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ISOLATION AND CHARACTERIZATION OF *METARHIZIUM* ISOLATES FROM THE SOIL OF AFGHANISTAN AND THEIR MYCOINSECTICIDE EFFECTS AGAINST SUBTERRANEAN TERMITE (*ANACANTHOTERMES VAGANS*)

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Fallahzadeh H., Karimi J., Moravvej G.H., Tarasco E. – Isolation and characterization of *Metarhizium* isolates from the soil of Afghanistan and their mycoinsecticide effects against subterranean termite (*Anacanthotermes vagans*).

The entomopathogenic fungi are widely used as a biocontrol agent against many insect pests. In the present study, strains of *Metarhizium* were isolated and identified from Badakhshan province of Afghanistan. The taxonomic identity of the isolates was confirmed by molecular data, using (ITS) rDNA gene sequence, and morphology: *Metarhizium robertsii* and *Metarhizium anisopliae.* The virulence and the mass conidial production of these strains were compared with two Iranian isolates of *Beauveria* (*B. bassiana* and *B. varroae*) using four different raw substrates (broken rice, broken wheat, green gram, and saw dust) by solid-state fermentation. The *M. robertsii* strain had the highest total conidial yields obtained after 18 days on broken rice and the least conidial growth was observed on saw dust. For *B. bassiana,* the highest conidial yields were obtained after 18 days on green gram, and also like to *M. robertsii,* the least conidial growth was observed on saw dust. The conidia suspension was applied against subterranean termite, *Anacanthotermes vagans,* at four different concentrations (1×10^5, 1×10^6, 1×10^7, 1×10^8 conidia/ml^{-1}). The highest mortality rate was obtained from 1×10^8 conidia/ml^{-1} for both *M. robertsii* and *B. bassiana*. In general, the indigenous isolate of *M. robertsii* was more virulent than *B. bassiana* while *B. varroae* had not effect on termites.

KEY WORDS: Entomopathogenic fungi, Identification, Phylogenetical analysis, Termites, Substrate.

INTRODUCTION

Termites are medium-sized, soft-bodied, light-colored, polymorphic and cellulose-eating social insects living in large communities of several hundred to several million individuals. The fossil record indicates the long history of termite's evolution (SINDHA *et al*., 2011; AZMI *et al*., 2016). Due to the feeding function, the worker casts cause widespread destruction resulting in major economic losses in tropical and subtropical areas by destroying agricultural crops, live trees, and wooden structures in the houses. About 300 species have been reported to cause significant damage to agriculture crops and have been recorded as pests (ABONYO *et al*., 2016; JAMIL *et al*., 2017). Termites also feed and often destroy various other structures or materials that people use, *i.e.* wooden portions of buildings, furniture, books, utility poles, fence posts, many fabrics, and other useful materials. The termites also attack telephone pole, boats, and other finished goods (*e.g.* paper and fabric), valuable manuscripts and paintings (CHERAGHI *et al*., 2012). Control of termites has depended on the use of insecticides. However, due to relatively high human toxicity and unacceptable environmental consequences, this situation has required research to find effective and safe alternatives for termite control (GHAFFARI *et al*., 2017). It is therefore essential that alternative safe management strategies be developed to replace chemical agents and reduce risks of environmental contamination (HUSSAIN *et al*., 2010; BRUNNER-MENDOZA *et al*., 2019).

Biological control agents can be used as a component of integrated pest management. Several natural enemies, including entomopathogenic fungi (EPF), have been under consideration as potential biological control agents. Under natural conditions, fungal pathogens are frequent and often cause natural mortalities within the insect populations (KEPPANAN *et al*., 2018a).

Mitosporic fungi in the genera *Metarhizium* Sorokīn and *Beauveria* Vuillemin are widespread in soil and are known with virulence to most arthropods (PEREZ-GONZALEZ *et al*., 2014). These genera have shown great potential for management of various insect pests (SINGHA *et al*., 2010). *Metarhizium anisopliae* (Metch.) Sorokin is being developed as insecticide for use against locusts and grasshoppers in Australia and Africa (DONG *et al*., 2007). Development of similar biopesticides is being undertaken in many countries. In this paper, isolation and characterization of two *Metarhizium* strains obtained from soils of Afghanistan were carried out and the pathogenicity of the Afghan strain *Metarhizium robertsii* Bischoff *et al*., compared with Iranian isolates of *Beauveria bassiana* (Bals.-Criv.) Vuill. and *Beauveria varroae* S.A. Rehner & Humber, on subterranean termite (*Anacanthotermes vagans* Hagen) was surveyed.

MATERIALS AND METHODS

Galleria melonella L. (Lepidoptera: Pyralidae) was reared in laboratory. Adult wax moths and larvae were kept

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in 3L plastic bottles and stored at room temperature in an incubator (28 ± 1 °C). The cultures were maintained by a continuous supply of nutrient media made with wax, flour, bran, yeast, glycerin and honey (LEPHOTO, 2013).

COLLECTION OF SOIL SAMPLES AND BAITING TECHNIQUE

One hundred and 58 samples of soil from Badakhshan province with a geographic of longitude: 71° 00' 0.00" E, and latitude 38° 00' 0.00" N, located in the northeast of Afghanistan, were collected. The soil samples, collected from depth of 25-30 cm were transferred to Biocontrol and Insect Pathology Lab., Ferdowsi University of Mashhad. The samples were kept in separate plastic containers and stored at 22 ± 3 °C during transport to the laboratory. Prior baiting, water was added to give a moisture content not greater than 10% moisture and the samples were then stored at room temperature overnight.

Extraction of the fungi from the soil samples has done using *Galleria* larvae as bait insect. About 6 insect larvae were placed on top of each sample. Each container was turned over and stored in an incubator set at 28 ±1 °C (KEPPANAN *et al*., 2018b). Observations were done daily to monitor the infection and dead larvae (SEPULVEDA *et al*., 2016).

All the dead larvae were collected after 96 hours. The dead larvae were removed and surface sterilized with 1% sodium hypochlorite for 1 min, then washed three times in sterile distilled water, placed on damp filter paper within a sealed medium petri dish (9 cm diameter) and incubated at 28±1 °C for 6 days. After incubation period, fungi were isolated from the dead larvae showing external mycelia growth on potato dextrose agar (PDA) with antibiotics (tetracycline at 40 µm/ ml) for suppression of bacterial growth. After growth of mycelia on PDA, they transferred to the liquid potato dextrose (PD) and were incubated for 10 days at 25 ± 1 °C on a shaker at 250 rpm (SAYED *et al*., 2018).

DNA EXTRACTION AND MOLECULAR CHARACTERIZATION

Fungal genomic DNA was extracted from the hyphae using a partially chemical lysis method. Approximately 30 mg of crushed mycelium was used for DNA extraction, and the rest of the sample was stored at -20 °C until needed. The DNA extraction was done using DNeasy Blood & Tissue Kits. The extracted DNA was stored at -20 °C until use as a template for PCR (FERNANDES *et al*., 2009).

The PCR mixture was carried out in a reaction volume of 25 µl, containing 2.7 µl of 10×PCR buffer, 14.4 µl of H2O, 1 µl of Mgcl2 (25mM), 0.5 ul of dNTPs (10 Mm), 0.3 µl *Taq* polymerase (5 units/ µl), 1 µl of forward primer (10 pmol/ µl), 1 µl of reverse primer (10 pmol/ µl), and 4 µl of template DNA (DARSOUEI *et al*., 2015). The ITS gene was amplified using the ITS4 and ITS5 primers (WHITE *et al*., 1990). The condition for PCR was as follows: denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 1min, annealing at 52 °C for 1min, and extension at 72 °C for 5min. A final extension was performed at 72 °C for 10 min. The PCR products were electrophoresed on 1% agarose gels and subsequently, the gels were stained using Green viewer (SYBR) (KARIMI *et al*., 2018). The PCR products were sequenced by Bioneer Company of South Korea. The sequences chromatograms were checked using BioEdit software. Forward the reverse sequence was assembled in BioEdit software.

For phylogenetic analysis of the recovered population, the DNA sequences were compared with those of other available in the GeneBank using the BLAST homology search program. The ITS rDNA sequences of some valid strains of *Metarhizium* isolates available in the GeneBank were retrieved and aligned using MEGA7 (KUMAR *et al*., 2018). The ITS sequence of *Armillaria gallica* Marxm. & Romagn (KP288483.1) was used as outgroup. The MEGA7 program (KUMAR *et al.,* 2016) was used to calculate nucleotide distances. Phylogenetic analyses were performed using the maximum likelihood with 10000 relocations of bootstrap.

MORPHOLOGY AND MASS PRODUCTION OF CONIDIA

The morphology of the conidia taken from the cadaver of *Galleria* larvae was studied by light microscopy. Culture of the fungus was grown on potato dextrose agar in the dark for 10, 15 and 20 days. The morphology of the conidial clumps taken from the potato dextrose agar medium was studied by light microscopy. The colonies were evaluated according to the technique described by HUMBER & STEINKRAUS (2005). Slides were prepared with lactophenol cotton blue, and micromorphologies observed at 100× magnification. Thirty conidia were measured by eyepiece measuring system as presented in the table1.

Four different solid culture which including broken rice, broken wheat, green gram and sawdust were used for evaluating the mass production of *M. robertsii* and Iranian strain of *B. bassiana* at 28 ±1 °C. One hundred and 50 g of each sample was washed well. The broken rice and broken wheat were soaked in water for two hours and green gram and saw dust for seven hours prior to starting the experiments (MIGIRO *et al.*, 2010). The excess water was drained by decanting and shade drying for 30 minutes to further remove the excess moisture. Three replications were maintained for each sample. The samples were packed in individual 1000 ml bottle for everyone separately.

The bottles were plugged with cotton wool and autoclaved for 1 h. After cooling, 3 ml of the spore suspension of the fungal pathogen was inoculated into each bottle, separately. All these procedures were done under laminar air flow chamber (PRASAD & PAL, 2014). The bottles were incubated in the incubator at 28 ±1 °C, separately for 18 days. To avoid clumping, after 9 days of inoculation, the bottles were shaken vigorously to separate the media and break the mycelial material. After 18 days of incubation, 450 ml sterilized water containing Tween 80 (0.05%) was added to each bottle and then shake for 10 min (AGALE *et al*., 2018). The suspension was filtered through double-layered muslin cloth. Counting of spores was made after serial dilution of the suspension using double ruled Neubauer hemocytometer for determining the number of conidia in 1g of each sample.

EVALUATION OF FUNGAL PATHOGENICITY AGAINST TERMITES IN LABORATORY BIOASSAYS

The insecticidal activity of three entomopathogenic fungi, *Metarhizium robertsii* (Afghan isolate), *Beauveria bassiana*

Table 1 – Conidia morphometrics of *Metarhizium robertsii* and *M. anisopliae* strains collected in the current work compared with relative isolates of *Metarhizium.*

Species name	isolate	measurement (µm)
M. robertsii	current work	7.6 (7.0 - 8.9) × 3.6 (3.4 - 3.8)
M. robertsii	NR_132011.1	7.9 (7.5 - 9.0) × 3.7(3.5- 4.0)
M. guizhouense	Hkd25-2	11.6 (9.3 - 13.6) × 4.2 (3.4 - 4.7)
M. anisopliae	Current work	7.0 (6.4 - 7.8) × 3.6 (3.2 – 3.8)
M. anisopliae	Kgs15-1	9.0 (7.5 - 10.1) × 3.4 (2.8-3.8)
M. anisopliae	Sag6-1	9.3 (8.0 - 10.3) × 3.7 (3.4× 4.2)
M. majus	Kkj2-1	9.4 (8.4 - 10.1) × 3.5 (3.3 – 4.0)

(FUM102) and *Beauveria varroae* (FUM121) (Iranian isolates) were determined on *Anacanthotermes vagans.* The *Beauveria* strains came from Ferdowsi University of Mashhad Insect Pathogens Collection. The fungal strains were tested at a series concentration of 1×10^5, 1×10^6, 1×10^7 and 1×10^8 conidia/ml^{-1}. Six hundred µm suspension of each fungal concentration was poured by a pipet into sterile 9 cm petri dish and allowed to dry partially. Termites were allowed to walk on the partially dried fungal suspension for 1 min. One milliliter 0.05 Tweem-80 solution was added on the filter paper in Petri dishes as the control (Keppanan *et al*., 2018b). Three replicates were performed for each concentration of conidial suspensions with ten individual termites which maintained. Mortality was observed at different day's intervals (every 24 h) for 10 days. The infected insect cadavers were transferred aseptically to another sterile Petri dish. At each observation, dead termites were removed, and surface sterilized with 1% sodium hypochlorite for 1 min, then washed three times in sterile distilled water and placed in plastic petri dish (90 mm) with wet filter paper and maintained at 28 ±1 °C in growth chamber for mummification and sporulation to confirm the pathogenicity.

Evaluation of fungal Pathogenicity against Termites in Semi-Fields Conditions

For semi-field experimental setup, plastic dishes were prepared at 14cm on the level of the balcony. The soil with saw dust and added moisture was placed in container. The open shape plastic dishes were laid out on the soil and saw dust to prevent termite's escape and ant attack and also protect the termites from direct sunlight. In this test, two fungal strains, *Metarhizium robetsii* (Afghan isolate) and *Beauveria bassiana* (Iranian isolate) were used. The fungal concentrations were 1×10^5, 1×10^6, 1×10^7 and 1×10^8 conidia/ml^{-1}. One ml suspension of *M. robetsii* and *B. bassiana* was sprayed by small sprayer into each plastic container and put 10 termites in each plastic container. Three replicates were performed on each concentration of conidial suspensions with ten individual termites which maintained and mortality was recorded at different day's intervals (every 24 h) for 12 days. Sterile distilled water was sprayed at the inner side of the container every day to maintain the humidity. One milliliter 0.05% Tween-80 solution was sprayed on the plastic container as the control. Similar to lab assay, the infected insect cadavers were transferred aseptically to another sterile petri dish. At each observation, dead termites were removed, counted and placed in a plastic petri dish (90 cm) lined with wet filter paper and maintained at 28 ±1 °C in a growth chamber for mummification and sporulation.

Data Analysis

Mortality data were corrected according to Abbott's formula (Abbott, 1925). Differences between the fungal isolates and control group, with respect to mortality, was determined by analysis of variance (ANOVA). And subsequently by LSD multiple comparison test. All analyses were performed using program SAS (Gupta *et al*., 2016).

RESULT

Two species of *Metarhizium* were identified from the collected soil samples. The general morphology of the fungal mycelia was similar to *Metarhizium* genus. The infected *Galleria* larvae turned from grayish green and grey to brown or black in color and the cadavers were abundantly covered with white mycelia, grayish green and green conidial spores. The first isolate had ovoid conidia formed in a chain, which is characteristic in *M. robertsii.* The conidia average length was 7.6 µm and the average conidia width was 3.6 µm. The second isolate had cylindrical conidia formed in a chain, which is characteristic in *M. anisopliae*. The conidia average length was 7 µm and the average conidia width was 3.6 µm as indicated in the table1. Preliminary confirmation of microscopic study revealed that the morphological appearance of the isolated fungi such as conidial shape and size of the spores were identical to those of *M. robertsii* and *M. anisopliae*. To confirm the identity of the *M. robertsii* and *M. anisopliae*, the partial sequence (550 fragments) of the ITS4-ITS5 gene was sequenced and its identify compared with representative valid sequences of *Metarhizium* isolates (Fig. I). According to phylogenetic analysis, the new *Metarhizium* isolates made a single clade along with other isolates of *M. robertsii* and *M. anisopliae* with high bootstrap. The Afghan strains of *Metarhizum* had the lowest genetic distance with those of other isolates of *M. robertsii* and *M. anisopliae.* The partial DNA sequence was deposited in the genebank.

Effect of media on Fungal Growth

In the present study, several naturally available substrates of solid media were tested for mass production of *B. bassiana* and *M. robertsii.* Those isolates were grown under standard conditions on different solid substrates and harvested after 18 days. There were significant difference between substrates ($F= 2061.04$, df= (1, 20), $P<0.0001$), fungal species ($F= 4706.74$, df= (4, 20), $P<0.0001$) and the interaction between them ($F= 655.93$, df= (4, 20), $P<0.0001$). For *M. robertsii*, the broken rice substrate with 1.4×10^8 conidia/g showed maximum conidial production and the saw dust substrate with 1.8×10^7 conidia/g showed minimum conidial production (Fig. II).

For *B. bassiana* after 18 days, the maximum conidial production was obtained on the green gram and broken wheat at 1.4×10^8 and 1×10^8 conidia/g, respectively, and the minimum conidial production with 4×10^7 conidia/g was observed on saw dust (Fig. II).

Pathogenicity of *M. robertsii*, *B. bassiana* and *B. varroae* under laboratory conditions

Four different conidial concentrations (1×10^5, 1×10^6, 1×10^7 and 1×10^8 conidia/ml^{-1}) of *M. robertsii*, *B. bassiana*, and *B. varroae* were administrated for insecticidal activity against *A. vagans* in laboratory conditions. After application of each conidial concentration they cause few mortality so during the 24h, although termites were very weak and hardly moving. It is certain that *M. robertsii* was virulent and pathogenic to the termite *A. vagans;* the termite mortality increased with the indigenous newly identified Afghani species of *M. robertsii*. In the laboratory, all isolates caused significant different mortality rate ($F=104.08$, df= (2, 30), $p<0001$) in comparison to control groups. All concentration caused significant different mortality rate in comparison to each other's ($F=28.98$, df= (4, 30), $p<0.0001$). The interaction between fungal species and concentrations were significantly different ($F=7.10$, df= (8, 30), $p<0.0001$). Conidia from the *M. robertsii* were highly virulent for *A. vagans* causing approximately 100 % mortality 4 days after inoculation in the concentration of 1×10^8 conidia/ml^{-1}; other concentration respectively caused 100% mortality approximately 5-6 days post inoculation.

The significant effect of fungal filtrates, observation time intervals were recorded in laboratory assay for

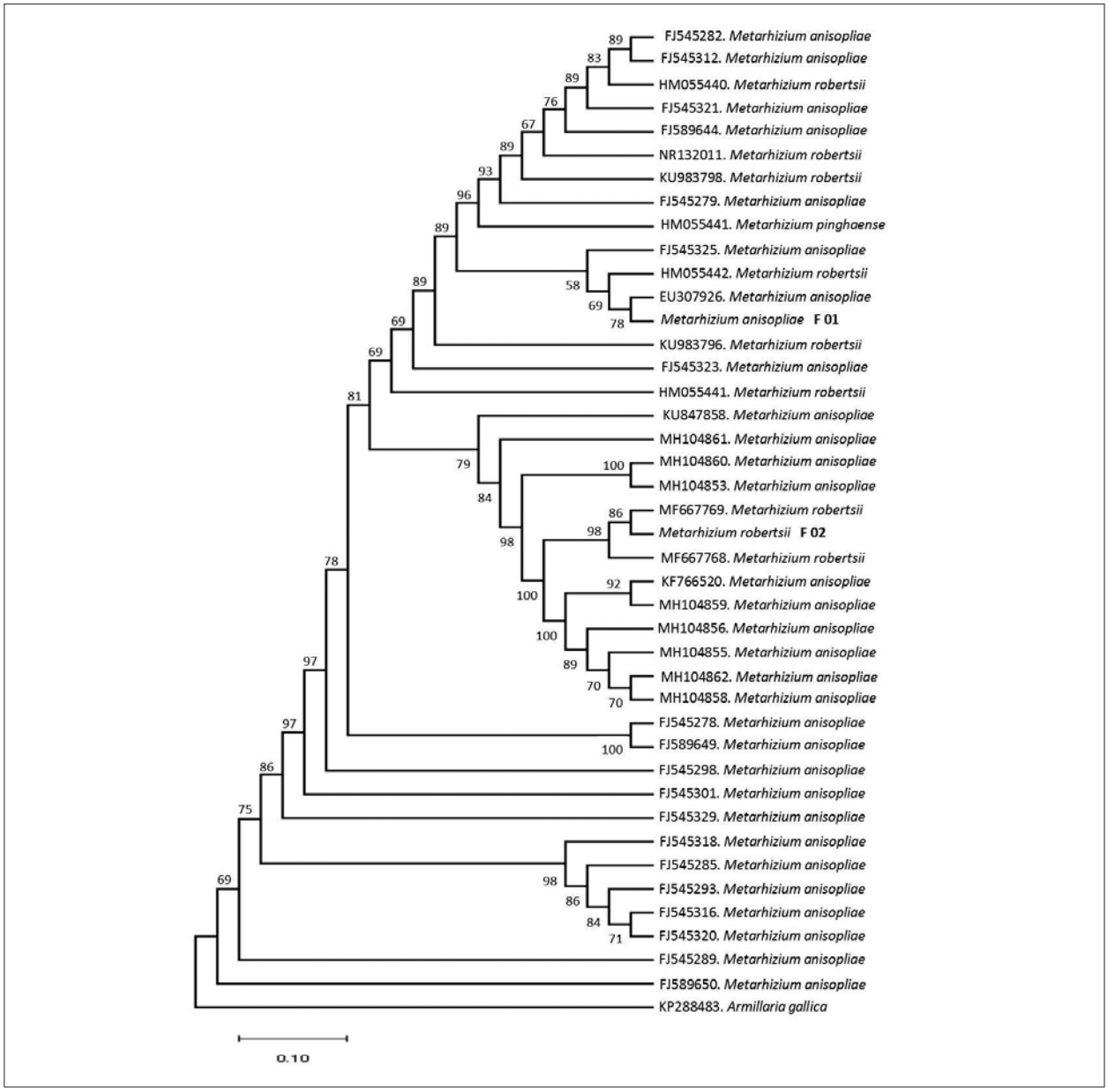

Fig. I – Phylogenetic analysis of new Afghan strains of *M. robertsii* and *M. anisopliae* and closely related species/strains based on ITS gene sequences using maximum likelihood.

concentration of 1×10^8 6 days of treatment of *B. bassiana*. Other concentration respectively caused 100% mortality approximately 7-10 days post inoculation. On the other hand, minimum mortality (so less than others) was recorded on 10th days by filtrate application of *B. varroae* (table 2). The results showed that indigenous *M. robertsii* was very virulent, it had significant highly mortality 4 day post inoculation (F=33.34, df= (4, 30), p<.0001), *B. bassiana* also significant effect 4 days post inoculation (F=9.47, df= (4, 30), p<.0001) and *B. varroa* was not significant effected 4 days post inoculation (F=.0.38, df=(4, 30), p<0.8246). *Metarhizium robertsii* and *B. bassiana* were selected for semi-field bioassay for their high pathogenicity.

Pathogenicity of *M. robertsii* and *B. bassiana* under Semi-Field Condition

Metarhizium robertsii and *B. bassiana* were tested for insecticidal activity against *A. vagans* in semi-fields bioassays. There were significant mortality differences between both isolates (F= 64.22, df= (1, 20), p<0001) in comparison to control groups. Mortality of *A. vagans* at different doses of the selected *M. robertsii* and *B. bassiana* isolates was significantly different (F= 65.22, df= (4, 20), p<0001) and the interaction between fungal species and concentration were not significant different (F=7. 2.56, df= (4, 20), p<0.0706). The results showed that the Afghan *M. robertsii* was much virulent with highly mortality 5-day post inoculation (F= 45.72, df=(4,30), p<.0001), *B. bassiana* also with a significant effect 5 days post inoculation (F= 22.06, df=(4,30), p<.0001). In both species the higher concentration (1×10^8 conidia/ ml^{-1}) had achieved maximum mortality compared to 1×10^5, 1×10^6 and 1×10^7 conidia/ml^{-1} (Fig. III). Dead termite infected by entomopathogenic fungi would develop mycosis in five to six days after they were placed in wet condition. For the dead termites infected by *M. robertsii* they were covered by green conidia, while for *B. bassiana* the white conidia were fully grown on termite cadavers. In pathogenicity test,

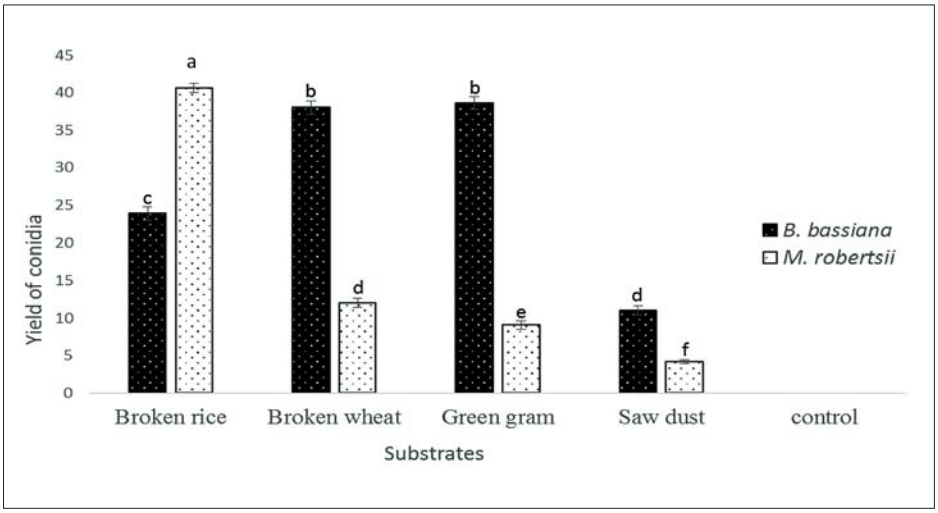

Fig. II – Mean number of conidia (± SE) produced by four solid substances (broken rice, broken wheat, green gram and saw dust) for *M. robertsii* and *B. bassiana.* Letters denotes significant differences between interactive fungal species and substance; Turkey's test; p<005.

Table 2 – Pathogenicity of three entomopathogenic fungi strains *Beauveria bassiana, B. varroae* and *Metarhizium robertsii* on termite *Anacanthotermes vagans* under laboratory condition.

Days after treatment	2	4	6	8	10
Fungal species [Concentration (conidia/ml-^{1})]	% Mortality (± SE)				
B. bassiana					
0.05% Tween-80	0.0 ± 0.0	0.0 ± 0.0	3.33 ± 3.3	6.7 ± 3.3	6.7 ± 3.3 ^{f}
1× 10^{5} conidia ml^{-1}	3.3 ± 3.3	13 ± 6.7	43.3 ± 6.7	63 ± 3.3	100 ± 0.0 ^{ef}
1× 10^{6}	3.3 ± 3.3	20 ± 5.7	63.2 ± 3.3	90 ± 5.7	100 ± 0.0 ^{efd}
1× 10^{7}	6.7 ± 3.3	23 ± 8.8	70 ± 11.5	100 ± 0.0	100 ± 0.0 ^{efd}
1× 10^{8}	13.4 ± 3.3	40 ± 5.7	100 ± 0.0	100 ± 0.0	100 ± 0.0 ^{bcd}
B. varroae					
0.05% Tween-80	0.0 ± 0.0	0.0 ± 0.0	3.3 ± 3.3	6.67 ± 3.3	6.67 ± 3.3 ^{f}
1× 10^{5} conidia ml^{-1}	3.3 ± 3.3	3.3 ± 3.3	3.3 ± 3.3	10.0 ± 3.3	23.3 ± 3.0 ^{f}
1× 10^{6}	3.3 ± 3.3	3.3 ± 3.3	6.7 ± 3.3	13.3 ± 3.3	26.7 ± 3.3 ^{f}
1× 10^{7}	3.3 ± 3.3	3.3 ± 3.3	10.0 ± 5.7	23.3 ± 3.3	30.0 ± 0.0 ^{f}
1× 10^{8}	0.0 ± 0.0	10.0 ± 3.3	20.0 ± 5.7	30.0 ± 7.7	40.0 ± 7.7 ^{ef}
M. robertsii					
0.05% Tween-80	5.0 ± 5.0	10.0 ± 0.0	10.0 ± 0.0	10.0 ± 0.0	10.0 ± 0.0 ^{ef}
1× 10^{5} conidia ml^{-1}	10.0 ± 6.7	40 ± 10.0	100 ± 0.0	100 ± 0.0	100 ± 0.0 ^{ecd}
1× 10^{6}	13.3 ± 3.3	56 ± 10.0	100 ± 0.0	100 ± 0.0	100 ± 0.0 ^{bc}
1× 10^{7}	23.3 ± 8.8	76 ± 8.8	100 ± 0.0	100 ± 0.0	100 ± 0.0 ^{ab}
1× 10^{8}	53.3 ± 12	100 ± 8.8	100 ± 0.0	100 ± 0.0	100 ± 0.0 ^{a}

Insecticidal effects of fungal species against termite at four different conidial concentrations. Values expressed as mean of three replicates. Different upper case letter indicates the significant difference (F= 27.21, df= 30, P<0.0001) at Tukey HSD.

melanization spots were observed around the thoracic and abdominal segments after inoculation of conidial suspension. The fungal infection changed the color of the insect body with the progressive symptom of sluggishness (slow movement) when compared to control. The highest concentration (1 ×10^{8} conidia/ ml-^{1}), caused quick sporulation when exposed to individual termites.

DISCUSSION

In recent years, some studies have focused on developing fungal insecticides to control insect pest. Four to five hundred species of fungi have pathogenic effects on insects. For the basal fungi the largest numbers of EPF species occurs in the *phylum* Entomophthoromycota. Most species within this group are obligate entomopathogens, occurring in five of the six known families. These fungi are often highly specialized parasites that show distinct eco-morphological adaptation to the life cycles of their hosts, including the production of conidia (spore's production from asexual reproduction), discharged actively from host cadavers and timed to take advantage of favorable environmental condition, manipulation of host behavior, and the production of resting spores to survive adverse condition (Chandler, 2017). This fungal virulence mostly has been associated with intra and extracellular synthesis of different substances including cuticle-degrading enzyme, and low molecular weight toxic compounds (Mishra *et al*., 2015; Luangsa-ard *et al*., 2017). However, most of the

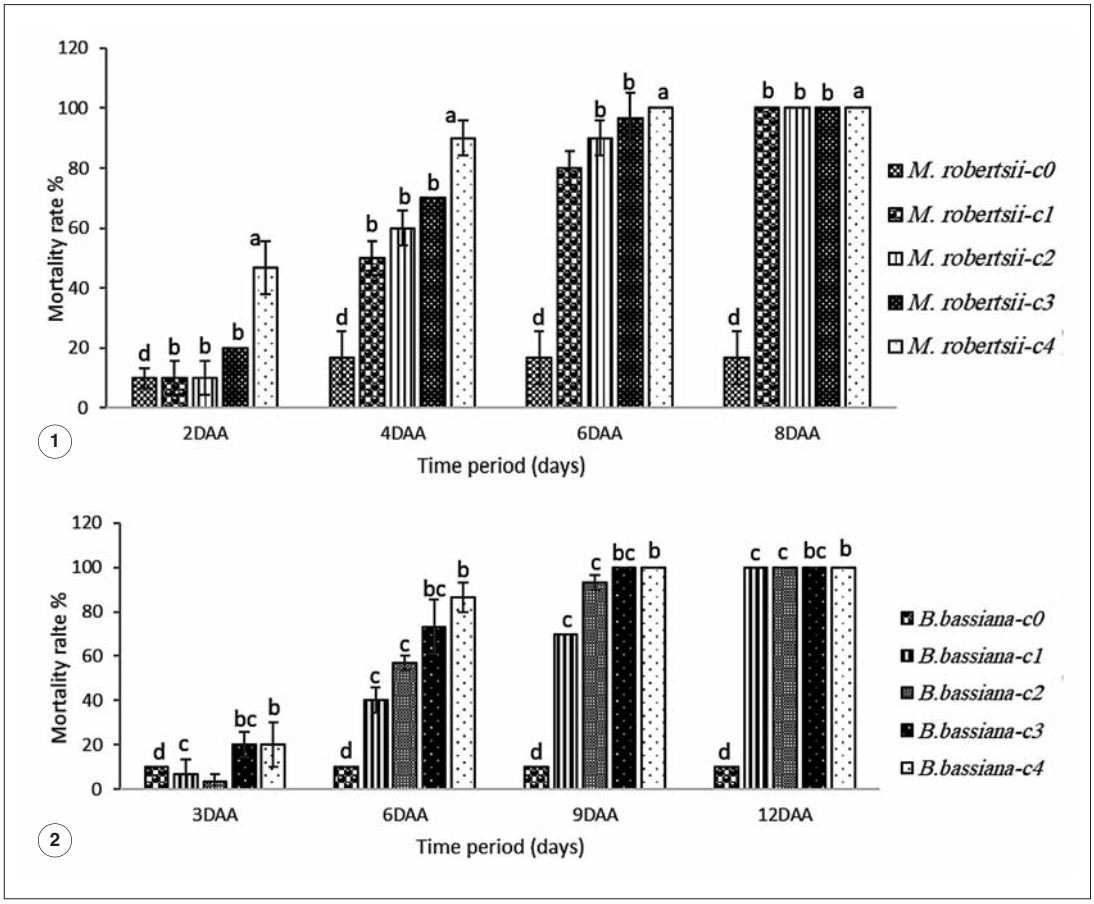

Fig. III – Virulence of difference concentrations (c0, c1, c2, c3, c4=concentrations) of *M. robertsii* (1) and *B. bassiana* (2) on the termite, *Anacanthotermes vagans* at 1-12 days (DAA: Days After Application). Bars indicate standard deviation of mean. Different letter indicates significant differences between effect of species and concentrations according Turkey's test.

literature has focused on low molecular Weight fungal metabolites (secondary metabolites), whereas the high-molecular Weight compounds, such as proteins, remain poorly studied (Keppanan *et al*., 2018a; Nishi *et al*., 2011). The host can be infected by direct treatment and by the transportation of inoculums from treated insects to untreated ones (Singha *et al*., 2011). In the present study, we identified indigenous isolates of *M. robertsii* and *M. anisopliae*. Identification based on molecular and morphological species identification of the fungal isolates were confirmed by phylogenetic analysis (Fig.1). From two indigenous isolates which were identified, only *M. robertsii* was applied with *B. bassiana* and *B. varroae* as a biological agent to replace chemical insecticides for control of *A. vagans* in laboratory. *M. robertsii,* with *B. bassiana* were tested against *A. vagans* under semi-field conditions. In laboratory, all isolates caused different mortality rate in comparison to control groups (Habtegebriel *et al*., 2016). All isolates caused different mortality rate in comparison to each other's.

The highest mortality rate was obtained by indigenous isolate *M. robertsii,* more than 95%, the second mortality obtained by *B. bassiana* and the lowest mortality recorded by *B. varroae,* confirming the data obtained by Singha *et al*. (2011) with the termite *Microtermes obesi* Holmgren. While others have reported the fungal effect of termites under laboratory conditions, this assessment shows that these fungi have also effect under semi-field condition. In semi-field condition only *M. robertsii* and *B. bassiana* were applied and both isolates caused different mortality confirming what Azmi *et al*. (2011) reported for the termite *Globitermes sulphureus* Haviland. We examined conventional parameter used to produce conidia on different solid substrates, with optimized sporulation (Keppanan *et al*., 2018a; Lopes *et al*., 2018).

The *Galleria* bait method's, *i.e*. using *G. mellonella* for EPF recover from soils, was described by Zimmermann (1986). Since then it has been used quite often in numerous studies as the only method for EPF isolations, in the past three decades (Sharma *et al*., 2018). The result of this study showed that the isolate *M. robertsii* infected most of the *Galleria* larvae in insect baiting assay and the progression of external symptoms was found at 48h of post inoculation when compared to the other EPFs. This data suggest that soil is an important reservoir of entomopathogenic fungi, with potential effect for controlling insect pests (Keppanan *et al*., 2018b).

Entomopathogenic fungi can control insect pests and, as a result, have the potential to be used in the biological control system (Nazir *et al*., 2018; Groth *et al*., 2017; Oreste *et al*., 2016; Tarasco *et al*., 2016). They offer the advantages of no pollution, safety and avoiding pesticide resistance; and they can sustainably control insects during their reproductive phase, thereby reducing pest population and the risks of epidemics. However, in the long-term, the effectiveness of entomopathogenic fungi as biological agents can decline as a result of strain degradation, variation, rejuvenation, and contamination (Meng *et al*., 2017; Migiro *et al*., 2010).

Most of the basic studies in the literature have suggested

that solids fermentation has emerged as an appropriate technology for the self-enhancement of fungal virulence and killing speed of the host (Keppanan *et al*., 2018b). Four different solid-substrates were used in the present study with the mass production of new isolate *M. robertsii* comparing an Iranian isolate of *B. bassiana* under *in vitro* conductions (Gurlek *et al*., 2018). We observed that the activities of endogenous isolate (*M. robertsii*) had a more potent effect on killing fast and quick sporulation on *A. vagans* insect cadaver. A time of killing of 4-6 days post inoculation in laboratory condition for *M. robertsii* and 6-10 for *B. bassiana* was observed. In semi-field condition the potential effect of killing 5-8 days post inoculation for *M. robertsii* and 8-12 days for *B. bassiana* was observed. Time of killing at four concentrations was different and quick mortality was seen at a concentration of 1×10^8.

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