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In vitro Screening of Natural *Metarhizium spp.* Isolates for Bioinsecticidal and Biofungicidal Activity

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Authors' contributions

This work was carried out in collaboration among all authors. Authors TD, ST, SB designed the study. Authors ST, SB performed the experiment and collected the samples. Author SB performed the DNA and RNA extractions. Authors SB, TD analyzed the amplicon sequencing data. Authors TD, ST wrote the paper. All authors read and approved the final version of the manuscript.

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ABSTRACT

Aims: To increase conidia production of local *Metarhizium* spp—strains by selecting the low-cost media to test obtained bioformulation against the sucking pests and fungal plant pathogens.
Study Design: The phytophages like black bean aphid (Aphis fabae), a scale insect (Aspidiotus nerii) and spider mite (Tetranychus urticae) are economically essential pests in Kyrgyzstan. The current requirement is to use environmentally friendly protection to reduce the number of these pests. The entomopathogenic fungus from the *Metarhizium* genus was used to reduce harmful pests *in vitro* and *in vivo* experiments.

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Place and Duration of Study: Department of Plant Protection, Kyrgyz-Turkish Manas University between May 2021 and May 2023.
Methodology: Natural *Metarhizium* spp. strains were isolated from dead insect bodies of the Lepidoptera and Apidae families. A laboratory bioassay was conducted to evaluate isolated *Metarhizium* spp.strains against nymphal stages and adults of sucking pests like A. fabae ((Blackfly), A.nerii (Scale insect) and T.urticae (Spider mite). The inhibitory effect of *Metarhizium* spp.against *Fusarium oxysporum*, *Alternaria* spp. and *Rhizoctonia solani* was detected using a dual culture technique.
Results: The results of in vitro bioassay tests against sucking pests have revealed the LC_{50} and LT_{50} values of TLK-1 isolate. The LC_{50} of this isolate for A. fabae adults was 1×10^6 conidia\ mL-1; for A.nerii, it was lower - 7×10^5 conidia\ ml-1; while for adults T.urticae it was higher- 13×10^6 conidia \ ml-1. Such mortality occurred after 49.63 hours in adults of A. fabae (LT_{50}), after 46.30 hours in A.nerii (LT_{50}), and after 75.87 hours in deutonymph and adults of T. urticae (LT_{50}). Three isolates, TLK-1, TLA-2 and BZTK1, have exhibited biofingicide activity in pathogen fungi like Fusarium oxysporum and Alternaria spp.
Conclusion: The found isolates can be simultaneously used against pathogenic fungi, essential for developing inexpensive biological formulas based on these fungi.

Keywords: Entomopathogenic fungus; Metarhizium spp.; bioinsecticide activity; biofingicide activity; bioformulation for plant protection.

1. INTRODUCTION

Entomopathogenic fungi in nature are essential in keeping the number of agriculture and forest pests at an economically low-damaged level. The main natural habitats for these fungi are the soil, plant parts, plant rhizosphere, and insect body [1,2,3].

Fungi in the genus *Metarhizium* (*Hypocreales: Clavicipitaceae*) are considered a relevant biological agent for reducing pests' population and mass death. This entomopathogenic fungus affects about 200 types of insects from orders like Lepidoptera, Homoptera, Hymenoptera, Coleoptera, and Diptera. Among them are plant aphids, mites, thrips, greenhouse whiteflies, tomato whiteflies, and larvae of the butter beetle [4,5,6].

The fungus can survive saprophytically in the soil and be isolated from the soil using the 'Galleria' bait method [7]. Some studies recorded that *Metarhizium* species are often found in European, Central and South American, African and Asian countries [5]. According to the authors, southeastern Asia is the probable origin of the evolution of *Metarhizium spp.* [8,9]. *Metarhizium species* have asexual, cylindrical conidia, which are produced by spores. The conidia stick together to form chains and make large masses during asexual development [9,10,11,12].

Abiotic factors like humidity, sunlight and temperature (25°C) can be affected by the growth and mycelium formation of these fungi to efficiency to harmful insects. There are various facts about the effect of water on the fungus; *Metarhizium spp*. has a hydrophobic future and does not mix well with water, forming float suspensions on the water. *Metarhizium spp*. mixed with Tween 80 can stored at room temperature for six weeks. The influence of humidity and temperature on the medium-term (3-4 months) storage of *Metarhizium* species conidia was studied. Conidia harvested after a 24-day culture on rice showed greater resistance to long-term storage than conidia from 12-day cultures. Dried conidia stored as a powder maintained a germination rate of 95% at 10-14°C but only up to 27% at 28-32°C. In another experiment, dried conidia maintained over 90% germination for 128 days, with or without silica gel at 10 - 14 ^{0}C or 15 - 18^0 C [13,14,15].

Like other entomopathogenic fungi, *Metarhizium* spp. produces conidia that penetrate the cuticle of host insects and mites, infecting them by adhesion, germination, invasion, colonization, and dissemination [10,16,17]. Hence, *Metarhizium* spp. may have an advantage over other biological agents (i.e., bacteria and viruses) for some applications as a contact biopesticide. Because of its ability to rapidly produce infectious conidia when incubated on inexpensive nutrient media, there is significant interest in the commercial mass production of highly effective bioproducts with broad ranges of activity based on *Metarhizium* spp. [2,3,18]. In vitro tests have recorded the ability of *M. anisopliae* to infect mite

eggs of the spider mite *Tetranychus urticae.* At the same time, the mortality of mature stages (deutonymphs and imago) of this mite depended on the highest conidial concentration of fungi [19]. *M. anisopliae* could infect other species, like the citrus rust mite *Phyllocoptruta oleivora* [20].

The *M.anisopliae* was tested against second and third larval instars of tomato pinworm *T.absoluta* [21,22] *in vitro* conditions, and LT50 results were of 1.93 and 2.8 days at high (10^8 spore/ ml−1) spore concentration. Based on other research results *of M. anisopliae*, strain Egy-5 infected adult females of *Aulacaspis tubercularis* (Diaspididae), mortality rates were significantly higher (P =04) [23,24,25, 26]. *M. anisopliae* was effective against a severe pest, such as aphids [27,28,29, 30].

Over the last two decades, studies have recorded a multifunctional lifestyle of *Metarhizium* species; their colonization of plants may exert growth promotion, and immunity modulation affects them. Except as insect pathogens, these fungi can benefit their host plant through growth promotion and protection against stresses [31,32,33]. Plant growth promotion has been observed for multiple species of *Metarhizium* spp. in tomato [33,34], maize [32,35,36,37], soybean [38], peanut [39], potato [40], cassava [41], sweet pepper [42], switchgrass and haricot beans [43].

The phytophages like black bean aphid (Aphis fabae), a scale insect **(***Aspidiotus nerii)* and spider mite (Tetranychus urticae) are economically essential pests in Kyrgyzstan. The current requirement is to use environmentally friendly protection to reduce the number of these pests. Therefore, the search for fungi biosources and assessing fungi virulence and conidia productivity are relevant to formulating a bioproduct based on *Metarhizium* spp. This study aims to increase conidia production by local *Metarhizium* spp strains by selecting the low-cost media to test obtained bioformulation against the sucking pests and fungal plant pathogens.

2. MATERIALS AND METHODS

2.1 *Metarhizium Anisopliae* Strains

In this study *Metarhizium* spp. strains newly isolated from insects' cadavers were used.

2.2 Methods of Isolation from Dead Insects

To remove the external microflora, the dead insect body was carefully washed in running water, then rinsed again with sterile distilled water and placed in a sterile cup (in some cases, if the studied object is resistant to alcohol, it can be rinsed with a small amount of ethyl alcohol), then 9 ml of sterile physiological solution added to the body, placed in a sterile porcelain container and crushed. The resulting suspension is diluted. The optimal dilution for fungi is 10^2 or 10^3. A dilution of 10^2 was used in this work, the prepared solution of 0.3 mL was poured into sterile Petri dishes using a pipette, and 500 ml pre-cooled nutrient medium (Czapek agar (CZA), PDA) was added, and thoroughly mixed. Inoculated dishes were incubated at 25°C for 7-10 days for colonies to grow (Fig. 1).

2.3 Cultivation and Morphological Study of *Metarhizium spp. Strains*

Metarhizium spp. can grow in two conditions: on solid and liquid media, showing different growth phases. Its chlamydospore predominates in the liquid medium, and conidia in the dry mass are common in the solid medium. The result can be provided using liquid and solid media to produce chlamydospores and conidia. One per cent from the liquid or solid medium of *Metarhizium* spp.conidia production will be sufficient.

The morphology of *Metarhizium* spp. depends on the compounds contained in the medium. During the research stages, three types of cultivation for *Metarhizium* spp. isolates were used:

1. Cultivation in a liquid medium;
2. Cultivation in a solid medium;
3. Grown on the grain surface.

The morphology of *Metarhizium* spp. isolates were identified following the methods [44]. The growth characteristics of isolates on the culture medium (PDA) were inspected continuously for 20 days, and their colony size, mycelial colour, colony reverse, and colour of conidial mass were visualized. Pure fungal cultures were obtained and examined microscopically (400× and 1000×) to characterize morphological structures using lactophenol blue staining.

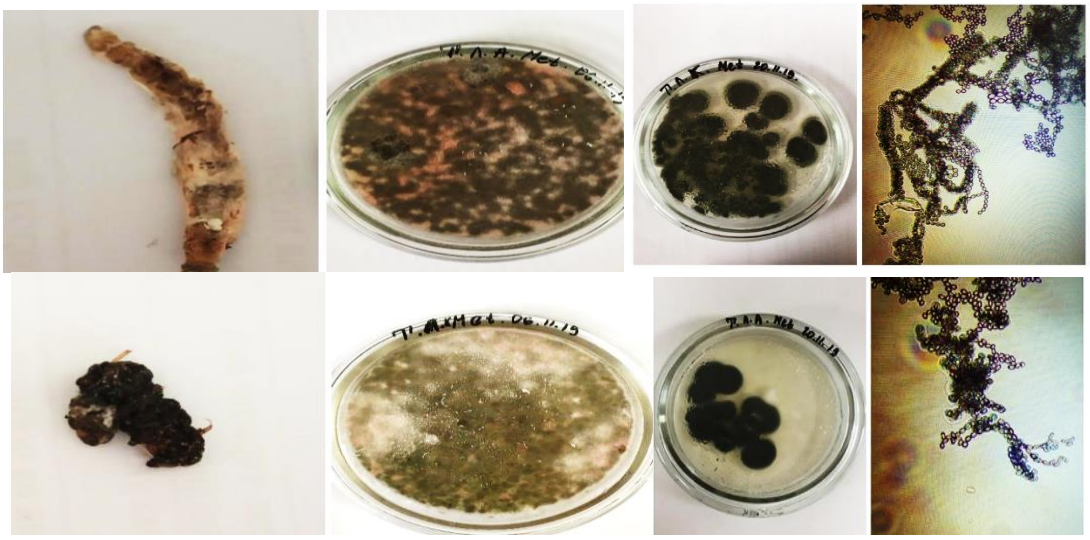

Fig. 1. The scheme of primary isolation of natural *Metarhizium* spp. strains from insects' cadavers

The stained slides were observed under 400× with a binocular light microscope (Leica, USA). The fungal isolates were identified based on the shape of the conidia and the arrangement of spores on the mycelia. Photomicrographs were obtained using a camera (CMEX) on the microscope (Euromex Oelphi-X, Observer series). When used for bioassays, the number of conidia spotted in the centre of plates and conidial concentrations were determined using a haemocytometer. The selected concentration of cell suspensions was obtained by diluting in 0.01%.

2.4 Production of Fungi Biomass during Cultivation in Liquid Medium

CSYE liquid medium (g/l^: Glucose -40; KNO_3 - 10; KH_2PO_4- 5; MgSO4- 2; $CaCl_2$- 0.05; Yeast extract -2.3; Distill. water 500ml, pH-5.5-6) was used. The prepared nutrient medium was autoclaved and poured into 100 ml flasks. Fungi culture was added and incubated with shaking for 48 h. As a result, a green mass appears on the surface of the medium.

2.5 Growth of Fungal Biomass in Solid Medium

Several solid agar mediums were used for the cultivation of *Metarhizium spp*. natural isolates :

Potato Dextrose Agar (PDA): Potatoes, infusion from: 200.0 g; Dextrose: 20.0 g, Agar: 15.0 g, distilled water (D/W):1000 mL. Final pH (at 25°C) - 5.6±0.2.

Czapek Agar (original), the composition of Cane sugar: 30 gm, monopotassium phosphate: 1 g, magnesium sulfate: 0.5 g, potassium chloride: 0.5 g, Iron sulfate: 0.01 g, distilled water (D/W): 1000 mL.

Wrot Agar: Malt extract 15.0 g; peptones 0.75g; maltose 12.75g; dextrin 2.75g; glycerin 2.35g; potassium phosphate monosubstituted 0.4g; ammonium chloride 1.0g; agar-agar 20.0g.

Oat Agar medium (Oat – 100g; agar- 15g; dis. water -1000 mL; pH- 6.2).

A suspension was prepared from growing fungi conidia in distilled water, and 0.01% Tween 80, vortexed and the concentration of conidia was counted using a Neubauer hemacytometer.

2.6 Growth of Fungal Biomass on the Grain Surface

Growing in a solid medium on a rigid substrate takes up much space for aeration and grows according to the quality of the medium. Mass production of conidia varies depending on the various compounds in the medium. Fungi production is challenging; most industries want to grow in low-cost mediums. Plant grain wastes are the primary source for the surface cultivation and mass production of fungi biomass. Oat bran was used for the surface cultivation of fungi. The

oat bran was simmered at 212°F (100 °C) for 10 min, then it was placed in the polyethene packets (37.5 x 26.3 cm) and sterilized at 270 °F (132 °C) for 60 min. After autoclaving, the oat bran media was weighed on 180 mg, and the wet media was different, from 20 % to 70%. The ready oat bran was inoculated with the fungi culture grown on a CSYE liquid medium. The polyethene packets were incubated at 24°C for 7 d. After 48-54 h incubation, the culture appeared with green mycelium, and during the incubation, it was weighed (growing from 1d 180 mg to 7d 255mg). The obtained bioproduct can be stored for eight months (Fig. 2).

2.7 Bioassay for Entomopathogenic Activity of the *Metarhizium spp* Isolates

Metarhizium spp isolates' conidia are produced on the oat-bran medium, harvested after 7d cultivation and used for inoculum to infect the pests. Four concentrations of suspension: 7.2×10^1, 7.2×10^2, 7.2×10^3 and 7.2×10^4 conidia/mL with adding 0.01% Tween 80 were prepared and vortexed. The plant leaves were washed with sterile distilled water. The insects' deutonymphs and adults of A. fabae, A. nerii and T.urticae (Table 1) were released on the leaf surface of host plants and directly sprayed with different concentrations (above mentioned) *Metarhizium spp.* conidia suspensions.

In control, the leaves were sprayed with sterile water. Each treatment was replicated three times. Thirty insect deutonymphs and adults for each treatment were tested. Observations for infected insects have lasted within seven days at 25.0± 2.0°C.

2.7.1 Assessment of mortality

Treatments were recorded daily and continued for seven days. The leaf was counted every 24h under a stereo microscope (Euromex Oelphi-X, Observer series) in the Petri dishes and compared with control treatments. The mortality was calculated with Probit analysis (Probit analysis software in SPSS version 25).

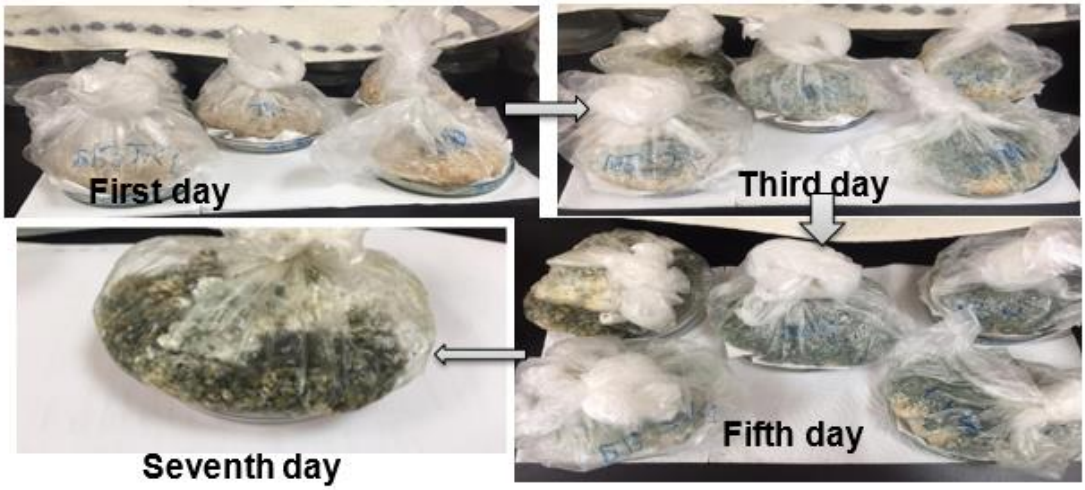

Fig. 2. The growth and mycelium formation of *Metarhizium* spp. on the oat bran during seven days after inoculation

Table 1. Insect population from the Chui region, Bishkek, was used in screening tests

Host	Hosts class	Stage of host	Month collected	Host plant	Region
Aphis fabae (Black bean aphid)	*Insecta, Aphidoidea* (arthropods)	adults, deutonymphs	December 2021	*Cucumis sativus*	Chui, Bishkek
Aspidiotus nerii (Scale insect)	*Insecta, Diaspididae* (arthropods)	deutonymphs	March 2022	*Citrus limon*	Chui, Bishkek
Tetranychus urticae (Spider mite)	*Arachnida Tetranychidae(* arthropods*)*	adults, deutonymphs	December 2021	*Citrus limon*	Chui, Bishkek

2.8 Antagonistic Tests

The inhibitory effect of *Metarhizium* spp.against *Fusarium oxysporum, Alternaria* spp. and *Rhizoctonia solani* was detected using a dual culture technique [45] on the YPG plate. One mycelial plug (6 mm diameter) of *Metarhizium* spp. was placed on a new YPG plate 1 cm away from the border of every Petri dish, and this was incubated at 25^0C for two days. Then, a mycelial plug (6 mm diameter) of *Fusarium oxysporum, Alternaria* spp. and *Rhizoctonia solani* colony was put on the other side of the plate. Plates were incubated for 4-6 days until a clear inhibition zone was quantified at 25 ± 2.0^0C, and three replicates were tested. Control tests were also carried out using *Fusarium oxysporum, Alternaria* spp. and *Rhizoctonia solani* alone. The percentage inhibition of growth of the tested pathogen in the presence of antagonist isolates was calculated over control. The percentage inhibition of radial growth (PIRG) was calculated by using the formula developed by [45]: Inhibition %: = [(R − r)/R] × 100], where R = diameter of the fungal colony in control, r = diameter of the fungal colony in dual inoculation. Interactions were assayed by giving ranking according to Bell's ranking scale [46], where R1= complete overgrowth of the pathogen by antagonist; R2= 75 % overgrowth; R3= 50% overgrowth; R4= growth inhibition at the line of contact; R5= pathogen over the growing antagonist.

2.9 Molecular Characterisation of *Metarhizium* spp

For DNA extraction, fungal isolates were grown on Yeast Extract Agar at 25°C. Approximately 100 mg of mycelium was transferred to a screw-capped 2 mL microcentrifuge tube containing Lysing matrix C (MP Biomedicals) and DNA was extracted using the DNeasy Plant kit (Qiagen). Purified DNA was eluted in 100 µL elution buffer AE and stored at -20 °C. The following DNA sequences were amplified from fungal DNA samples using standard Taq DNA polymerase (GoTaq (Promega). The internal transcribed spacer (ITS) region of nuclear rDNA was PCR amplified from genomic DNA by using ITS5 (GGAAGTAAAAGTCGTAACAAGG) and ITS4 (TCC TCC GCT TAT TGA TAT GC) primers [47,48]. The generalised PCR protocol consisted of one initial denaturation step of 95°C for 2 min, 35 cycles of 45 sec at 95°C, 45 sec at the primer-specific annealing temperature and an elongation-step at 68 °C for an amplicon-specific elongation time, followed by a 5 min final elongation step at 68 °C . PCR product size was controlled by agarose gel electrophoresis, and PCR products were purified using the Qiaquick PCR purification kit (Qiagen). A commercial provider, Macrogen (Seoul, Korea), performed Sanger sequencing of PCR products using PCR and additional sequencing primers. Raw sequence data were combined into a single consensus sequence for each marker using version 6 of the MEGA software package [49]. The search for homologous sequences was done using Basic Local Alignment Search Tools at the National Center for Biotechnology Information.

2.10 Statistical Analysis

Spore germination, sporulation date, and screening test results were analysed using one-way variance (ANOVA) using SPSS software version 25 statistical programs. Multiple mean comparisons were made using the Tukey HSD test when statistical differences were found between data sets ((P < 0.05). Lethal concentration (LC_{50} and LC_{90}) was analysed 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).

3. RESULTS AND DISCUSSION

3.1 Morphological and Cultural Future of Natural *Metarhizium anisopliae* Isolates

Table 2 shows the source and site of isolation *of Metarhizium spp.* strains. As results have shown, natural fungi isolates were found in dead insect bodies from *the* Lepidoptera and Apidae families.

Morphological characteristics of *Metarhizium* spp. isolates were proved by detailed microscopy studies of colonies grown on different solid mediums. When observed under x40 magnification in Petri dishes, all of these isolates produced typical greenish *Metarhizium* conidial masses on the back and front sides of the culture dish, which is an essential preliminary distinguishing feature for identifying this putative entomopathogen from other fungal relatives [50]. In addition, microscopic examination confirmed the partial identification of the isolates, as the isolates produced spores of medium and small size, as well as elliptical spores (Fig. 3).

3.2 Mycelium Growth Intensity and Conidia Production at the Solid Medium, per/ml

The intensity of mycelium growth of *Metarhizium spp*. strains was observed on solid nutrient media used in the experiment at 25 ^{0}C. The diameter of the radial circle of mycelium growth of fungal isolates was measured daily. All studied strains grew intensively on oatmeal agar since, on the seventh day, the diameter of mycelium growth in strain TLK-1 reached 7.5± 0.96 cm, in BZTK1 and TLA-2 - 7.0±0.95 cm, and A – 3.0±0.91cm. A significant growth rate was observed on the PDA medium, forming a wide circle of mycelium growth, the diameter in all strains reaching up to 5.0± 0.95 cm. The remaining two media observed no high fungal isolates' growth intensity (Fig. 4).

Table 2. The source and place of new *Metarhizium spp.* isolation

Collection number of *Metarhizium* spp. natural isolates		Source and site of isolation	Maintained medium types
1	TLK-1	Cadavers of (Lepidoptera) larvae found in Torken village, Jalal-Abad region. 41˚50'26.7"N73°09'13.7"E	PDA
2	A-3	Cadavers of insect from (Apidae) , found in Sokuluk village, Chuy region 42°51'02.7"N74°17'13.6"E	Czapek agar
3	BZTK1	Cadavers of insect from (Hemıptera),found in Sokuluk village , Chuy region	CSYE
4	TLA-2	Cadavers of (Lepidoptera) larvae found in Torken village, Jalal-Abad region. 41˚50'26.7"N73°09'13.7"E	PDA

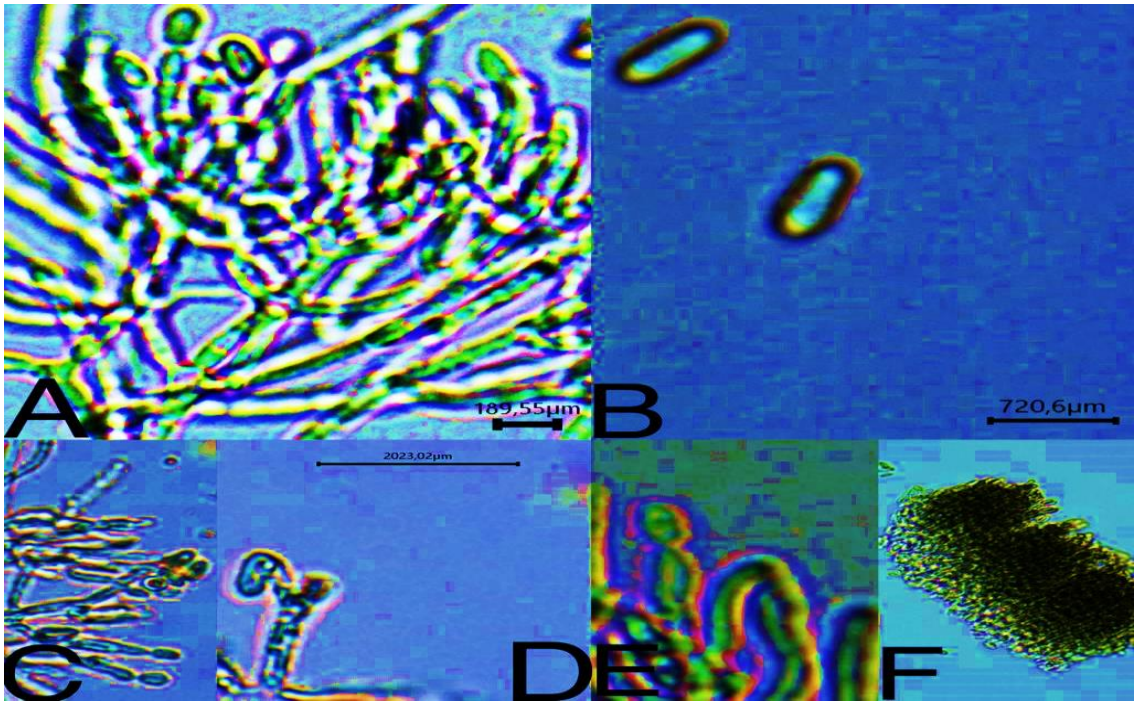

Fig. 3. A-F. Conidiogenous cells and conidia of *Metarhizium* species. A. Mature phialides with developing conidia (TLK-1), Black bar = 189.55µm (×400 magnification); B. Mature conidia (TLK-1). Black bar = 720.6µm (×400 magnification); C.Mature phialides (TLA-2); D. Black bar = 2023.02µm (×400 magnification) of mature phialides(TLA-2); E. Mature phialides with developing conidia(TLK-1); F. Distinctive ellipsoidal conidia in regular chains

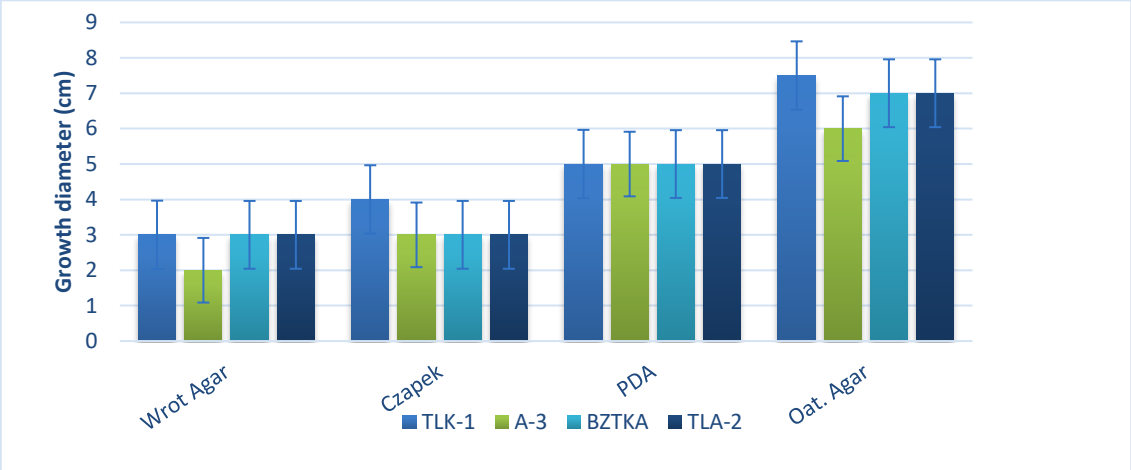

Fig. 4. Mycelium growth of TLK-1, A-3, BZTK1 and TLA-2 strains on different medium for seven days. The colony diameters were measured and subjected to statistical analysis. Error bars represent standard deviations. Differences was analyzed by analysis of variance (ANOVA), P<0.05)

Conidia production at different solid media was assessed using an improved Neubauer hemocytometer under a light microscope, then calculated using the formula M=x*104/0,1*1/400), per/ml. Fig. 5 shows a spore concentration of tested *Metarhizium spp* isolates on Czapek, oat, Potato Dextrose**,** and Wort Agar. The isolates used solid nutritional formulations differently. Almost all isolates preferred oatmeal agar, produced high concentrations of spores; for example, the TLA-2 isolate produced 7.2x 10^7 conidia, mL^{-1} of suspension, BZTK1 isolate - 5.3x 10^7, TLA-2 - 6.3x 10^7. Of all the isolates studied, A-3 produced few conidia on almost all media. All isolates produced low conidia on Czapek's media, Wort agar, and PDA. Thus, a cheap medium containing oatmeal was chosen to obtain the conidia production.

3.3 Mycelium Growth Intensity at Different Temperatures

Culture of tested new *Metarhizium* spp. isolates were incubated at 5 ^{0}, 15^0, 25^0 and 36 ^{0}C on a PDA medium. The results have demonstrated that the growth rate of fungal isolates at 5 ^{0} C was slow, so during seven days of incubation, the diameter of TLA-2 and TLK-1 colonies reached 3.5-3.8 ± 0.96 mm; the other two isolates grew more slowly than the strains mentioned above. At 15^0 C, the growth intensity was higher than at 5^{0}C; for example, the diameter of TLA-2 and TLK-1 colonies made 10.2 – 13.0 ± 0.96 mm for seven days of incubation. The temperature optimum for all tested *Metarhizium* spp. isolates were 25^0 C, their colony diameter reached from 12. 2± 0.95 mm to 24.5 ±0.96 mm for seven days. The low growth rate was reported at 36 ^{0}C on the PDA medium (Figs. 6 and 7).

3.4 Insecticidal Activity of *Metarhizium* Spp Isolates' Conidia Produced on the Oat-Bran Medium

Considering that new isolates have preferred the oat-containing medium, oat bran was used to develop a low–cost formulation based on newly obtained *Metarhizium spp*. isolates.

Dried formulation with high content of tested isolates conidia was used for assessment of their insecticidal activity against adults and deutonymphs of *A. fabae*, *A.nerii* and *T.urticae* and using the doses 7.2× 10^{-1}, 7.2× 10^{-2}, 7.2× 10^{-3}, 7.2× 10^{-4} conidia ml.

Fig. 8 shows the insecticidal activity of different concentrations of the tested *Metarhisium* spp. TLK isolate towards the nymphalid stage of the pests mentioned above. The dose of conidia 7.2 x 10 $^{-1}$ showed low pathogenicity for the nymphalid stage of all three pests, and the mortality ranged from 22.0 ± 0.96 % for *T.urticae;* 29.0 ± 0.95 % for *A. fabae* and 40.0± 0.95 % *A.nerii*. While the dose 7.2 x 10^{-2} of TLK-1 has 50.0± 0.95 % mortality in *T.urticae*, 60.0 ± 0.95 % in *A.nerii* and 60.0± 0.95 % in the population of *A. fabae*. A dose of 7.2 X 10^{-3} conidia has

already shown a more effective activity concerning the nymphalid stage of all three pests; death has reached 78.0 ± 0.95 % in *T.urticae* nymphalid population, 90. 0 ± 0.95 % in the population of *A. fabae,* 97.0 ± 0.95 % in *A.nerii*. The most active insecticidal dose was 7.2 x $10^{-4,}$ at which the TLK isolate caused 97.0% ± 0.95 % death in *T.urticae and* 98.9% ± 0.95 % in *A. fabae 99.9* ± 0.95 % in *A.nerii* nymphalid population.

The results of in vitro bioassay tests against sucking pests have revealed the LC_{50} and LT_{50} values of TLK-1 isolate (Table 3). The dose that caused 50.0% mortality in the population of used pests was different. The LC_{50} of this isolate for A. fabae adults was 1.0 × 10^6 conidia\ mL^{-1}; for A.nerii it was lower – 7.0 × 10^5 conidia\ ml^{-1}; while for adults T.urticae it was higher - 13.0 × 10^6 conidia \ $ml^{-1.}$. Such mortality occurred after 49.63 h in adults of A. fabae (LT_{50}), after

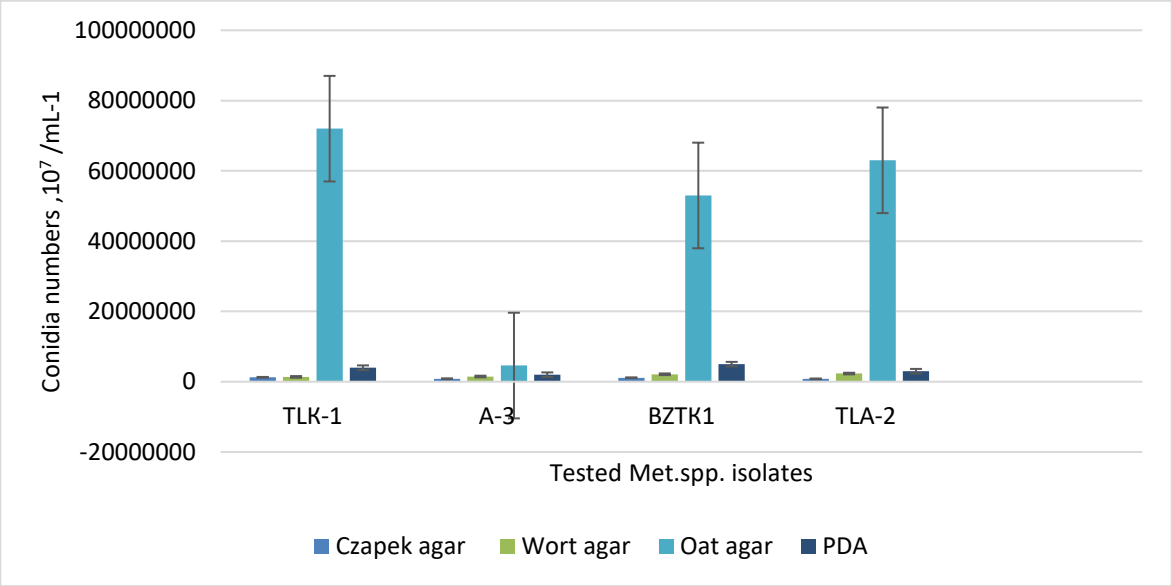

Fig. 5. Conidia production by *Metarhizium spp* isolates in a solid medium for seven days. Error bars represent standard deviations. Differences were analyzed by analysis of variance (ANOVA, P<0.05)

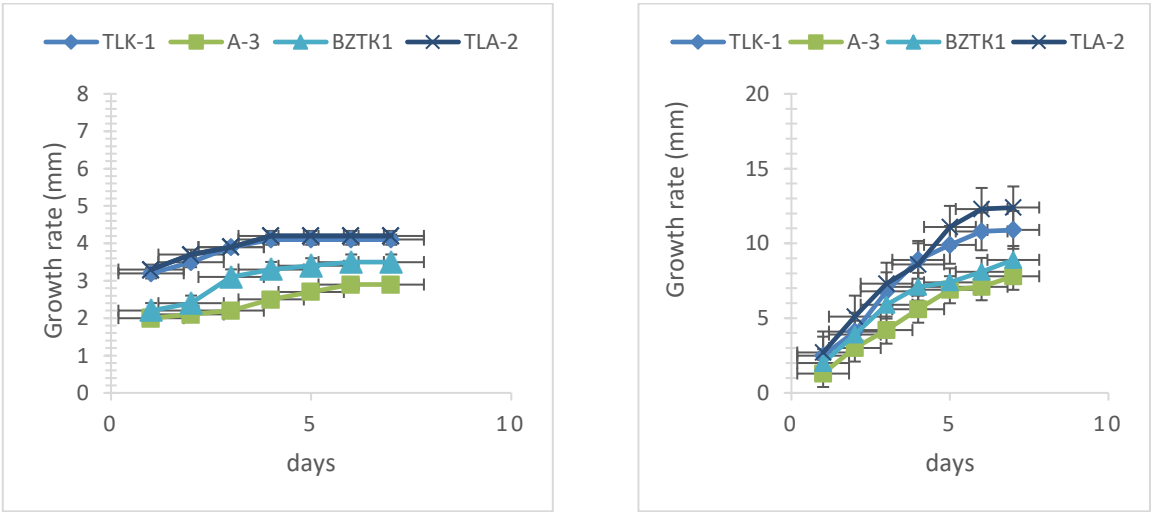

Fig. 6. The growth intensity of *Metarhizium* spp isolates on a PDA medium for 8 days, at 5⁰C and 15 ⁰ C. Error bars represent standard deviations. Differences were analyzed by analysis of variance (ANOVA, P<0.05)

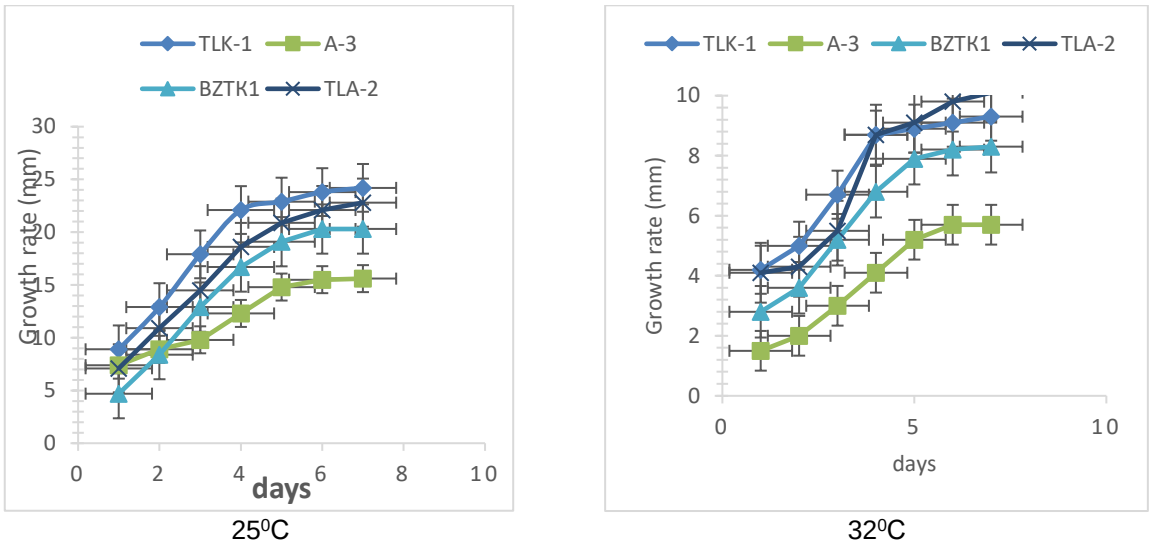

Fig. 7. The growth intensity of *Metarhizium* spp, isolates on a PDA medium for 8 days, at 25⁰C and 36 ⁰ C. Error bars represent standard deviations. Differences were analyzed by analysis of variance (ANOVA, P<0.05)

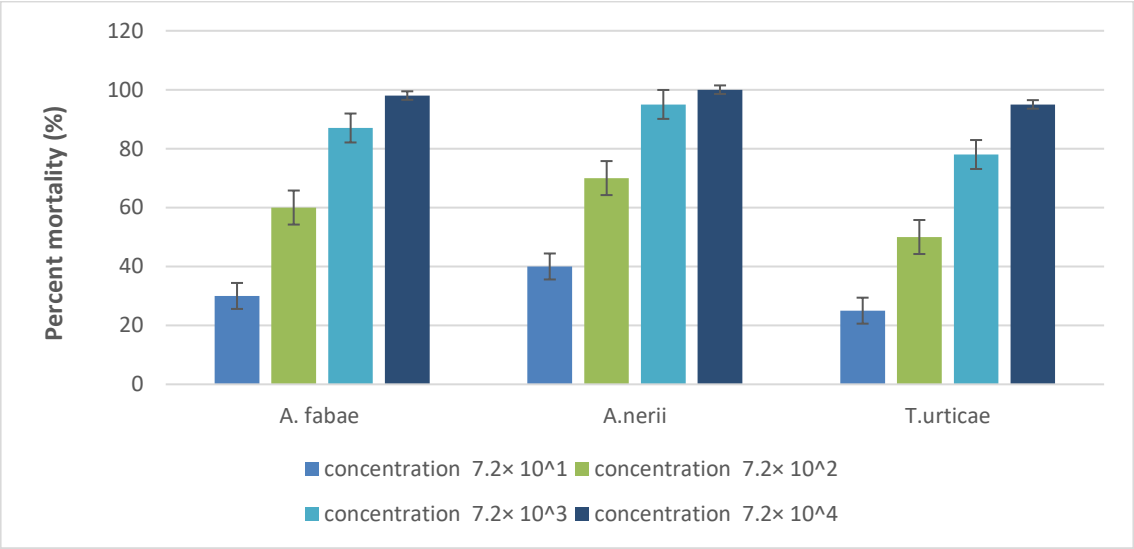

Fig. 8. The insecticidal activity of natural *Metarhizium spp*. TLK -1 isolate to the nymphalid stage of pests for seven days after applying different conidia doses. Error bars represent standard deviations. Differences were analyzed by analysis of variance (ANOVA, P<0.05)

46.30 h in A.nerii (LT50), and after 75.87 h in deutonymph and adults of T. urticae (LT_{50}). These data indicate that sucking pests have shown susceptibility to infection caused by the *Metarhizium spp*. isolate TLK-1, while the smallest dose of 1.0 × 10^6 conidia could cause death in 50.0% of *the Aphis fabae* adult population after 49.63 h. The shortest time during which death occurred in 50.0% of the population was noted in A.nerii deutonymphs at a dose of 7.0 × 10^5conidia ml-1. After 46.30 h was on the adults green- white mycelium, the lower mortality the time was adults of T.urticae, was hatched after 75.87 h, green mycelium grew out of the cuticle of the adults.

LC_{50} and LT_{50} values and 95% confidence limits (CL) expressed as conidia (×10^4) (Proc Probit (SPSS). LT_{50} values for mortality were estimated by analysis (Proc Probit (SPSS) data for >72 h for *A. fabae*, *A.nerii* and *T. urticae*, corresponding to the last day of incubation in bioassay>10 days.

The optimal climatic conditions created during the experiment (humidity 60-65%, temperature 25^0 C) ensured the fungus's pathogenicity and its conidia could form hyphal bodies on the cuticle of infected pests. At the same time, hyphae produced different pigments on the cuticles of different insects. On the cuticle, deutonymphs of A.nerii were white-green hyphae (Fig.9). On the cuticle of *Aphis fabae* adults the fungus produced grey-green hyphae (Fig.10). At the adult stage of T. urticae, the fungal isolate had produced dark green hyphae. According to [51], insect tolerance to infection with *Metarhizium* spp. may change depending on environmental stresses. The production of fungal pigment depends on many factors.

3.5 Biofungicide Activity of New Natural *Metarhizium* Isolates

Three isolates, TLK-1, TLA-2 and BZTK-1 have exhibited biofungicide activity in two pathogen fungi like, *Fusarium oxysporum and Alternaria spp.* According to Bell's ranking scale [46], interactions between *Metarhizium i*solates and plant pathogens were assayed by ranking R4= growth inhibition at the line of contact. The TLK-1 isolate has shown significant activity against the pathogenic fungus *Fusarium oxysporum*, strain Fus:85 (Fig.11D) and relatively strong activity against *Alternaria spp.,* strain1.3 (Fig. 11F). The zone of inhibition of these pathogens from the antagonistic effect of the TLK-1 isolate is visible in the figures, which was 1.8-2.0 mm on the fifth day of the joint incubation of these two types of fungi.

Other strains: TLA-2 and BZTK-1 also showed antagonistic activity against pathogenic *Fusarium oxysporum and Alternaria* spp. strains; their zone of inhibition was 1.7-1.8 mm (Fig.11 G, I, J, L, M and O). At the same time, these three isolates of *Metarhizium spp* .did not show any antagonistic activity against the pathogen *Rhizoctonia solani (*Fig.11.E, H, K and N*).* Colonies of these two different species of fungi grew together when co-cultivated.

Selected fungal isolates were subjected to DNA sequencing for identification. The ITS1 and ITS4 DNA fragments of select fungal isolates were amplified by PCR using the primer pairs ITS1 (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (CAGGAGACTTGTACACGGTCCAG) and genomic DNA as the template. Based on their morphological characteristics, including the production of ellipsoidal conidia, and molecular characteristics (ITS, partial 18S [SSU rDNA] and EF1-α sequences), the isolates were identified as *Metarhizium anisopliae* (belonging to Clade E from Asia (Fig. 12).

Fungi in the genus *Metarhizium* (Hypocreales: Clavicipitaceae) are considered a relevant biological agent, that affect about 200 types of insects from orders like Lepidoptera, Homoptera, Hymenoptera, Coleoptera, and Diptera [5,6,52]. *Metarhizium* fungi have an advantage in rapidly producing infectious conidia when incubated on inexpensive nutrient media to produce the commercial mass production of highly effective bioproducts with broad ranges of activities [2,3,18]. A multifunctional lifestyle of

Table 3. Mortality (LC_{50} values) and (LT_{50} values) for adults and deutonymphs of *A. fabae*, *A.nerii* and *T. urticae* and after inoculation of *Metarhizium* spp.

	LC_{50} values (conidia ml)					LT_{50} values (h)				
Target pests	**n**	**x^2**	**df**	**Slope±SE**	**LC_{50} conidia ml**	**n**	**x^2**	**df**	**Slope±SE**	**LT_{50}(h)**
Aphis fabae adult,and deutonymphs	10	0.60	1	-2.53±1.5	1.0×10^6	10	1.71	1	-6.9±5.52	49.63
Aspidiotus nerii adult,and deutonymphs	10	1.023	1	3.2±1.7	7.0×10^5	10	0.86	1	-9.5±5.68	46.30
Tetranychus urticae adult,and deutonymphs	10	0.30	1	-6.4±4.42	13.0×10^6	10	0.6	1	-19.0±16.1	75.87

Fig. 9. Mycosis on the cuticle of Aspidiotus nerii produced by *Metarhizium* spp.TLK-1 isolate (×100 magnification)

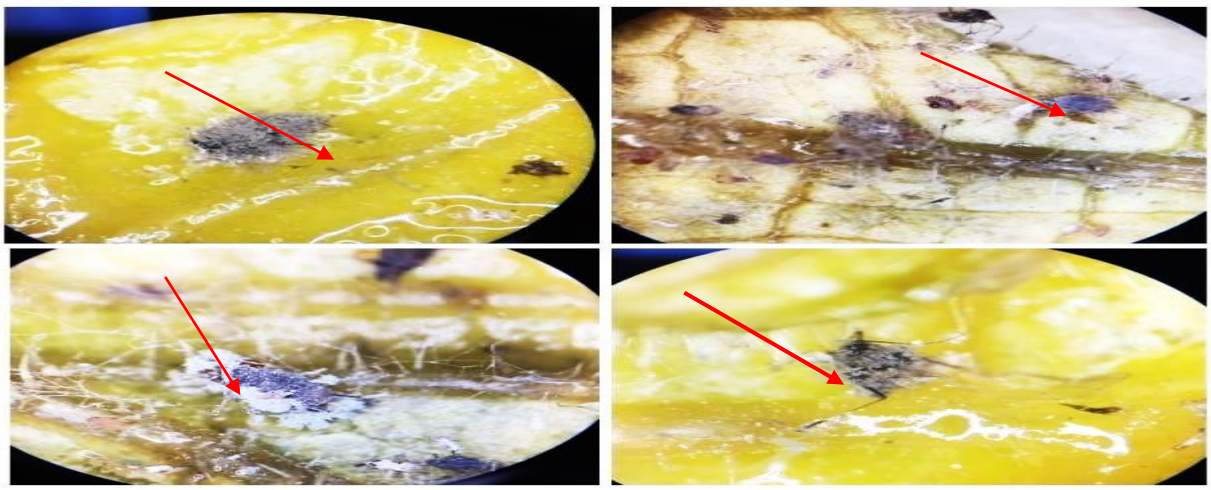

Fig. 10. Mycosis on the cuticle of Aphis fabae adults produced by *Metarhizium* TLK-1 isolate (×100 magnification)

Metarhizium.spp. species is recorded by several studies, except as insect pathogens, these fungi can benefit in inhibiting plant pathogens, in growth promotion and protecting against stresses [31,32]. The studies have noted that native fungal isolates may offer a better alternative than imported strains. They may be better adapted to kill local insects and survive local environmental conditions as vectors for biological control [53]. The level of this fungus virulence depends on environmental conditions, and biotic and abiotic factors can affect the effectiveness of their application. Therefore, it is necessary to consistently screen the local strains against pathogenic pests to increase the chances of obtaining effective microbial biopesticides.

In this study, in vitro experimental screenings were carried out to identify and select local *Metarhizium* spp.strains with several properties, namely, bioinsecticidal and biofungicidal activity. To cause mycosis or infectious disease of harmful insects in natural conditions, in addition to relevant abiotic factors, these fungi must have a sufficient dose of their conidia. Studies have shown that the most pathogenic fungal isolates produced spores on the corpses of insects with a minimum (3.2×10^6 spores ml–1) to a maximum (5.4×10^7 spores ml–1) concentration. Considered to be rapid sporulation, with a large amount of conidia on corpses, it can cause increased horizontal pest infestation due to rapid self-propagation [54]. These conidia can attach to insects' covering tissues in a high dose. Moreover, they secrete enzymes that dissolve

insects' cuticles when they stand on the cuticle cover. This study selected various compositions of cheap nutrient media to obtain a high titer of this entomopathogenic fungus, which provided the fungi with intensive conidia formation. According to studies, among *Metarhizium,* there are species with a narrow spectrum of activity; for example, *Metarhizium acridum* is a specific locust pathogen [55,56].

In our studies, the local strains have shown an entomopathogenic activity against sucking pests from various taxonomic groups, for example, against Aphis fabae (Insecta, Aphidoidea), Aspidiotus nerii (Insecta, Diaspididae) and Tetranychus urticae (Arachnida, Tetranychidae), which can be recommended as a universal biological control for the regulation of the number of the above economically important

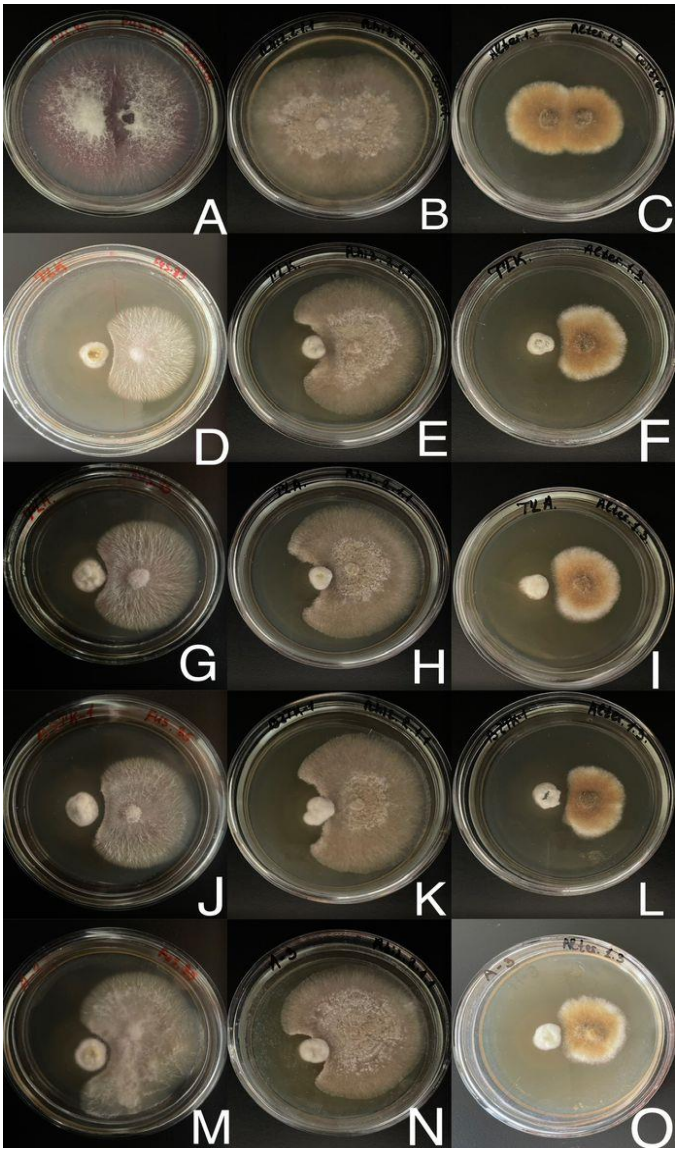

Fig. 11. Dual plate assays is showing an antagonistic activity of natural new *Metarhizium* spp.isolates. A- Control, *Fusarium oxysporum* (strain Fus.85). B- Control, *Rhizoctonia solani* (strain Rhiz.2.1.1). C- Control, *Alternaria* spp. (strain Alter.1.3.). D- Antagonistic activity of TLK-1 on Fus.85. E- Antagonistic activity of TLK-1 on Rhiz.2.1.1.; F- Antagonistic activity of TLK-1 on Alter.1.3. G. Antagonistic activity of TLA-2 on FUS.85. H. Antagonistic activity of TLA-2 on Rhiz.2.1.1. I. Antagonistic activity of TLA-2 on Alter.1.3. J. Antagonistic activity of BZTK-1 on Fus.85. K. Antagonistic activity of BZTK-1 on Rhiz.2.1.1. L. Antagonistic activity on Alter.1.3. M. Antagonistic activity of A-3 on Fus.85. N. Antagonistic activity of A-3 on Rhiz.2.1.1. O. Antagonistic activity of A-3 on Alter.1.3

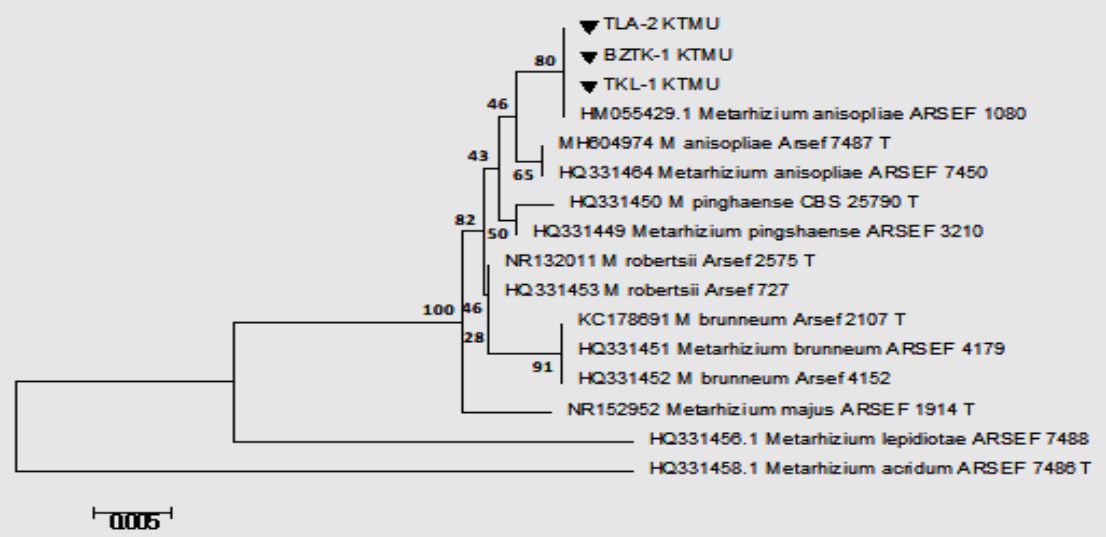

Fig. 12. Neighbors-joining phylogenetic tree based on 18SrRNA gene sequences showing the position of new local strains TLA-2KTMU, BZTK-1 KTMU and TKL-1 KTMU among other closely related *Metarhizium anisopliae* species from the database

greenhouse pests, as well as in open ground conditions (Fig. 7, Table 3). Other authors have also obtained data showing the efficacy of *Metarhizium* to flies (Insecta: Diptera: Tephritidae); for example, [57] have shown the effect of *Metarhizium anisopliae* inoculation even on the mating behaviours of three fruit fly species: Ceratitis cosyra, C. fasciventris and C. capitata. Mating duration was reduced in males treated with these fungi. Some research has shown sub-lethal and lethal effects of *M. anisopliae* on the cotton bug Oxycarenus hyalinipennis, showing that this entomopathogenic fungus can have an effect not only on the parent generation but on the next generation (F1) [58].

Most entomopathogenic fungi used as biocontrol agents require high humidity to germinate their spores. However, high humidity can also promote the growth of phytopathogens, causing rot, powdery mildew and others. Therefore, in recent years, research has focused on identifying entomopathogens that have dual effects on both pests and pathogens to resolve this issue.

The dual control potential of *Metarhizium anisopliae* SD3 isolate for pest and plant pathogens was reported [59,60]. This fungus has shown virulence against green peach aphids (Myzus persicae and antifungal activity against grey mould (Botrytis cinerea). The most virulence was observed in the simultaneous treatment with blastospores and cultural filtrate.

Our studies also confirmed that the studied local strains had shown insecticidal and biofungicidal activity against plant pathogens; vigorous activity was shown against the phytopathogenic fungus *Alternaria* and then against *Fusarium oxyporum.* At the same time, these entomopathogenic fungi do not show any activity *Rhizoctonia solani* (Fig. 10). Many researchers reported that *Metarhizium* species had a high virulence against such plant pathogens as *B. cinerea, Fusarium spp., Phytophthora megasperma, Podosphaera fuliginea, Pythium spp., Rhizoctonia solani, Sphaerotheca fuliginea and Verticillium dahliae* [59,61,62,63,64].

Our study is the first report of the dual activity of native entomopathogenic fungi against dangerous sucking pests from different taxonomic groups and against essential pathogens of many crop species such as *Fusarium oxyporum* and *Alternaria spp*. These new local strains can be recommended for bio-formulation development to protect plants from diseases and as bioinsecticides and bioacaricides against harmful mites and insects.

4. CONCLUSIONS

Biological control agents (BCAs) as an alternative to synthetic products remain the focus of researchers and several commercial ventures worldwide. These alternative products are already integrated into conventional growing methods, many of which are available to farmers.

Our results are significant as they point the way for other Integrated Pest Management (IPM) methods to be combined with these fungi to improve the effectiveness of insect control. We have identified and begun preliminary characterization of local isolates of *Metarhizium spp.,* which have the potential to be used in complex protection systems against various important pests, in particular suckers. The found isolates can be simultaneously used against widespread pathogenic fungi, essential for developing and producing inexpensive biological formulas based on these fungi. Optimizing cheap nutritional compositions for producing fungal biomass with a high titer of conidia opens up new opportunities for creating low-cost, high-efficiency biopreparations with a multifunctional spectrum of action.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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