






RESEARCH ARTICLE

REVISED Isolation, morphological characterization, and screening virulence of *Beauveria bassiana* and *Metarhizium robertsii* fungal isolates on *Galleria mellonella* [version 5; peer review: 2 approved]

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Abstract

Background

Entomopathogenic fungi exists naturally in plants as an asymptote and have the potential to reduce the population of insect pests through indirect interactions. This study was conducted to detect and characterize the endophytic fungi *Beauveria bassiana* and *Metarhizium robertsii* from the rhizosphere soil of tomato plants and their virulence effect on *Galleria melonella*.



Methods

From the rhizosphere soil of 40 tomato fields, three *Beauveria bassiana* and seven *Metarhizium robertsii* isolates were isolated using the galleria bait method. All fungi isolate were morphologically characterized by their colony color, shape, and surface texture. Isolates with the highest percentages of germination, conidial yield,

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and radial growth were selected, and their virulence was evaluated on second instar larvae of *Galleria melonella* under laboratory conditions.

Results

In this study, *Beauveria bassiana* showed white colony color and aseptate hyphae, whereas *Metarhizium robertsii* showed dark green to light green colony color and septate hyphal structures. Maximum spore production and conidial length were obtained by *Beauveria bassiana* isolate APPRC-27 with 2.67×10^7 spores ml^{-1} and $3.24 \mu\text{m}$, respectively. Colony radial growth rates ranged from 1.73 to 3.24 mm day^{-1} . The results revealed that the highest mortality rate of *Galleria melonella* (100%) was obtained by *Metarhizium robertsii* isolates K-61 and K-102 at a concentration of 1×10^8 conidial ml^{-1} at 7 days post-inoculation. The lowest mortality rate was registered by *Metarhizium robertsii* isolate RST-11.

Conclusions

In the present study, isolates that produced the most spores and had the highest germination rates were the most virulent to *Galleria mellonella* second instar larvae. Therefore, virulent entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium robertsii*, are promising bioagents for the control of insect pests.

Keywords

Conidia, colony color, endophytic, entomopathogenic fungi, *Galleria melonella*, isolates

Eldoret, Kenya

Any reports and responses or comments on the article can be found at the end of the article.



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REVISED Amendments from Version 4

In the new version some sentences added in the discussion.

Any further responses from the reviewers can be found at the end of the article

Introduction

Entomopathogenic fungi, *Beauveria bassiana*, (Bals. -Criv.) Vuill. and *Metarhizium robertsii* (Metchnikoff) Sorokin, live in plant tissues without showing pathological symptoms (Dutta *et al.*, 2014). They have the potential to limit the feeding capacity, preference, and oviposition of stem boring, sap-sucking, and leaf mining insects on many crops by producing toxic compounds that disturb the insects performance. Additionally, they increase the plant defense mechanisms against pests or abiotic factors (McCormick *et al.*, 2016; Hokkanen and Menzler-Hokkanen, 2017). Entomopathogenic fungi are capable of penetrating their insect hosts through cuticular exoskeletons (Zimmermann, 2007).

Entomopathogenic fungi have become more promising than chemical pesticides for the control of pests in Ethiopia due to pesticide resistance. This is due to the misuse of pesticides, which causes the incrementation of pesticide-resistant biotypes (Negash *et al.*, 2020), and a lot of pests have developed resistance due to the high use of pesticides (Tabashnik *et al.*, 2009). Consequently, using eco-friendly biopesticides like *B. bassiana* and *M. robertsii* as bioagents against agricultural pests is the best option to increase crop production and productivity. They are symbiotic with their hosts, reduce pest populations, and require little inoculum for their systemic transmission within plant tissues (Nair and Padmavathy, 2014).

Identification and characterization of entomopathogenic fungi, *B. bassiana* and *M. robertsii*, are highly required for entomological research and essential for the selection of virulent isolates against several pests (Boucias *et al.*, 2000). Currently, the selection of virulent strains of entomopathogenic fungi as the biological control agent of pests is of great interest to many researchers (Lacey *et al.*, 2015). However, there is a limit to the work on the isolation, morphological characterization, and screening of virulent isolates of entomopathogenic fungi, particularly *B. bassiana* and *M. robertsii* in Ethiopia, to control agricultural pests. Hence, this study was made to identify, characterize the morphology, and screen the virulence of *B. bassiana* and *M. robertsii* isolates on *Galleria melonella* collected from rhizosphere soil in tomato plant farms at Koka and Guder areas in Ethiopia.

Methods

Description of the study site

The experiment was conducted at the Ambo Agricultural Research Center under laboratory conditions. Ambo is far away from Addis Ababa, 110 km to the west, at a geographical coordinate of 8°59'N latitude and 37.85°E longitude, with an altitude of 2,100 m a.s.l. The daily temperature ranges from 15 to 29°C, with an annual temperature of 22°C on average. The soil sample was collected from two sites, namely Guder and Koka in Ethiopia. Guder is located at a latitude and longitude of 8°58'N and 374°6E, respectively, with an altitude of 2,101 m a.s.l., whereas Koka is found at 8°25' N latitude, 39°02' E longitude, and an altitude of 1,605 m a.s.l. The minimum and maximum temperatures ranged from 12.14 to 27.39°C, respectively (Etefa and Dibaba, 2011).

Sampling of rhizosphere soil from tomato plants

Rhizosphere soil was collected from tomato farmlands by uprooting vegetative-stage tomato plants. During the collection, the upper layer of soil was removed. A total of 40 rhizosphere soil samples were taken from two locations. Five samples were taken from each spot and mixed in a plastic bag before being taken to the bio-control laboratory of the Ambo Agricultural Research Center for the isolation of *B. bassiana* and *M. robertsii*. Finally, one kilogram (kg) of the soil sample was used for the isolation by removing unnecessary parts from the soil (Sevim *et al.*, 2010).

Isolation of *B. bassiana* and *M. robertsii* from Rhizosphere soil samples

From 40 soil samples, *B. bassiana* and *M. robertsii* were isolated by using the Galleria baiting method as follows (Zimmermann, 1986; Belay *et al.*, 2017).

One kilogram of soil sample was moistened by sterilized water and added to a 1.5 kg screw-capped glass jar. A total of 10 wax moth larvae were separately introduced into jars filled with soil samples and incubated at 26°C for 10 consecutive days under dark conditions.

Larval deaths were inspected and recorded every three days. The moisture of the soil was maintained by moistening it with sterile water daily. The cadavers were taken off the soil and surface sterilized with 1% sodium hypochlorite for 2 minutes, followed by 70% ethanol for 10 seconds, and rinsed three times with sterile water. Surface-disinfected larval cadavers were placed on sterile plastic plates with slightly wetted filter paper and incubated at room temperature up to the sporulation of outgrown mycelia.

The sporulation of *B. bassiana* and *M. robertsii* over the dead cadavers was examined, and the spores were harvested by a sterilized inoculating wire loop, transferred onto Sabouraud Dextrose Agar (SDA) Microexpress[®] media, and incubated at 27±1°C for 14–21 days. Isolates were purified by subculturing on newly prepared SDA media, and pure cultures were preserved with glycerol and maintained on agar at -19°C for further work.

Morphological identification and characterization of isolates

Fungal isolates were morphologically identified and characterized using the identification key of Humber (Humber, 1997), depending on fungal structures acquired from pure culture (Gürlek *et al.*, 2018). In addition to the new isolates, three *B. bassiana* (APPRC-44BC, APPRC-44BC-1, and APPRC-27) and one *M. robertsii* (S-26) isolates were also characterized from the Ambo Agricultural Research Center as standard checks for the comparison of isolates.

Consequently, the morphologies of *B. bassiana* and *M. robertsii* were characterized by the culturing of pure cultures on the SDA media, and inspection was done for 14–21 days. Both macroscopic and microscopic characterization of isolates was conducted by observation of morphological features of isolates. Macroscopic features such as mycelial color, colony reverse, colony shape and color, colony elevation, and surface texture were observed. Microscopic characterization of isolates was also done under an Olympus SC50 (CX33RTFS2, Olympus Corporation) microscope with ×40 magnification (Barra *et al.*, 2013) by measuring conidial length and diameter, hyphal growth, and conidiogenous cells using the slide culture technique.

Slide culture preparation

The technique was conducted by autoclaving a 9-cm-diameter Petri dish with filter paper and a bent glass rod. A 15 g⁻¹ of water agar was cut by a 1 cm × 1 cm block with a sterilized scalpel blade and put on a glass slide. Fungal spores were inoculated on the four sides of the agar blocks by a sterile inoculating needle, and coverslips were placed on the block. Moistened filter paper with 2 ml of sterile water was placed over plates, sealed with parafilm, and incubated at 27±1°C.

Agar blocks were discarded after incubation for 72 hours, and the slides were moistened with a drop of 97% ethanol and enabled to heat fix by passing on an alcohol lump. A drop of lactophenol blue was used to stain the slide with a coverslip, which was later sealed with nail polish. The slide was placed under a SC50 microscope with ×40 magnification for the observation and characterization of conidiophore structure (Rosana *et al.*, 2014). Finally, the slides were permanently preserved for further characterization. The characterization and measurement of spore features such as conidial length and diameter were also done using the Sc 50 microscope (magnification, ×40).

Spore germination test for the viability of fungal isolates

The spore germination test was verified by the conidial germination test technique according to standard procedures (Goettel and Inglis, 1997). Fungal spores were harvested from a 21-day-old culture by scraping with a sterilized spatula. Harvested spores were added to 10 ml of sterile water with Tween 80 (0.001% v/v) as a surfactant in a falcon tube and vortexing. Fungal spores were adjusted to 1 × 10⁶ conidia ml⁻¹ by using a Neubauer hemocytometer (USA) under a SC50 microscope.

In total, 100-μl of suspended spores was spread over fresh Potato Dextrose Agar (PDA) (Himedia[®]), and two sterilized glass slides were placed on the media and incubated at 28°C for 18 hours. After 18 hours, germination of the spores was halted by dispensing with 70% ethanol. Both germinated and non-germinated cells were counted by using a Neubauer hemocytometer under a SC50 microscope (magnification, ×40).

The experiment was conducted under laboratory conditions in CRD with 15 treatments and three replications. A germ tube with more than half the diameter of the spore was considered germinated, and *vice versa* for non-germinated. Isolates with more than 95% germination were selected against *G. melonella* to test their virulence. The percentage of spore germination was calculated according to the method developed by Vega *et al.* (2008).

$$\text{Percent spore germination} = \frac{\text{number of spores germinated}}{\text{Total spore counted}} \times 100$$

Radial growth rate

Conidia of 14-day-old cultures were harvested from plates and spread on a 90 mm Petri dish containing fresh SDA and PDA media separately. Isolates were incubated for three days at $27\pm 1^\circ\text{C}$, and the 5 mm diameter of three-day-old cultures mycelium was taken by using a cork borer and placed in the center of a 90 mm petri dish containing SDA and PDA media individually. Cultures were incubated at $27\pm 1^\circ\text{C}$ (Membang *et al.*, 2021).

The experiment was conducted in CRD with 11 treatments and replicated three times. Data on radial growth were recorded in mm/day from the 4th to the 15th days and calculated by:

$$\text{RG/day} = \frac{\text{Colony diameter at the end of incubation period} - \text{Fungal disc diameter}}{\text{Total incubation days}}$$

Where, RG/day is radial growth per day.

Estimation of conidial yield

Spore production was estimated as follows (Schemmer *et al.*, 2016). A 21-day-old culture of 10 mm-diameter circular agar was cut with a cork borer, and the disc was added to 10 ml of sterile 0.001% Tween 80 solution. The solution was well vortexed to evenly mix, and the conidial concentration was counted by using a Neubauer hemocytometer. The conidial yield was expressed in terms of conidial ml^{-1} . The experiment was conducted under laboratory conditions in CRD with eight treatments and three replications.

Conidial suspension preparation

Conidia were obtained from pure cultures grown on SDA, which was incubated for 14 days at $27\pm 1^\circ\text{C}$ under dark conditions. Conidia were harvested by disposable cell scrapers and added into test tubes containing Tween 80 (0.001% v/v). Suspensions were vortexed well for 2 minutes and adjusted to 1×10^8 conidia ml^{-1} using a Neubauer hemocytometer (Gurulingappa *et al.*, 2010).

Rearing of *Galleria mellonella*

Galleria mellonella larvae (Lepidoptera: Pyralidae) were reared at the Ambo Agricultural Research Center in the biocontrol room following the methods described by Meyling (2007). Adult moths were kept in 500-ml flasks containing folded tissue paper to fasten their mating and egg-laying capacities. To hatch second-instar larvae, the eggs were laid on folded tissue paper, transferred to plastic containers filled with 80 g honey, 50 g wheat bran, and 180 g glycerol, and incubated at 20°C for four weeks in the dark.

Virulence test of isolates using *G. mellonella*

The potential virulence of fungal isolates was tested using 2nd instar *G. mellonella* larvae by the galleria dipping method. Spores collected from 14–21 days of old culture by sterilized spatula scraping were adjusted to 1×10^8 spores' ml^{-1} and 10 ml were prepared from the suspension of sterilized water with Tween 80 (0.001% v/v) in a sterilized falcon tube and vortexing well. A total of 10 second instars of *G. mellonella* larvae were dipped into spore suspensions for 15 sec. Treated larvae with fungal isolates were incubated at 26°C for 10 days under dark conditions. Cadavers were collected every day 3 days post inoculated, disinfected on their surfaces, transferred into sterilized plastic plates lined with moistened filter paper, and incubated in a dark place. Dead larvae were daily inspected and moistened with sterilized water to promote the outgrowth of mycelial on the cadavers. A control was treated with Tween 80 (0.001% v/v) and incubated under the same conditions (Ayele *et al.*, 2020). Isolates with greater than 90% mortality of larvae were selected, considered to be virulent isolates, and preserved for further work.

Data analysis

Germination test, radial growth, conidial yield and size, and virulence test of isolates data was analyzed using analysis of variance (ANOVA) followed by Tukey's test with a statistical difference of $P < 0.05$. Statistical Analysis System (SAS) (RRID:SCR_008567) software 9.4 version was used for data analysis. Means were separated using Tukey's honestly significant difference (HSD) at $P < 0.05$.

Results

Morphological identification and characterization of *B. bassiana* and *M. robertsii* isolates

This study revealed that three *B. bassiana* and seven *M. robertsii* isolates were identified from rhizosphere soil samples of tomato plants. *B. bassiana* isolates showed white colony color, wide round to round colony shape, smooth powdery to smooth cottony surface texture, and raised colony elevation, as indicated in Table 1.

Table 1. Macroscopic features of *B. bassiana* and *M. robertsii* isolates.

Isolate name	Isolate code	Source	Location	Host plant	Colony Color		Colony shape	Surface texture	Colony elevation
					front	back			
<i>B. bassiana</i>	RST-8	Rh. soil	Guder, Ethiopia	tomato	white	yellowish	wide round	smooth powder	raised
<i>B. bassiana</i>	APPRC-27	soil	Ambo ARC collection	unknown	white	yellowish	round	smooth, powder	raised
<i>B. bassiana</i>	K-91	Rh. soil	Koka, Ethiopia	tomato	white	yellowish	round	smooth, cottony	raised
<i>B. bassiana</i>	K-5	Rh. soil	Koka, Ethiopia	tomato	white	yellowish	round	smooth, cottony	raised
<i>B. bassiana</i>	APPRC-44BC	soil	Ambo ARC collection	unknown	white	yellowish	round	smooth, powder	raised
<i>B. bassiana</i>	APPRC-44BC-1	soil	Ambo ARC collection	unknown	white	yellowish	round	smooth, powder	raised
<i>M. robertsii</i>	RST-11	Rh. soil	Guder, Ethiopia	tomato	dark green	brown	round	thick	flat
<i>M. robertsii</i>	RST-12	Rh. soil	Guder, Ethiopia	tomato	light green	brown	medium round	thick	flat
<i>M. robertsii</i>	S-26	soil	Ambo ARC collection	unknown	light green	brown	round	thin, cottony	flat
<i>M. robertsii</i>	K-61	Rh. soil	Koka, Ethiopia	tomato	dark green	brown	round	thin, addressed	flat
<i>M. robertsii</i>	K-63	Rh. soil	Koka, Ethiopia	tomato	dark green	brown	round	thin, addressed	flat
<i>M. robertsii</i>	K-101	Rh. soil	Koka, Ethiopia	tomato	light green	brown	medium round	thick, cottony	slightly raised
<i>M. robertsii</i>	K-7	Rh. soil	Koka, Ethiopia	tomato	light green	brown	round	thin, addressed	slightly raised
<i>M. robertsii</i>	K-102	Rh. soil	Koka, Ethiopia	tomato	light green	dark brown	medium round	thick, cottony	slightly raised

Note: Rh=Rhizosphere, Ambo ARC=Ambo Agriculture Research Center.

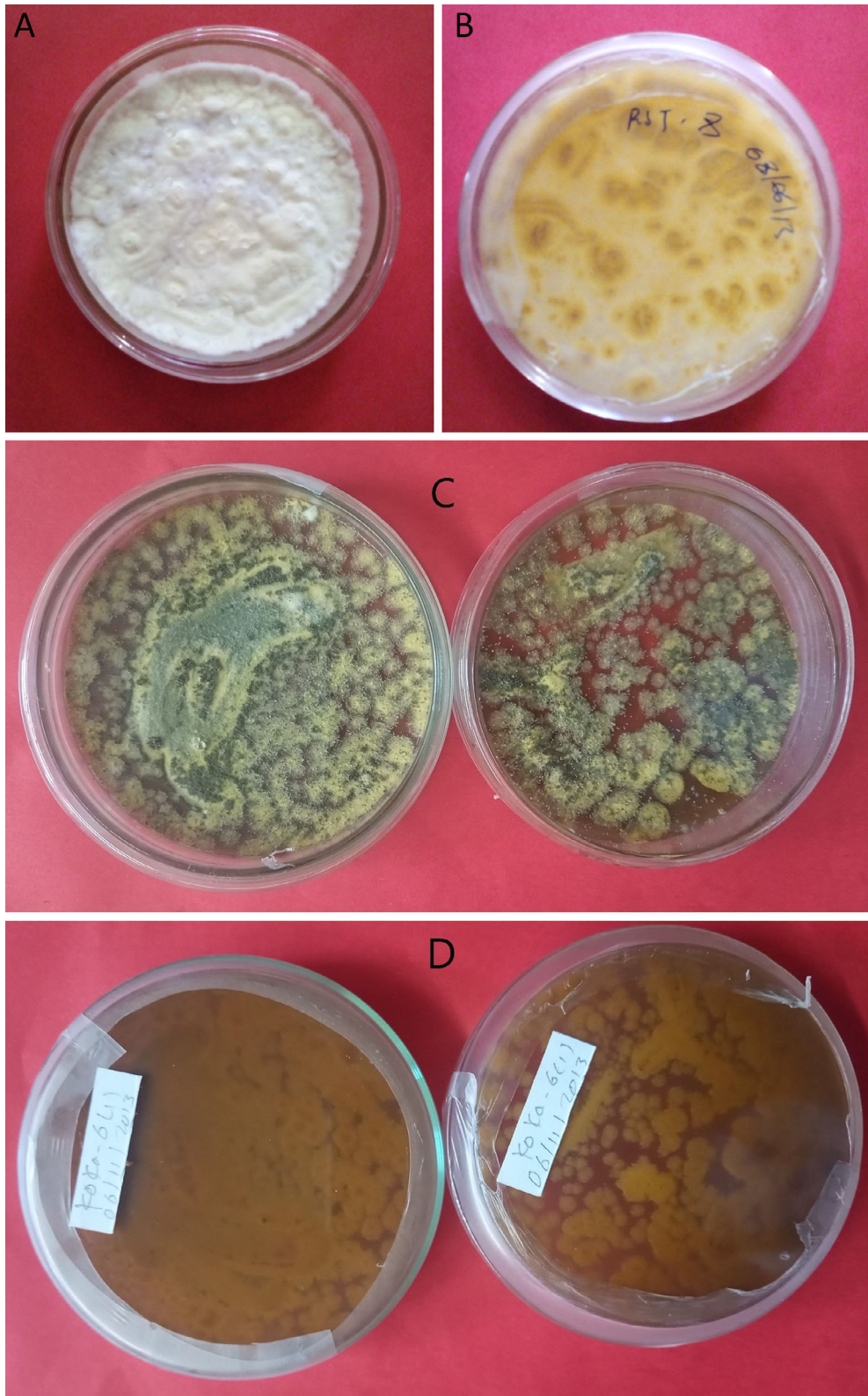


Figure 1. Morphology of *B. bassiana* and *M. robertsii* isolates. (A) Colony color (front) and (B) colony color (back) of *B. bassiana*. (C) Colony color (front) and (D) colony color (back) of *M. robertsii*. No manipulations were made to the image.

Metarhizium robertsii isolates showed dark green to light green colony color at the front and brown at the backside, medium round to round colony shape, and flat to slightly raised colony elevation. Isolates K-101, K-102, RST-12, and S-26 showed a light green colony color, whereas K-61 and K-63 showed a dark green (Figure 1). The surface texture was thin, thick, and cottony.

Results from microscopic features indicated that *B. bassiana* isolates showed a flask-like shape in a conidiogenous cell, a branched conidiophore, and hyaline and smooth conidia with a spherical to subglobose arrangement (Figure 2) and aseptate hyphae (Figure 3),

M. robertsii isolates revealed cylindrical in spore shape, branched, and densely intertwined conidiophore (Figure 2) and septate hyphal structures (Figure 3).

Spore viability

Spore germination was ranging from 79.33 to 99.03% within 18 hours of incubation at $27 \pm 1^\circ\text{C}$ (Table 2). The results showed that there was a statistically highly significant difference among isolates in the percentage of mean germination ($F=54.6$; $DF=14$; $P<0.0001$). Isolate K-61 showed a maximum mean percentage of germination, whereas the minimum mean percentage was recorded by isolate RST-12.

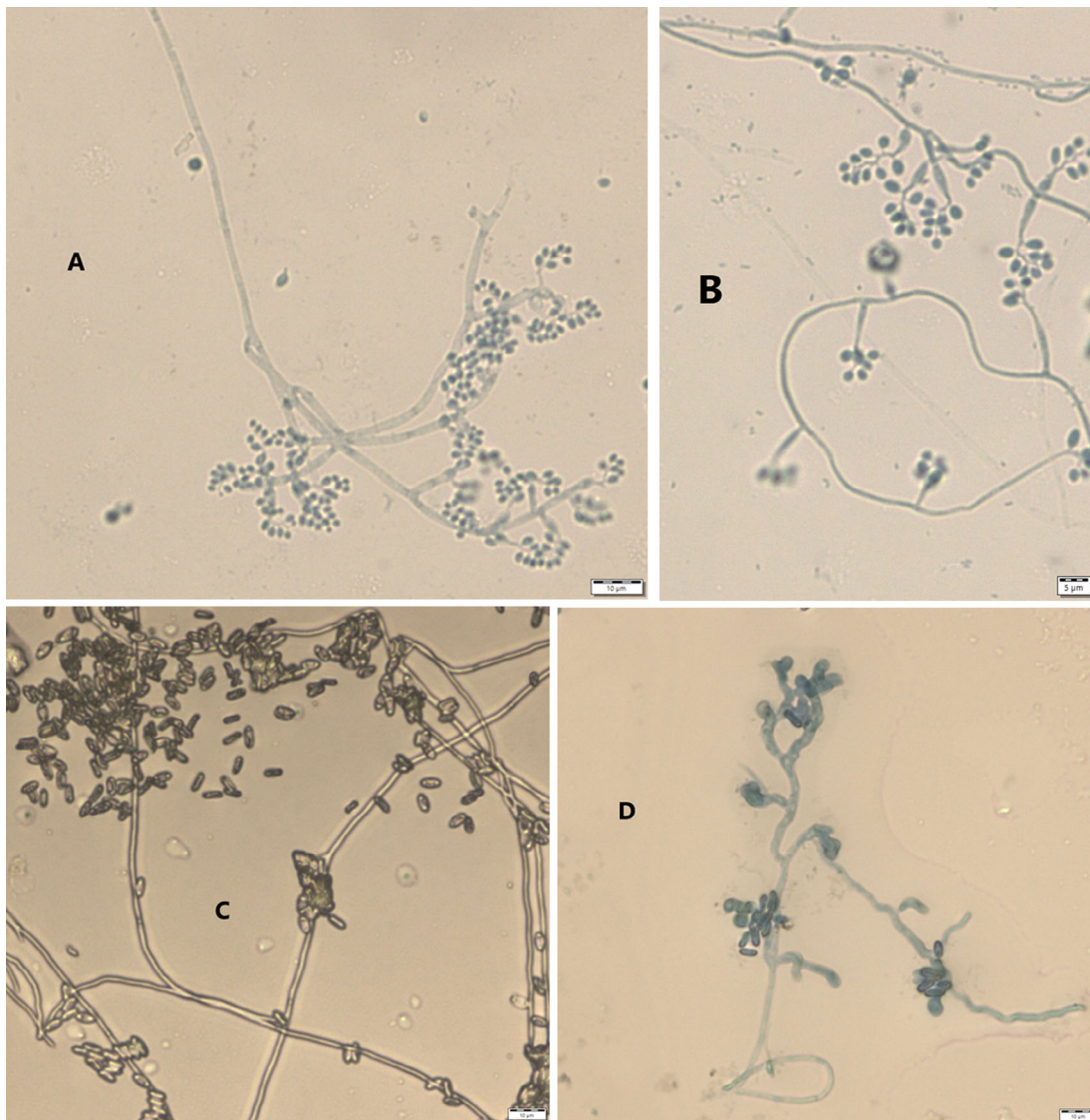


Figure 2. Microscopic feature of *B. bassiana* and *M. robertsii* isolates. (A and B) *B. bassiana*. (C and D) *M. robertsii*. No manipulations were made to the images.

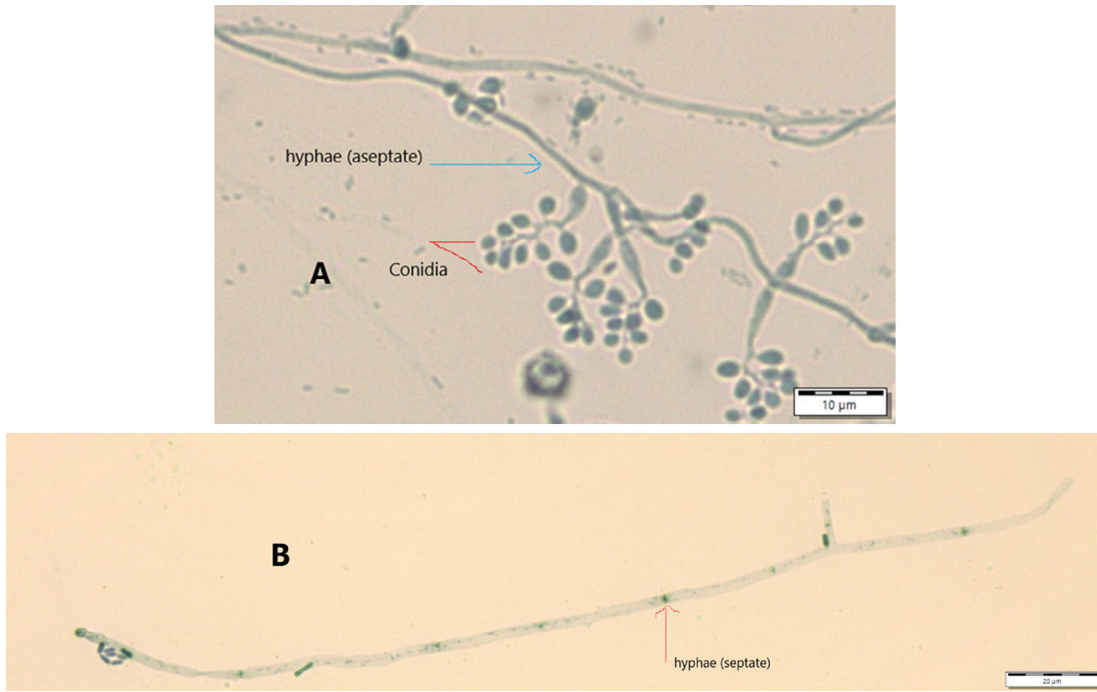


Figure 3. Conidial structure and hyphae of *B. bassiana* and *M. robertsii* isolates. A=*B. bassiana*, B=*M. robertsii*. No manipulations were made to the images.

Table 2. Germination percentage of *B. bassiana* and *M. robertsii* isolates.

Fungal isolates	Germination % ± SE
<i>Beauveria bassiana</i> RST-8	97.13±1.95 ^{ab}
<i>Beauveria bassiana</i> APPRC-27	98.1±0.59 ^{ab}
<i>Metarhizium robertsii</i> K-61	99.03±0.78 ^a
<i>Metarhizium robertsii</i> K-102	97.56±1.36 ^{ab}
<i>Metarhizium robertsii</i> RST-11	95.40±2.91 ^{abc}
<i>Metarhizium robertsii</i> K-63	97.63±1.82 ^{ab}
<i>Beauveria bassiana</i> K-5	97.03±1.63 ^{ab}
<i>Beauveria bassiana</i> APPRC-44BC	95.86±1.07 ^{abc}
<i>Beauveria bassiana</i> K-91	96.40±1.53 ^{abc}
<i>Metarhizium robertsii</i> K-101	97.67±1.24 ^{ab}
<i>Metarhizium robertsii</i> K-7	88.36±4.85 ^{abc}
<i>Metarhizium robertsii</i> S-26	80.80±9.83 ^{bc}
<i>Beauveria bassiana</i> APPRC-44BC-1	87.60±5.03 ^{abc}
<i>Metarhizium robertsii</i> RST-12	79.33±3.84 ^c
Tween 80 (Control)	0.00±0.00 ^d
HSD (0.05)	17.7
CV	6.71

Note: Means with the same letter, in the same column, are not significantly different according to Tukey's studentized range (HSD) test at, α=0.05. CV, Coefficient of Variance.

Table 3. Conidial yield and size of isolates.

Isolate	Conidial yield/ml ± SE	Conidial length (µm) ± SE	Conidial width (µm) ± SE	Length/width ratio ± SE
<i>B. bassiana</i> APPRC-44BC	$0.11 \times 10^7 \pm 0.16^e$	2.85 ± 0.03^{cd}	1.86 ± 0.16^c	1.56 ± 0.12^c
<i>M. robertsii</i> K-63	$1.67 \times 10^7 \pm 0.02^{cb}$	7.10 ± 0.24^a	2.49 ± 0.06^a	2.86 ± 0.13^{ab}
<i>B. bassiana</i> K-5	$0.17 \times 10^7 \pm 0.01^e$	2.23 ± 0.02^d	1.45 ± 0.05^d	1.54 ± 0.07^c
<i>M. robertsii</i> K-102	$1.81 \times 10^7 \pm 0.18^{cb}$	6.43 ± 0.11^{ab}	1.93 ± 0.03^c	3.32 ± 0.03^a
<i>B. bassiana</i> APPRC-27	$2.67 \times 10^7 \pm 0.17^a$	3.24 ± 0.11^c	1.99 ± 0.06^c	1.63 ± 0.05^c
<i>B. bassiana</i> K-91	$1.53 \times 10^7 \pm 0.01^{cd}$	2.08 ± 0.07^d	1.36 ± 0.03^d	1.53 ± 0.03^c
<i>B. bassiana</i> RST-8	$1.28 \times 10^7 \pm 0.04^d$	2.51 ± 0.05^{cd}	1.64 ± 0.11^{cd}	1.54^c
<i>M. robertsii</i> K-61	$1.85 \times 10^7 \pm 0.15^b$	5.78 ± 0.48^b	2.36 ± 0.04^{ab}	2.44 ± 0.20^b
HSD at (0.05)	0.29	0.99	0.40	0.54
CV (%)	7.46	10.40	8.93	11.15

Note: Means with the same letter, in the same column, are not significantly different according to Tukey's Studentized Range (HSD) test at $\alpha=0.05$.

Conidial yield and size

Statistical analysis showed a highly significant difference among isolates at $P<0.0001$ in their conidial production. Conidial production varies from 0.11×10^7 to 2.67×10^7 spores ml^{-1} . Maximum spore production was attained by isolate APPRC-27, followed by isolate K-61, whereas the minimum conidial yield was recorded by isolate APPRC-44BC (Table 3). The length of the conidia showed a mean value of 2.08 to 3.24 µm for *B. bassiana* isolates, and a higher conidial length was achieved by isolate APPRC-27 with 3.24 µm. The highest and lowest conidial diameters were recorded by isolates APPRC-27 and K-91, respectively.

Regarding *M. robertsii* isolates, maximum conidial length and diameter were registered by isolate K-63, while minimum conidial length was obtained by isolate K-61 (Table 3). The lowest conidial diameter was recorded by isolate K-102 (1.93 µm) when compared to others (Figure 3).

Comparison of colony radial growth on a different media

Colony radial growth rates ranged from 1.73 to 3.24 mm day^{-1} (Figure 4). The highest colony radial growth rate was achieved by *B. bassiana* isolate K-91 on SDA, which increased by 16.96% radial growth compared to PDA media. The minimum radial growth rate was scored by isolate 44BC on both media.

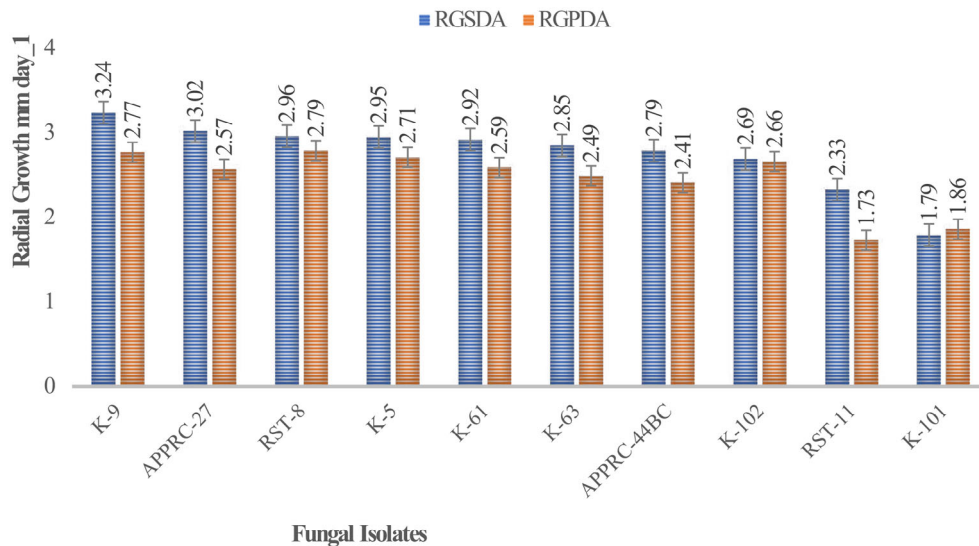


Figure 4. Mean radial growth of fungal isolates on SDA and PDA media. SDA, Sabouraud Dextrose Agar; PDA, Potato Dextrose Agar; RGSDA, radial growth on SDA; RGPDA, radial growth on PDA. No manipulations were made to the images.

Table 4. Mortality of *G. melonella* larvae by *B. bassiana* and *M. robertsii* isolates.

Fungal isolates	Percentage of mortality \pm SE
<i>Metarhizium robertsii</i> K-61	100 \pm 0.00 ^a
<i>Metarhizium robertsii</i> K-102	100 \pm 0.00 ^a
<i>Metarhizium robertsii</i> K-63	97.5 \pm 0.25 ^{ab}
<i>Beauveria bassiana</i> K-91	95.00 \pm 0.28 ^{abc}
<i>Beauveria bassiana</i> APPRC-27	92.5 \pm 0.25 ^{abcd}
<i>Beauveria bassiana</i> RST-8	90.00 \pm 0.28 ^{abcd}
<i>Beauveria bassiana</i> APPRC-44BC	85.00 \pm 0.28 ^{bcde}
<i>Beauveria bassiana</i> K-5	82.5 \pm 0.47 ^{cde}
<i>Metarhizium robertsii</i> K-101	80.00 \pm 0.40 ^{ed}
<i>Metarhizium robertsii</i> RST-11	75.00 \pm 0.28 ^e
Control (Tween 80)	7.5 \pm 0.25 ^f
HSD at (0.05)	1.37
CV (%)	6.31

Note: Means with the same letter, in the same column, are not significantly different according to Tukey's Studentized Range (HSD) test at, $\alpha=0.05$.

Concerning *M. robertsii* isolates, K-61 showed the highest radial growth with 2.92 on SDA media, followed by K-63 (2.85) mm day⁻¹. Isolate RST-11 showed the lowest colony growth on PDA media.

In this study, the maximum growth rate was recorded on SDA media rather than PDA after 15 days of incubation at 27°C \pm 1 for both species of isolates.

Virulence test of isolates on *G. mellonella*

Most isolates were virulent to Great Wax Moth (*G. melonella*) of second instar larvae, with 75–100% mortality after seven days of inoculation of treatments (Table 4). The mean percentage of mortality of *G. melonella* larvae by *B. bassiana* and *M. robertsii* was highly variable (F = 101.49; DF = 10; P < 0.0001). Maximum mean percentage mortality was achieved by *M. robertsii* isolates K-61 and K-102, while minimum mortality was recorded by *M. robertsii* isolate RST-11.

Discussion

From the fungal isolates collected from the soil rhizosphere of tomato plants, 70% of them had characteristics typical of the species *M. robertsii*. In the present study, six *B. bassiana* and eight *M. robertsii* were morphologically characterized to understand the potential virulence of entomopathogenic fungi. *B. bassiana* has a white colony color and is round in shape, whereas *M. robertsii* has a dark to light colony color and is medium-round to round in shape. Similar work was done by Deb and Dutta (2021). This result was confirmed by the previous findings of different researchers, they reported *M. robertsii* isolates indicating a round colony shape, thin to thick addressed texture, and flat to slightly raised elevation (Sepúlveda et al., 2016).

This study also detected a microscopic feature of isolates. Consequently, *B. bassiana* had aseptate hyphae and *M. robertsii* showed a cylindrical shape with septate hyphae. A similar study reported that microscopic characterization of *B. bassiana* isolates indicated round to slightly round conidial shape with glassy-like aseptate hyphae, while *M. robertsii* were described as having cylindrical conidial shape, septate hyphae, and branched conidiophores in the form of a candelabrum (Bich et al., 2021).

Germination is one component that affects the degree of virulence; the faster the germination rate, the higher the fungal infection of pests (Herlinda, 2010). This study showed that the highest conidial germination was achieved by *M. robertsii* isolate K-61 (99.03%) within 18 hours of incubation. This result is consistent with a study conducted by Gebremariam et al., (2021), in which spore germination percentage varied between 85.43 and 99.67% within 24 hours of incubation for 26 isolates. Likewise, conidial germination of *Beauveria* isolates ranged from 76.33 to 95.75% (Belay and Tenkegna, 2017), and 89.30 to 99% of spore viability were recorded by 22 isolates of *B. bassiana* and *M. robertsii* (Mkiga et al., 2020).

Maximum sporulation rate, radial growth, and fast germination rate were the most important parameters for determining virulent isolates of entomopathogenic fungi, and the variation in virulence among isolates may be due to the geographical source of isolates and the potential of fungal species (Dotaona *et al.*, 2015; Gebremariam *et al.*, 2021). This result revealed that the highest radial growth rates were obtained by *B. bassiana* isolate K-91 (3.24 mm/day) and *M. robertsii* isolate K-61 (2.92 mm day⁻¹) on SDA media at 27°C ±. This is consistent with *M. robertsii* developed on SDA media at 30°C, the temperature at which the rates ranged from 0.14 to 3.39 mm day⁻¹ (Rodríguez *et al.*, 2009). The present study revealed that all isolates showed maximum colony radial growth on SDA media rather than PDA. This may be due to the variation in nutrient contents between the media as SDA is selective. However, Gebremariam *et al.* (2021) reported that the radial growth of *B. bassiana* and *M. robertsii* isolates attained between 0.83 and 3.43-mm day⁻¹ by AAUEM-3 and AAUMFB-77 isolates, respectively, which were grown on PDA media at 25°C.

In the case of *M. robertsii* isolates K-61 and K-102 showed higher virulence levels than other fungal isolates to *G. melonella* under laboratory conditions at seven days post-inoculated. The result confirmed the findings of previous work (Ibrahim *et al.*, 2016). All isolates of *B. bassiana* and *M. robertsii* showed greater than 86% larval mortality, which indicated that isolates were virulent to *G. melonella*. These findings were agreed upon in the work of Kebede (2019), in which it was reported that the top eight isolates of *B. bassiana* and *M. robertsii* were virulent to *G. melonella* with above 75% larval mortality.

The results from microscopic features such as conidial length and width revealed that there was a highly significant difference among isolates. Accordingly, the highest conidial length was obtained with 7.10 and 3.24 µm for *M. robertsii* isolate K-63 and *B. bassiana* isolate APPRC-27, respectively. This agrees with previous work conducted by Doolotkeldieva *et al.* (2019), who reported the conidial length and width of *B. bassiana* were 2.27 and 1.85 µm, respectively, with a length to width ratio of 1.23 µm. Bich *et al.* (2021) also indicated that the conidial diameters of *B. bassiana* showed a mean value of 1.7 to 2.3 µm. Mongkolsamrit *et al.* (2020) also stated that *M. robertsii* showed a cylindrical to ellipsoid conidial shape with 5-7 and 2-3 µm in conidial length and width, respectively. However, Sepúlveda *et al.* (2016) reported that lower conidial lengths vary between 4.52 and 5.54 µm for *M. robertsii* strains. This may be due to the variation in the ecology of fungal species.

Conclusions

Correct identification of entomopathogenic fungi through morphological characterization is a baseline for the selection of virulent isolates for the management of agricultural pests. There was a variation in morphological features among all isolates in terms of colony color, shape, and texture. Isolates with the highest germination percentage and maximum spore production were virulent to second instar larvae of *G. melonella*. Accordingly, *M. robertsii* isolates K-61 and K-102 were highly virulent to *G. melonella* by the Galleria dipping method, with a 100% mortality rate at 10 days post inoculated at a concentration of 1×10^8 conidial ml⁻¹. This study showed that virulent *B. bassiana* and *M. robertsii* isolates collected from rhizospheric soil of tomato plant had the potential to control pests sustainable and cost-effective, and increasing crop production and productivity using this eco-friendly method. Hence, it is recommended to use *B. bassiana* and *M. robertsii* due to their endophytic properties for the management of agricultural pests, particularly insect pests. Moreover, further studies are needed on the factors attributed to the distribution, ecology, efficacy, and root association of *B. bassiana* and *M. robertsii* with tomato and other Solanaceae plants against pests.

Data availability

Underlying data

DANS-EASY: Entomopathogenic fungi characterization data, <https://doi.org/10.17026/dans-27p-epm2> (Geremew, 2023).

This project contains the following underlying data:

- Germination (germination test of entomopathogenic isolates)
- Radial growth PDA (radial growth of isolates on PDA media)
- Radial growth SDA (radial growth of isolates on SDA media)
- Screening (pathogenicity screening of isolates on *Galleria melonella*)
- Conidial length and width (conidial size of isolates)
- Conidial yield (conidial yield of isolates)

Data are available under the terms of the [Creative Commons Zero “No rights reserved” data waiver](#) (CC0 1.0 Public domain dedication).

Acknowledgments

The Ethiopian Institute of Agricultural Research (EIAR) acknowledged for the financial support. The authors are grateful to the Ambo Agriculture Research Center’s biocontrol research team for their assistance with laboratory experiments. Furthermore, the authors thank Mr. Denberu Kebede, Ms. Aberash Chala, and Mr. Tesfaye Abdisa for their assistance with the laboratory work. The authors also thank Tariku Hunduma (PhD) for his technical support in methodology development. The authors confirmed that the EIAR and individuals mentioned in this section permitted their names to be included in this publication.

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Linnet Gohole 

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Discussion

1. The first sentence of the discussion is still confusing. I suggest that it should be re-written to read "Of the fungal isolates collected from the soil rhizosphere of tomato plants, 70% of them had characteristics typical of the species *M. robertsii*".
2. The discussion is still largely missing on scientific explanations to the findings obtained. Had this been done, then the manuscript would have been scientifically richer.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Entomology, biological control of insect pests and integrated pest management.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 06 Aug 2024

Dereje Geremew

Dear Reviewer,
All comments are accepted and included in the new version of article.

Competing Interests: No competing interests were disclosed.

Version 3

Reviewer Report 08 November 2023

<https://doi.org/10.5256/f1000research.158147.r219340>

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I have looked at the revised version of the manuscript (Version 3) and going by the comments earlier made bullet by bullet, I note only 3 of the queries have been addressed. The purpose of giving feedback is to improve the manuscript and I strongly recommend that the comments are considered seriously and incorporated accordingly to improve the quality of the paper.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Entomology, biological control of insect pests and integrated pest management.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 15 Jan 2024

Dereje Geremew

General comment

Pathogenicity and virulence are not interchangeable, as the virulence of isolates tested in the experiment. I accepted the comment and modified it accordingly.

Competing Interests: No competing interests were disclosed.

Author Response 15 Jan 2024

Dereje Geremew

All necessary comments were accepted and included in the new version of the article. The title of the article was also modified accordingly.

Competing Interests: No competing interests were disclosed.

Reviewer Report 31 October 2023

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The authors have significantly improved the manuscript material; I, as a reviewer, confirm that the published article has undergone adjustments according to the reviewer's recommendations.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Biocontrol, entomopathogenic bacteria, fungi, soil microbiology, plant pathology, biopesticides, bioremediation

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 2

Reviewer Report 20 September 2023

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1. Suggestion for title: Isolation, morphological characterization and screening virulence of *B. bassiana* and *M. robertsii* fungal isolates in *G. melonella*.

Abstract

2. Background - the word asymptote should read asymptomatic.
3. Methods - *Galleria* bait method is used to isolate fungi and not to identify them.
4. Amended conclusion - what is the rationale for singling out sucking insects as candidate pests to be controlled using the virulent fungal isolates, why not consider insect pests in general?

Introduction:

5. The following sentence does not convey the intended message "Moreover, entomopathogenic fungi are the immediate solution for pests that feed inside leaves, stems, roots, and seeds because they reduce their exposure to synthetic insecticides and other control methods (Resquín-Romero *et al.*, 2016)."
6. Was there anything special about collecting the fungal isolates from the rhizosphere of tomato plants?

Methods

7. Under morphological characterization, there is mention of 3 *B. bassiana* and one *M. robertsii* characterized for comparison purposes with new isolates. Can it be clarified why these 4 isolates were included in the study.
8. How many isolates were eventually tested in the various experiments? The number of treatments keeps changing with each experiment and no explanation is given on how these isolates were selected.
9. "To hatch third- to fourth-instar larvae, the eggs were laid on folded tissue paper" Was this sentence meant to inform readers of the egg laying process, hatching and eventually obtaining 3rd - 4th instar larvae to be used in the pathogenicity experiments? Eventually 2nd instar larvae were used. Clarify the use of the 3rd - 4th instars in the section on rearing *Galleria*. It is confusing.
10. Replace the word deepened with dipped. Larvae were dipped in spore suspension.
11. Did collection of cadavers begin only after the larvae had been incubated for 10 days? If so, then for how long was this data collected? At what point was the greater than 90% mortality determined? i.e. after how many days? It cannot be indefinite.

Results

12. Fig 2. The hyphae in A look more septate than those in C. Better photos clearly showing the difference need to be taken.
13. The titles of the tables and figures need to be complete and more informative e.g. Table 4 is about % mortality caused by the various fungal isolates and not screening.

Discussion

14. The first sentence of the discussion is not clear.

15. The discussion section needs to go beyond repeating the results obtained and whether the agree or disagree with previous findings. It should focus more on scientific explanations of the interesting and unique findings of the current study. e.g. what could be the reason for some fungal isolates being more virulent than others; what do the findings imply when it comes to them being used as biological control agents; is there any connection between the isolate characteristics and virulence with where they were collected or the target crop - tomato?

16. The isolates were collected from two different locations with different ecological conditions, did this factor have some influence on the isolates collected and their performance?

17. Some already known isolates were added to the experiments, what does this finally mean?

Conclusion

18. The conclusion on reduction of usage of synthetic pesticides just because the fungal isolates showed high degree of pathogenicity was not a subject of investigation in this study. The conclusions should come directly from the study. Then the implications of these findings can then be speculated and areas of further research recommended.

General comments

1. The authors need to reconstruct some sentences to make them grammatically correct without losing the science being communicated.

2. Virulence and pathogenicity are used interchangeably in this manuscript. Do they mean the same?

3. The discussion and conclusion sections should be re-written to capture the suggestions given to help improve the paper.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?

No source data required

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.**Reviewer Expertise:** Entomology, biological control of insect pests and integrated pest management.**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Author Response 18 Oct 2023

Dereje Geremew

Question 9.

Response: *Galleria melonella* were reared to get a mass of 2nd instar larvae, and 3rd and 4th larvae instar larvae were not used in the experiment.**Competing Interests:** No competing interests were disclosed.

Author Response 18 Oct 2023

Dereje Geremew

Question 11. Response

Cadavers were collected every day after 3 days.

Competing Interests: No competing interests were disclosed.

Author Response 18 Oct 2023

Dereje Geremew

Question 17.

Response. The performance of some isolates were tested as standard checks with newly collected isolates

Competing Interests: No competing interests were disclosed.**Version 1**

Reviewer Report 09 August 2023

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Finding highly virulent entomopathogenic fungi remains an important challenge as an alternative to chemical pesticides. In this article, the authors tried to find two species of entomopathogenic fungi from tomato rhizosphere soil using classical methods of insect pathology, microbiology, entomology, etc.

However, the authors intended to isolate endophytic fungi, and not from the soil itself; in total, they isolated them from the rhizosphere soil, and not from plant parts.

Therefore, the authors should realize that they were dealing with soil fungi, this should be emphasized in the conclusions.

In conclusion, the authors suggest these strains for practical use, but it is too early to speak with such data, after extensive research by testing on other types of sucking insects and for the presence of endophytic properties, it will be possible to recommend them for practical purposes.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Biocontrol, entomopathogenic bacteria, fungi, soil microbiology, plant

pathology, biopesticides, bioremediation

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 10 Aug 2023

Dereje Geremew

Agreed with the comments, and the comments were included in the conclusion of the article. The conclusion was modified as follows:

"Correct identification of entomopathogenic fungi through morphological characterization is a baseline for the selection of virulent isolates for the management of agricultural pests. There was a variation in morphological features among all isolates in terms of colony color, shape, and texture. Isolates with the highest germination percentage and maximum spore production were virulent to second instar larvae of *G. mellonella*. Accordingly, *M. robertsii* isolates K-61 and K-102 were highly virulent to *G. mellonella* by the Galleria dipping method, with a 100% mortality rate at 10 days post inoculation at a concentration of 1×10^8 conidial ml^{-1} . This study showed that virulent *B. bassiana* and *M. robertsii* isolates collected from rhizospheric soil had the potential to control pests by reducing the use of synthetic pesticides, making them sustainable and cost-effective, and increasing crop production and productivity using this eco-friendly method. Hence, it is recommended to use *B. bassiana* and *M. robertsii* due to their endophytic properties for the management of agricultural pests, particularly sucking insects. Moreover, further studies are needed on the factors attributed to the distribution, ecology, efficacy, and root association of *B. bassiana* and *M. robertsii* with tomato and other Solanaceae plants against pests".

Competing Interests: No competing interests were disclosed.

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