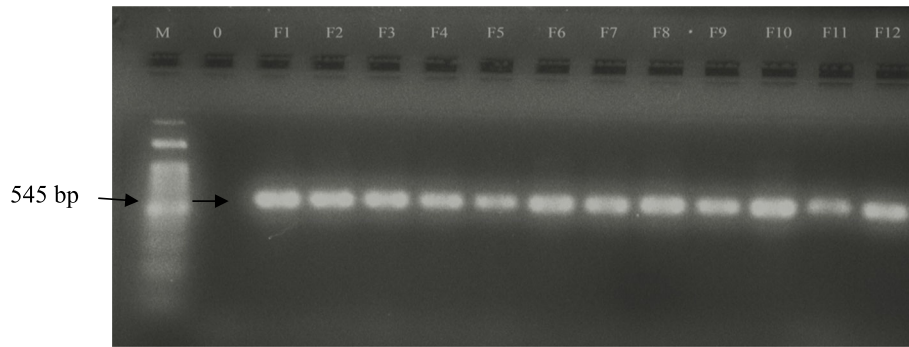[♦]Reselected isolates

Fig. 3 PCR products amplified using ITS primers (ITS1 and ITS4) from twelve potential isolates of *Metarhizium anisopliae*. M, 1000 bp ladder; 0, blank; F1 up to F12 was AAUM78, AAUM12, AAUM30, AAUM76, AAUM40, AAUM39, AAUM43, AAUM80, AAUM06, AAUM21, AAUM32, and AAUM20, respectively
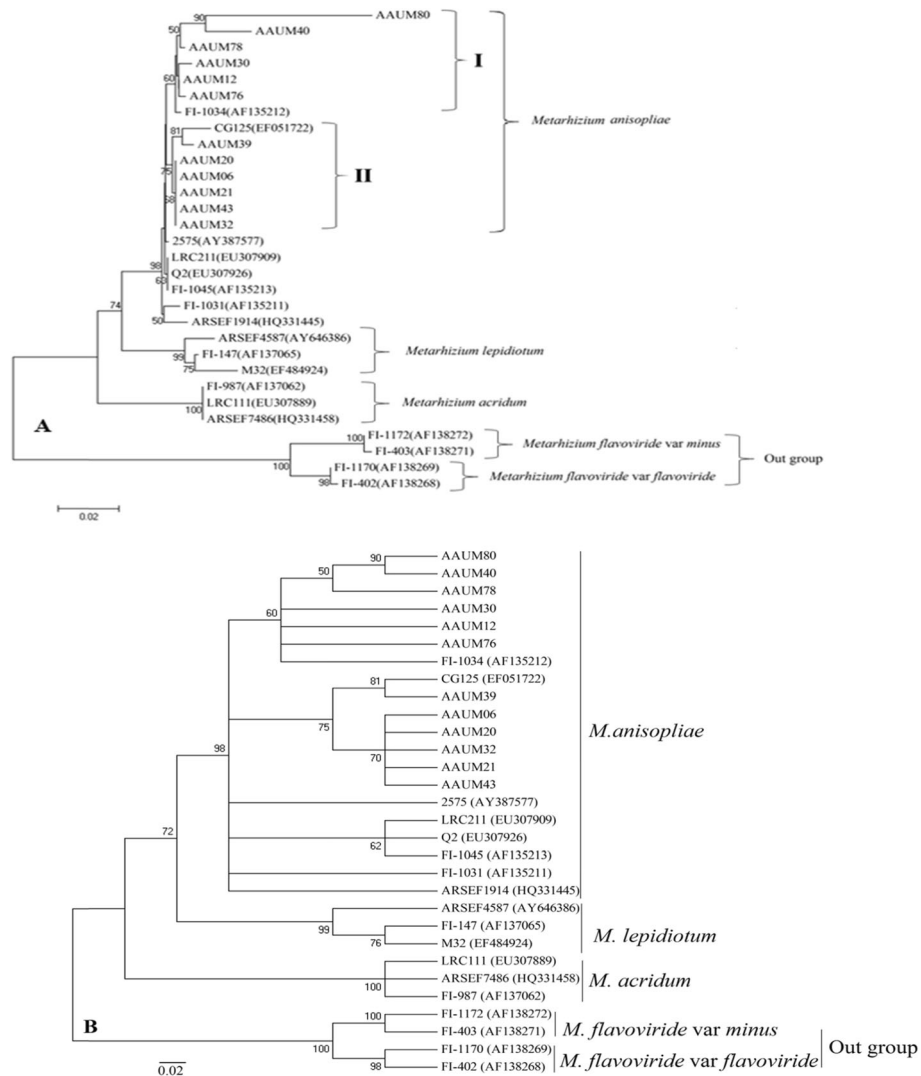

Fig. 4 Molecular phylogenic (**a**) and cladogram (**b**) analysis of ITS region of *Metarhizium anisopliae* from Ethiopia and other related sequences retrieved from gene Bank. Maximum likelihood phylogenetic tree (MEGA4.1) based on ITS sequences from *Metarhizium* species using K2pl model, with 1000 bootstrap replications. Bootstrap values above 50% shown and corresponding accession number of strains were presented in parenthesis

In this study, maximum and minimum mortality rates on both 2nd larval instar attained 95 and 93%, respectively, at the isolate AAUM39 and 48.47 and 50%, respectively, at AAUM30 (Table 2). Different studies showed that *Metarhizium* had a high virulence against 3rd larval instar of *T. absoluta* (Alikhani et al. 2019; Ndereyimana et al. 2019).

Meanwhile, the most pathogenic fungal isolates produced promising spores over larval cadavers with minimum $(3.2 \times 10^6$ spore ml^{-1}) to maximum$(5.4 \times 10^7$ spores ml^{-1}) concentration as indicated by a diamond (♦) in Table 2. The isolates that achieved high pathogenicity on both larval instars of the pest and had better sporulation are potential candidates to develop mycoinsecticides in the future. Especially, the mortality of *T. absoluta* at the early stage was vital to reduce extensive crop damages (Pires et al. 2010). Furthermore, fast sporulation entomopathogens with a large amount of spore production over the cadavers might give enhanced horizontal pest infection through swift self-dissemination (Conceschi et al. 2016).

Amplification of internal transcribed spacer (ITS1-5.8S-ITS4) rDNA regions of the 12 selected isolates showed DNA with an approximate size of 545 bp (Fig. 3). DNA sequencing and phylogenic analysis tentatively positioned these isolates in to *M. anisopliae* species (Fig. 4), in which the topology was similar to that described by other authors (Brancini et al. 2018). Relationship check was assured by BLAST search method and all of the isolates that have 99% and above similarity with sequence of *M. anisopliae* species quarried from GenBank. Interestingly, this main node is divided into 2 subgroups (I and II), with a percentage of statistical support bootstrap of 60 and 75%, respectively (Fig. 4a). Group I contained a sample of the data bank corresponding to AF135212 and group II to sample EF051722 (Fig. 4a, b). Although the statistical support is not as robust for both groups, it would be very interesting to see if the samples within each group have any genetic, biological, and/or entomopathogenic characteristics in common. Something similar could happen in the case of samples AAUM80 and AAUM40, which have a high bootstrap (90%) and sample AUM39 with the sample from data bank EF051722.

The efficacy of all isolates increased as concentration and exposure time was increased (Table 3). However, the median lethal time taken to kill 50% (LT$_{50}$) was significantly different between isolates at $P = 0.05$ (Table 3). The LT$_{50}$ of the isolates was between 3.5 and 5.33 days at low (10^4 spore ml^{-1}) spore concentration against 3rd larval instar of *T. absoluta*, whereas LT$_{50}$ of 1.93 and 2.98 days was scored at high (10^8 spore ml^{-1}) spore concentration, respectively. The two best isolates, AAUM78 and AAUM39, displayed LT$_{50}$ of 3.93 and 3.5 days at low (10^4 spores ml^{-1}) concentration and 1.93 and 2.8

Table 3 Summary of Probit analysis, calculated lethal time, and concentration-mortality response at 10 days of post inoculation to third larval instar of *Tuta absoluta*

Spore ml^{-1}	AAUM43	AAUM76	AAUM20	AAUM80	AAUM78	AAUM39
Mortality (mean ± SE)						
10^4	50.33 ± 4.41[e]	52.33 ± 4.41[e]	50.00 ± 5.77[e]	55.00 ± 2.89[e]	78.33 ± 4.41[a]	65.00 ± 10.41[c]
10^5	61.67 ± 10.1[c]	61.67 ± 6.01[c]	61.67 ± 4.41[c]	60.00 ± 5.77[c]	83.33 ± 1.67[ab]	73.33 ± 4.41[bc]
10^6	81.67 ± 3.33[b]	70.00 ± 5.77[d]	83.33 ± 6.01[bc]	76.67 ± 4.41[c]	86.67 ± 1.67[b]	83.33 ± 1.67[bc]
10^7	95.00 ± 1.89[a]	80.00 ± 5.00[bc]	89.00 ± 2.89[ab]	88.33 ± 1.67[b]	93.33 ± 1.67[a]	91.33 ± 1.67[b]
10^8	95.00 ± 0.00[a]	86.67 ± 1.67[c]	91.67 ± 1.67[b]	90.00 ± 2.89[b]	96.67 ± 1.67[a]	93.33 ± 1.67[ab]
LT$_{50}$ (mean ± SE)						
10^4	5.33 ± 0.58[c]	4.00 ± 1.00[b]	4.00 ± 1.00[b]	4.00 ± 1.00[b]	3.93 ± 0.90[ab]	3.50 ± 0.50[a]
10^6	3.00 ± 0.00[b]	3.60 ± 0.53[a]	3.33 ± 0.58[b]	3.87 ± 0.12[ab]	3.33 ± 0.58[b]	3.00 ± 0.00[b]
10^8	2.81 ± 0.00[b]	2.98 ± 0.00[a]	2.87 ± 0.12[b]	2.93 ± 0.12[a]	1.93 ± 0.00[a]	2.80 ± 0.20[b]
Summary of Probit analysis						
LC$_{50}$	1.4×10^4	7×10^4	4.3×10^4	4.1×10^4	1.2×10^3	2.9×10^3
95% FL	4×10^3–7.2×10^4	2.8×10^3–3.6×10^5	3.8×10^3–1.7×10^5	2.5×10^3–1.8×10^5	8.3×10^2–3.8×10^3	1.9×10^2–2.9×10^4
LC$_{90}$	8.2×10^6	1.7×10^8	1.6×10^7	2.8×10^7	3.5×10^6	7.1×10^6
95% FL	1.6×10^6–3.6×10^7	1.6×10^7–1.1×10^9	3.1×10^6–4.5×10^8	4.6×10^6–1.7×10^8	1.2×10^4–3.4×10^6	1×10^6–1.7×10^7
Int ± SE	1.93 ± 0.66	1.83 ± 0.66	2.32 ± 0.64	2.10 ± 0.63	0.59 ± 0.80	1.31 ± 0.66
S ± SE	4.6 ± 0.12	3.8 ± 0.10	5 ± 0.11	4.5 ± 0.11	3.4 ± 0.15	3.8 ± 0.12
P value	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

Letters in the columns (a, b, c, d, and e,) are ranks of the mean
FL fiducial limit, *SE* standard error, *LT* lethal time taken to kill 50% of experimental organisms, *LC* lethal concentration, *Int* intercept, *S* slope

days at high (10^8 spore ml^{-1}) spore concentration, respectively. This was a better score than the commercial *Metarhizium* (Metatech® WP) with LT_{50} of 3.9 days against larvae of *T. absoluta* at spore concentration of 10^8 spores ml^{-1} in Rwanda (Ndereyimana et al. 2019) and 5.21 days with 2.5×10^9 spore ml^{-1} of *M. anisopliae* in Ethiopia (Tadele and Emana 2017).

Although some variations among the strains, all of the selected isolates showed promising LC_{50} (1.2×10^3 spores ml^{-1} to 7×10^4 spores ml^{-1}) and LC_{90} (3.5×10^6 spores ml^{-1} to 1.7×10^8 spores ml^{-1}) values (Table 3). Particularly, AAUM78 and AAUM39 showed LC_{50} of 1.2×10^3 and 2.9×10^3 and LC_{90} of 3.5×10^6 and 7.1×10^6, respectively (Table 3), and this indicated the effectiveness of these 2 strains. Isolates, effective at the lowest lethal concentration, are promising for *T. absoluta* management (Alikhani et al. 2019) with economical conidial mass production. Generally, researchers revealed the importance of best concentration and lethal time determination methods to select effective isolates for mycoinsecticidal formulations for field application against target insect pest (Sain et al. 2019).

Conclusion

The preset study revealed the possibility of isolate *Metarhizium* strains from the local environment to manage *T. absoluta*. Most of the *M. anisopliae* strains, especially the two strains AAUM78 and AAUM39, showed a high pathogenicity against larvae of *T. absoluta*. The strains with short LT_{50} and low LC_{50} values are promising to control the target pest at early stage as well as produce economical conidia for pest management. Further field evaluation of these strains is needed to determine their potential against *T. absoluta*.

Abbreviations

EPF: Entomopathogenic fungi; APPRC: Ambo Plant Protection Research Center; AAU: Addis Ababa University; LT: Lethal time; LC: Lethal concentration; PCR: Polymerase chain reaction;
EDTA: Ethylenediaminetetraacetic acid; TBE: Tris-borate-EDTA;
ANOVA: Analysis of variance; BE: Biological efficiency; SG: Spore germination; DPCS: Date of plate culture sporulation; RSR: Relative sporulation rate; LM: Larval mortality; ITS: Internal transcribed spacer

Authors' contributions
BA designed the study, conducted the laboratory experiment, performed data analysis, and drafted the manuscript. JV involved in molecular work, processed DNA sequencing, and performed molecular data analysis. FA and DM reviewed the manuscript and added their inputs. The authors read and approved the final manuscript.

Availability of data and materials
The analyzed datasets used in this study were included in the manuscript and the primary data was found with the corresponding author.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
[1]Institute of Biotechnology, Addis Ababa University, Addis Ababa, Ethiopia. [2]Faculty of Medicine, Institute of Biomedical Sciences, Cellular and Molecular Biology Program, University of Chile, Santiago, Chile. [3]College of Natural and Computational Sciences, Department of Microbial, Cellular and Molecular Biology, Addis Ababa University, Addis Ababa, Ethiopia.

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