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Entomopathogenic Fungal consortium of *Beauveria bassiana* and *Metarhizium anisopliae* as Biodegradation from Residue Insecticide Profenofos by *In Vitro* Studies

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Abstract

Entomopathogenic fungi are microorganisms that can control pests, especially *Beauveria bassiana* and *Metarhizium anisopliae*. Secondary metabolite compounds released by entomopathogenic fungi have biodegradation properties. The study aimed to assess the ability of a single or consortium of entomopathogenic fungi *B. bassiana* and *M. anisopliae*, in vitro. Biodegradation testing used 1 kg of suppressive soil in each box with 30 x 30 cm size. The soil was applied with a synthetic pesticide with the active ingredient Profenofos 500 g L^{-1}. The entomopathogenic fungal isolates used were collections from the Food and Horticultural Plant Pest Disease Observation Laboratory with isolate codes *B. bassiana* (B-Tg1) and *M. anisopliae* (M-Tg1). Bioassay testing using a petri dish, in the 48-hour phase, the highest mortality rate of *Spodoptera litura* in treatment A3 (*M. anisopliae*) was 70% with a concentration of 4.8 x 10^{10} spores mL^{-1}. The best treatment in the biodegradation test (A3) can reduce the initial residue concentration (R0) from 2.78 mg kg^{-1} to 0.39 mg kg^{-1} using Gas Chromatography/Mass Spectrometry (GC/MS). Treatments A2 and A4 in the treatment of *B. bassiana* and the consortium of the two fungi have the potential to reduce pesticide concentrations. This study concludes that entomopathogenic fungi can degrade insecticide residues. This fungal ability needs to be developed for the implementation of sustainable agriculture.

Keywords – Action – Bioassay – GC/MS – Metabolism – Pathogenicity

Introduction

Entomopathogenic fungi are a group of fungi that can infect insect pests (Ferreira & Soares 2023, Sam-on et al. 2024). These fungi are abundant in the soil, and some insects are infected. Entomopathogenic fungi have different modes of action and entry than other microorganisms. Generally, entomopathogens infect insects by contacting the cuticle layer (Mannino et al. 2019).

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When the fungus infects the cuticle surface, spores will enter and develop. In contrast to entomopathogenic viruses that have a way of entering through digestion, the target is the majority of larval stages (Habriantono et al. 2023). Spore development will take 24 days to form hyphae and break spores. Then, the spore mycelium will develop, and the infected insect will become mummified (Islam et al. 2021).

These entomopathogenic fungi have enzymatic activities with different levels of virulence (Erawati et al. 2021). *Beauveria bassiana* and *Metarhizium anisopliae* are heterotrophic fungi and are parasitic on host insects. Entomopathogenic fungi are effective in controlling several insect pests. The pathway of entomopathogenic fungi infects insects by infection of the cuticle layer; secondary metabolites enter the body, causing pH in the insect hemolymph, then clotting and stopping blood circulation throughout the body (Ding et al. 2023). Enzymatic activity and secondary metabolites produced by entomopathogenic fungi are reported to degrade pesticide residues (Swathy et al. 2024).

Pesticide residues are a problem found in the environment (soil, water, and air) and agricultural products. Most farmers still use synthetic pesticides to increase crop production. Long-term effects cause environmental contamination on biotic and abiotic indicators (Maurya & Malik 2016, Pelosi et al. 2021). Pests that are infected continuously will cause resistance and immunity to one of the active ingredients (Siddiqui et al. 2023). Pesticide residues that are contaminated with biotic and abiotic species take a long time to decompose. The intensive application causes the microbial population in the soil to decrease (Ataikiru & Ajuzieogu 2023, Mu et al. 2023). However, it has never been proven how the mechanism (mode of action) of enzymatic compounds from entomopathogenic fungi can be used for the biodegradation of pesticide residue compounds. The purpose of this study was to assess the Profenofos biodegradation ability of entomopathogenic fungi *B. bassiana* and *M. anisopliae in vitro*.

Materials & Methods

Preparation and rejuvenation of entomopathogenic fungal isolates

A suspension of 0.1 mL was taken using a syringe needle and placed in a petri dish containing PDA media aseptically. The suspension in the petri dish containing PDA media was levelled by rotating. The petri dish lid was then wrapped around the petri dish to prevent contamination from outside. Furthermore, pure isolates of entomopathogenic fungi were propagated in test tubes of PDA media as a starter for the propagation of entomopathogenic fungi with solid media. The entomopathogenic fungal isolates used in this study were obtained from the Food and Horticultural Plant Pest Disease Observation Laboratory located in Tanggul District, Jember Regency, Indonesia, with isolate codes *B. bassiana* (B-Tg1) and *M. anisopliae* (M-Tg1) (Fig. 1).

Viability and Spore Density Calculation

Spore viability is one indicator of the ability of entomopathogenic fungi to germinate. In this study, we used SDB (Saborroud Dextrose Broth) media for testing. Viability was determined by suspension with incubation for 24 hours (Banu & Rajalakshmi 2014). One drop of suspension was dripped on de glass and covered with cover glass. Counted the number of spores that germinate under a microscope with a magnification of 400 times. Determination of spore density was taken as much as 1 mL using a haemocytometer.

Bioassay testing

Each treatment uses 10 instar III larvae of *S. litura*. Prepare a rearing box containing 250 g of soil covered with gauze. The larvae were then inoculated with entomopathogenic fungi with a variety of graded concentrations. In this study created some of a difference treatment was created with Profenofos and consortium fungi. After treatment is covered with paper and saved in the storage.

SEM (Scanning Electron Microscopy)-EDX Analysis

Samples of entomopathogenic fungi were placed on de glass and covered with sterile cover glass. De-alcoholization was carried out using ethanol at levels of 30%, 50%, 70%, 80%, 90%, and 96% by spraying. After the sample was dehydrated, it was placed in the sample holder. Then, the SEM-EDX working distance was 10.5 mm with a voltage of 5.00 kV.

Biodegradation testing

An insect-rearing box containing 250 g of soil was applied intensively with a synthetic pesticide with the active ingredient Profenofos 500 g L^{-1}. Several treatments applied synthetic pesticides, and the application of fungal consortium *B. bassiana* and *M. anisopliae*. Then, the initial residue analysis (R0) and the final residue (Rt) were carried out. Residue analysis in soil was carried out with GC-MS.

Gas Chromatography/Mass Spectrometry (GC/MS) Analysis

5 g of soil sample was put into a 50 mL QuEChERS tube containing $MgSO_4$, 1 and Na-acetate, 10 mL of distilled water, and 15 mL of Acetonitrile + 1% acetic acid solution was shaken for 1 minute. The mixture was centrifuged for 15 minutes at 6000 rpm. Transfer 1 mL aliquot of the centrifuge results into a quencher dSPE tube containing $MgSO_4$, PSA, and C18, then centrifuge for 1 minute at 6000 rpm. Transfer 1 mL of eluate into a vial and add TPP as an internal standard. The standard solution was diluted to at least 5 concentrations to make a standard curve. Dilution using acetonitrile solvent. Each of these concentrations is then put in a vial, ready to be injected into the tool. Before analysis using GC-MS, the instrument must be conditioned with parameters such as carrier gas consisting of Helium, flow rate of 1.0 mL/minute, injection volume of 5 μL, and split injection mode.

Experimental Design and Data Analysis

The experiment was designed using CRD (Completely Randomized Design), with five treatments and six replicates (Table 1). Data on the percentage of *S. litura* mortality were analyzed by analysis of variance (ANOVA), followed by DMRT (Duncan's Multiple Range Test) at the 5% real level.

Table 1 Experimental design.

Code	Description
A0	Kontrol (Water)
A1	Profenofos 5%
A2	Profenofos 5% + B-Tg1 (10^8 spora L^{-1})
A3	Profenofos 5% + M-Tg1 (10^8 spora L^{-1})
A4	Profenofos 5% + B-Tg1 (10^8 spora L^{-1}) + M-Tg1 (10^8 spora L^{-1})

Results

Entomopathogenic Fungi

The entomopathogenic fungi used in this study were *B. bassiana* and *M. anisopliae*. In Fig. 1a, it can be seen that the *B. bassiana* colony is white and has a soft, cotton-like texture. The hyphae of *B. bassiana* are white and branched and have septa. Meanwhile, for the *M. anisopliae* (Fig. 1b), the fungal colonies are dark green in color, with a smooth texture on the petridish media. Conidia are smooth, cylindrical green in shape. It has a transparent and branched hyphae shape.

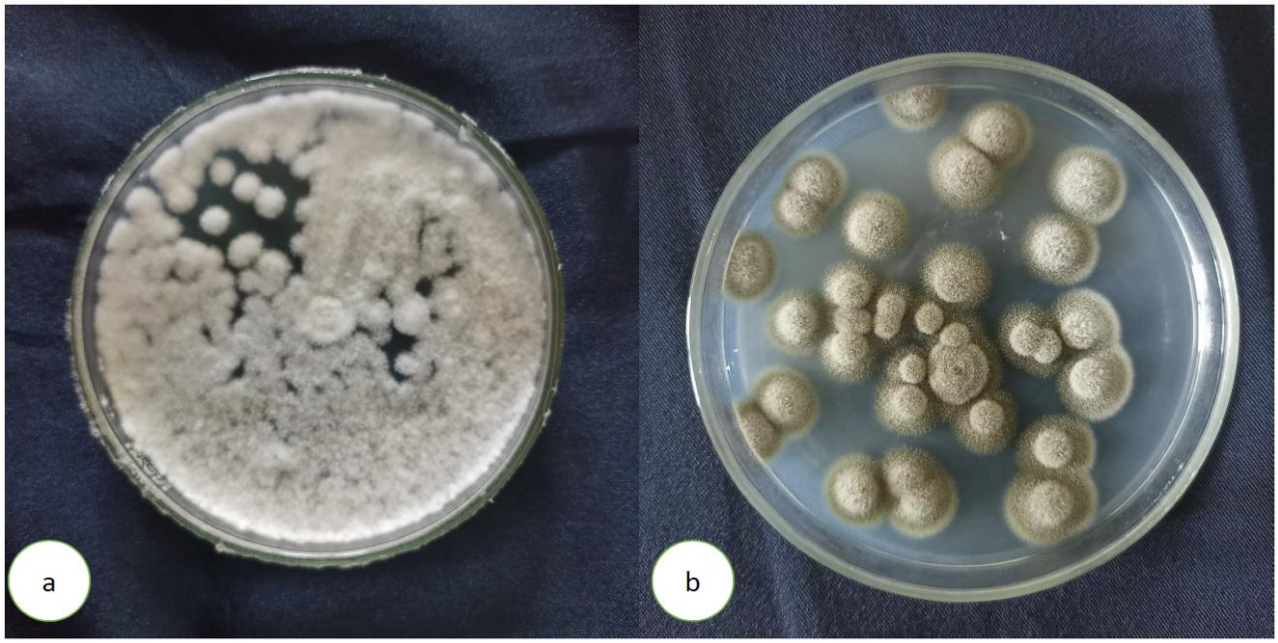


Fig. 1 – Isolates of entomopathogenic fungi from the exploration. Description: a) *B. bassiana*, and b) *M. anisopliae*.

Spore viability

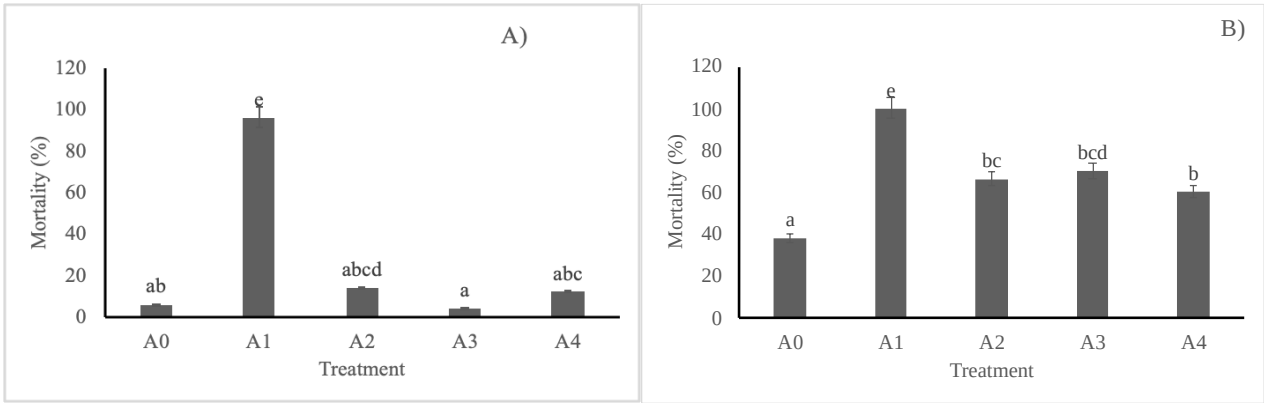
Viability is an indicator of the effectiveness of entomopathogenic fungiSpore viability is used to determine the ability to germinate and survive on a medium. In Table 2, each entomopathogenic fungus has different viability. In the sub-*in vitro* culture experiment, both fungi showed their existence at time intervals. *Beauveria bassiana* fungus at 24 hours viability reached 93%. At the same time, the *M. anisopliae* fungus at 24 hours viability reached 88%. There are several conditions for viability to decrease, such as nutrient content in sub-culture media, and the availability of carbon sources can reduce spore viability.

Table 2 Viability of entomopathogenic spores.

Types of entomopathogenic fungi	**Spore viability %**			
	4 hours	**8 hours**	**12 hours**	**24 hours**
B. Bassiana	14	39	57	93
M. anisopliae	12	13	52	88

Bioassay Test

Bioassay has a strong relationship with testing the effectiveness of entomopathogenic fungi. The purpose of this bioassay is to test the efficacy of entomopathogenic fungal candidates, namely *B. bassiana* and *M. anisopliae*. In Fig. 2a, Treatment A1 (Profenofos) had a mortality rate of almost 90% at 24 hours. At the same time, the A0 treatment had larvae that died due to the cannibal nature of *S. litura*, due to lack of food. The single and consortium treatments still did not show high mortality rates. In Fig. 2b, the mortality rate of *S. litura* as the test insect increased to 100%. This means that Profenofos, as one of the active ingredients of synthetic pesticides, can kill 10 larvae in 48 hours. The results of variance analysis using the Duncan Test can be significantly differentiated between treatments. Both single and consortium treatments were able to increase mortality above 75%. In the single treatment, *M. anisoplae* was higher than *B. bassiana*. In the control treatment, the mortality rate reached almost 40%. This is because during the test, there was starvation, so the events killed each other.

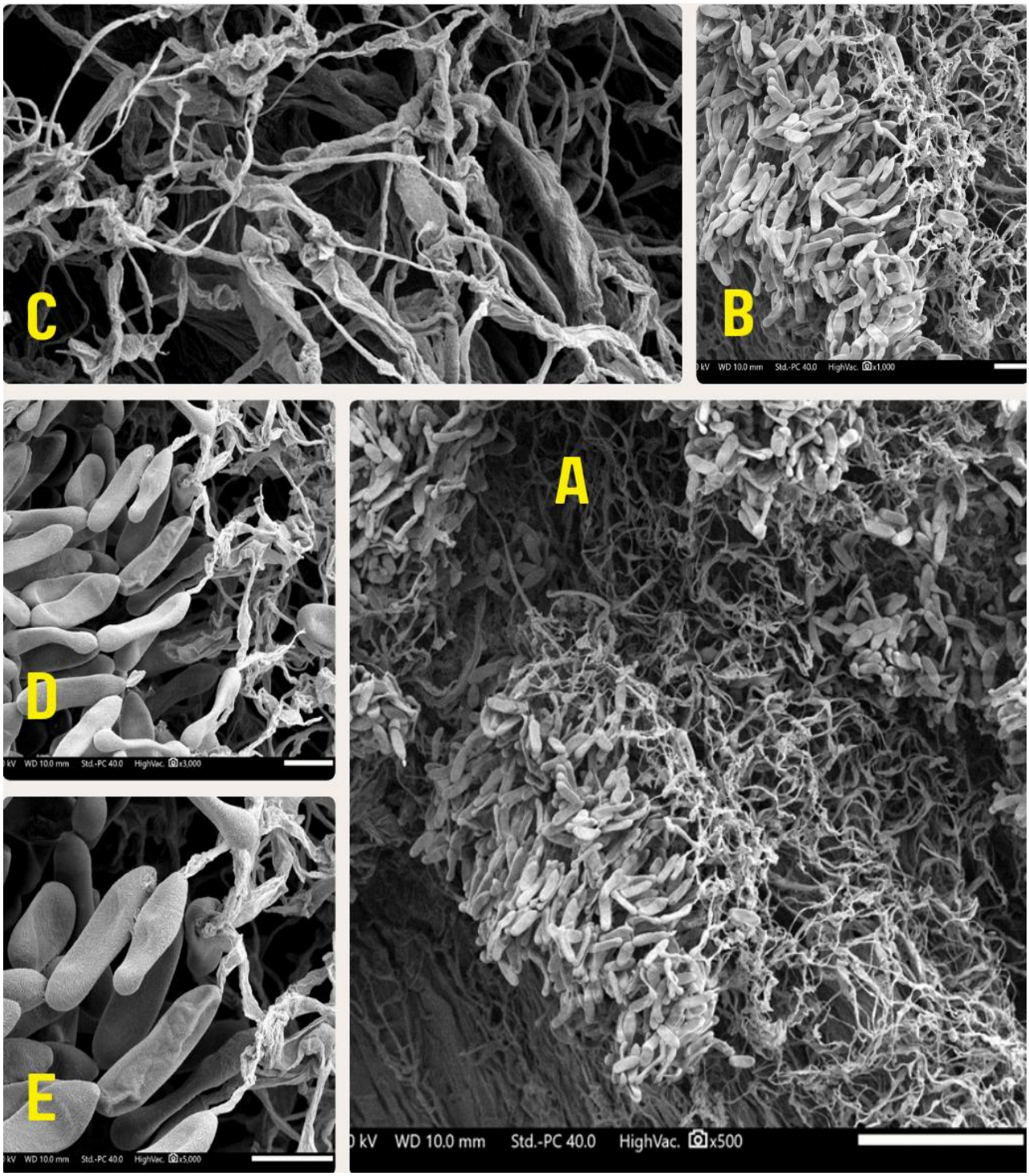


Figs 2 – Mortality of *S. litura* in various treatments. Notes: A Observation at 24 hours. B Observation at 48 hours.

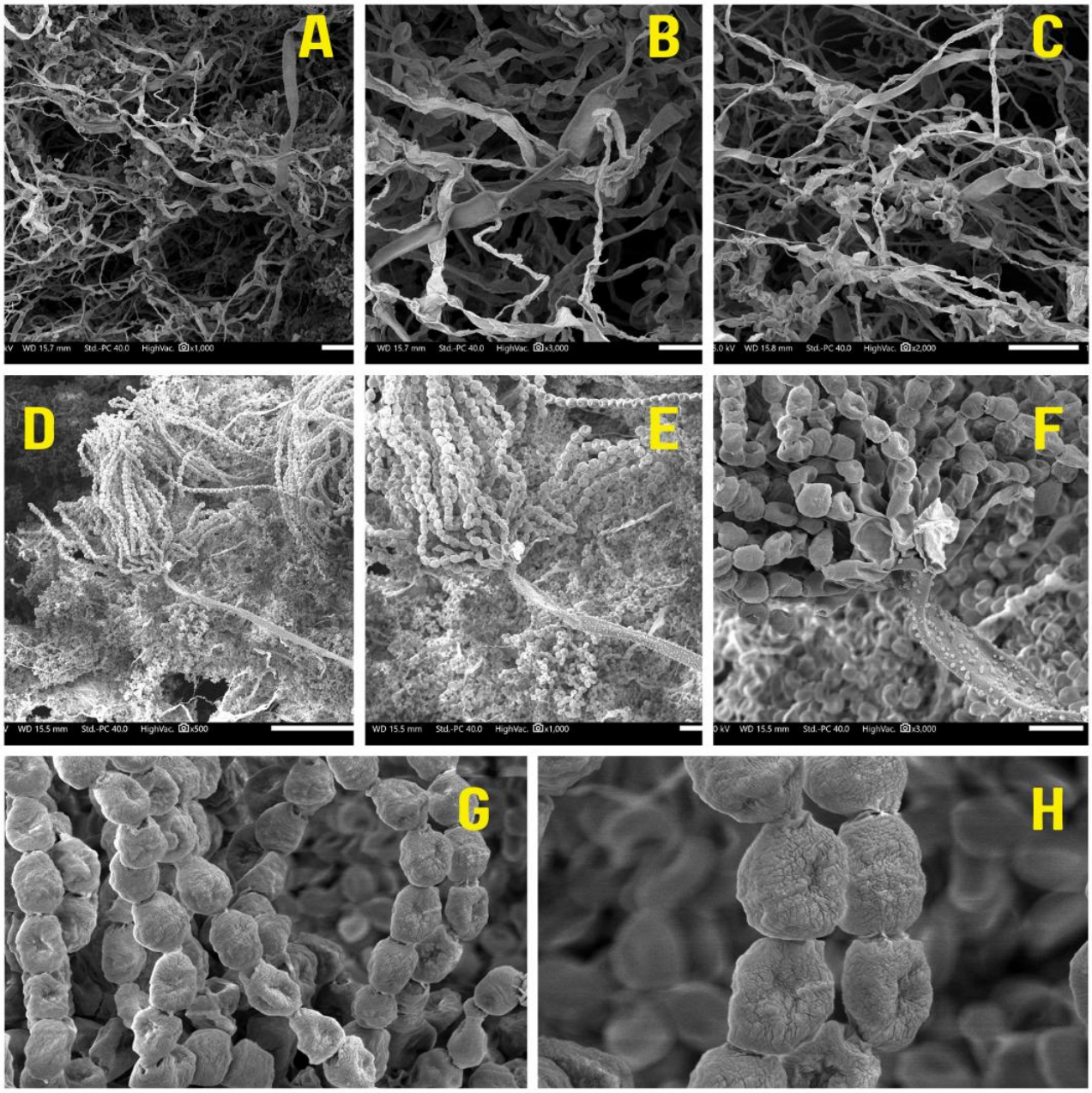
Figs 3 – Bioassay test of *T. molitor* in various treatments.

Scanning electron microscope analysis

SEM was used to identify the morphological characteristics and shape of *B. bassiana* (Figs 4, 5). The hyphal cells are clustered and form a single unit. Section C is part of the hyphae and mycelium. The fungal conidia in part E are round and slightly oval. Based on the reference (Humber 2012) on the denticles, there are fine scales on the surface. This is in accordance with the morphological characteristics in part E with 500X magnification. On the part of *M. anisopliae*, conidiophores are widely branched and colonized. The hyphae of *M. anisopliae* are flat and branched (parts A, B, C).

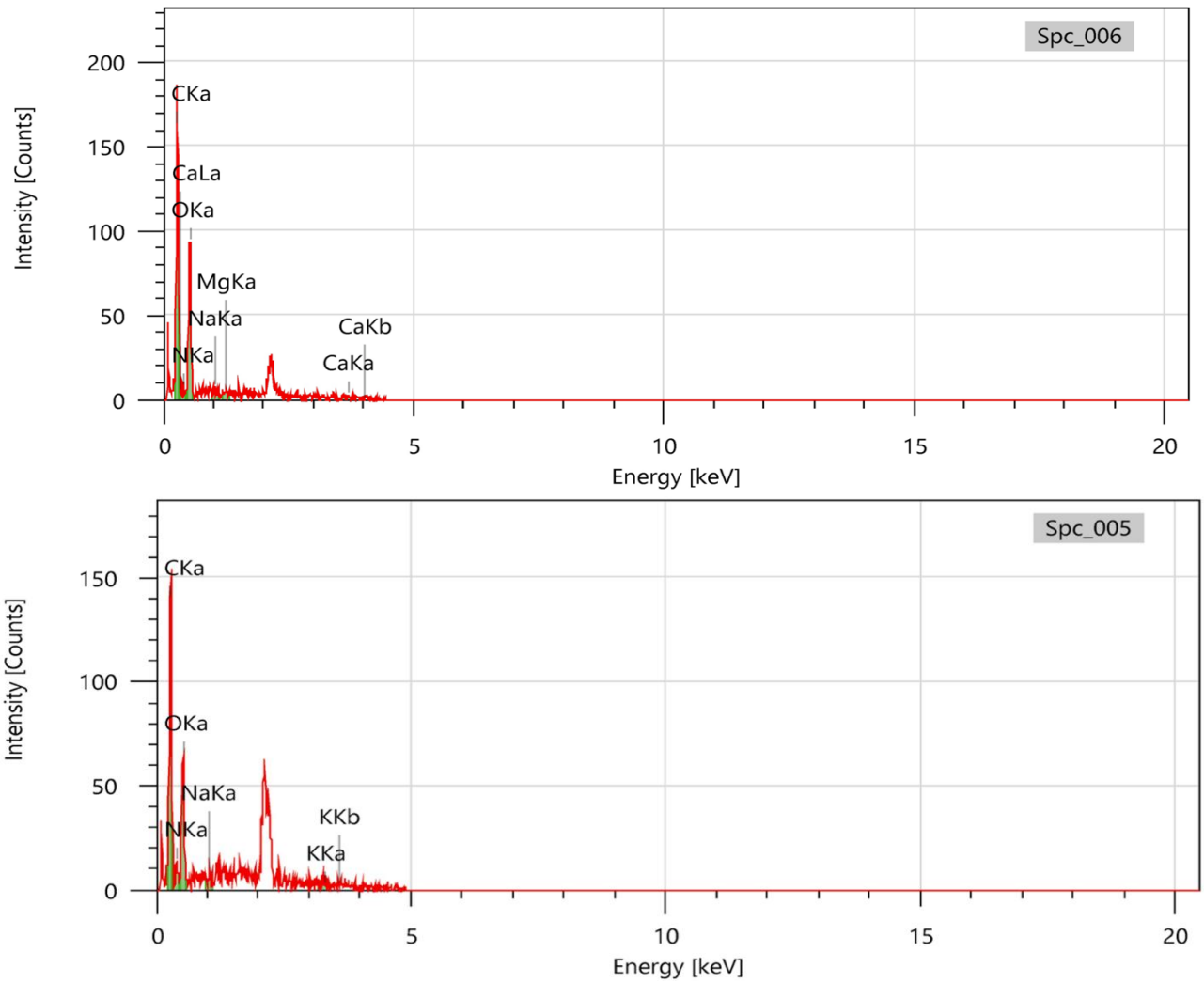


Figs 4 – *Beuvaria bassiana*. SEM analysis of *B. bassiana* A 500X magnification. B 1000X magnification. C Hyphae and mycelium with 2500X magnification. D Conidia with 3000X magnification. E Conidia with 5000X magnification.



Figs 5 – *Metarhizium anisopliae*. SEM analysis of *M. anisopliae*. A Hyphae and mycelium with 1000X magnification. B Hyphae and mycelium with 3000x magnification. C. Hyphae and mycelium with 2000X magnification. D Conidia with 500X magnification. E Conidia with 1000X magnification. F Conidia with 3000X magnification. G Conidia with 5000X magnification. H Conidia with 10000X magnification.

Fig. 6 is a graph showing the content material contained in entomopathogenic fungi. Based on the results of Energy Dispersive X-ray (EDX), the two fungi are dominated by elements C (carbon), O (oxygen), and K (potassium). This analysis aims to determine the composition of the chemical elements that make up the atomic structure, so that it has a relationship with electromagnetics. This EDX data can be used to measure and trace properties that can activate electrons. This EDX analysis (in Tables 3, 4). In the *B. bassiana*, the most dominant elements consist of C and O, with mass values of 50.01 ± 0.75% and 39.81 ± 1.51%. In contrast to the constituent elements in the *M. anisopliae*, it was found that the composition of the atomic structure consisted of C (carbon), O (oxygen), and K (potassium) with respective mass weights of 34.95 ± 0.58%, 28.60 ± 1.26%, and 23.92 ± 7.40%.



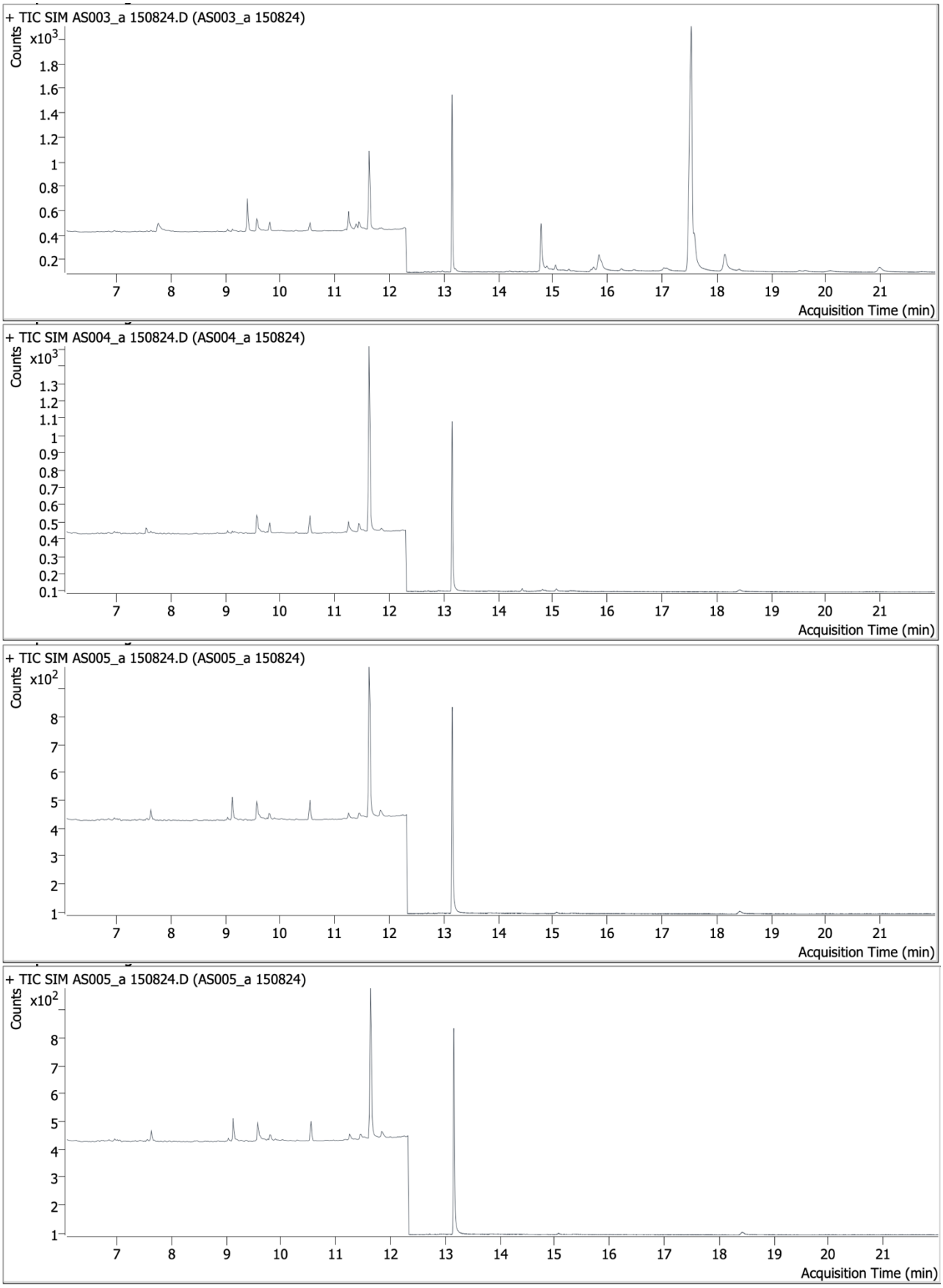
Figs 6 – Chemical intensity graph of entomopathogenic fungi.

Table 3 Mapping, structure, and content of elements in *B. bassiana*

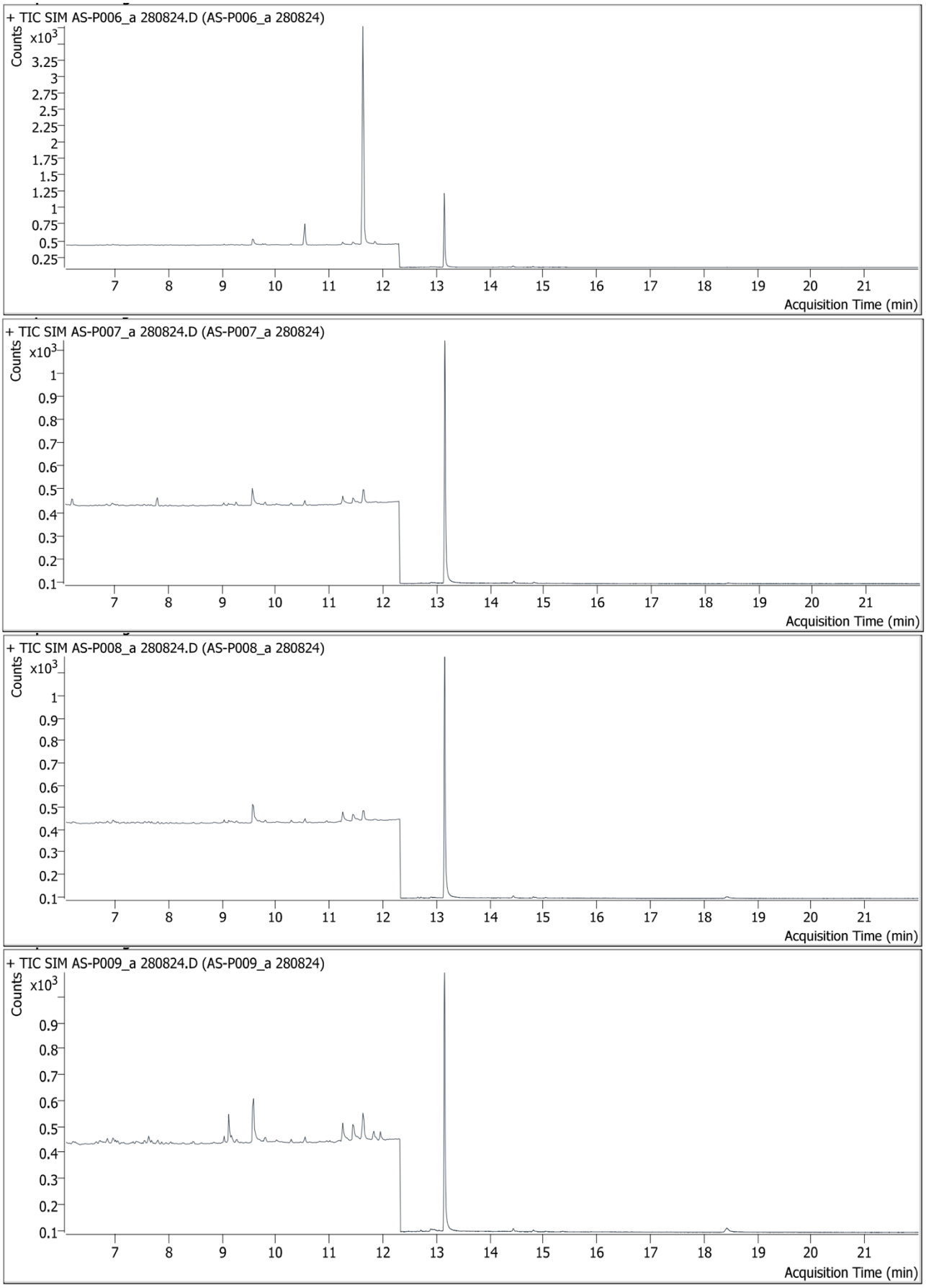
Element	Line	Mass%	Atom%
C	K	50.01 ± 0.75	57.78 ± 0.86
N	K	5.70 ± 0.90	5.65 ± 0.89
O	K	39.81 ± 1.51	34.53 ± 1.31
Na	K	1.14 ± 0.48	0.69 ± 0.29
Mg	K	0.90 ± 0.48	0.51 ± 0.27
Ca	K	2.44 ± 9.57	0.84 ± 3.31
Total		100	100

Table 4 Mapping, structure, and element content of *M. anisopliae*

Element	Line	Mass%	Atom%
C	K	34.95 ± 0.58	47.30 ± 0.78
N	K	10.70 ± 1.10	12.41 ± 1.28
O	K	28.60 ± 1.26	29.06 ± 1.28
Na	K	1.83 ± 0.51	1.29 ± 0.36
K	K	23.92 ± 7.40	9.94 ± 3.08
Total		100	100



Figs. 7 – Chromatogram of Profenofos at an early stage (R0). Notes: A1 (Profenofos), A2 (*B. bassiana*), A3 (*M. anisopliae*), and A4 (*B. bassiana* + *M. anisopliae*).



Figs. 8 – Chromatogram of Profenofos at an early stage (Rt). Notes: A1 (Profenofos). A2 (*B. bassiana*). A3 (*M. anisopliae*). A4 (*B. bassiana* + *M. anisopliae*).

Table 5 Quantification of biodegradation of Profenofos pesticide residues.

Treatment	Active ingredients	Test Method	Results		LOQ	Units
			R0	Rt		
A1	Profenofos	IKP. P-18 (GC-MS)	1.36	22.91	0.01	mg kg^{-1}
A2			1.12	0.59	0.01	mg kg^{-1}
A3			2.78	0.39	0.01	mg kg^{-1}
A4			1.79	1.60	0.01	mg kg^{-1}

Fig. 7 and 8 illustrate the detection results using GC-MS of the active ingredient Profenofos located at 11.6 minutes. All soil samples treated with Profenofos were detected on the chromatography column. The sample with code (R0) is the initial data as the standard of the biodegradation test. Bioassay applications were intensively conducted in treatments A1 to A4. However, A1 was periodically applied continuously until the end of the application. A2-A4 was only applied once for the active ingredient Profenofos. The rest were applied with entomopathogenic fungi, according to Table 1. Conversion results from the detection of the active ingredient Profenofos with GC-MS showed that the initial data (R0) showed the highest residual value in the soil was 2.78 mg kg^{-1} in treatment A3. The final data (Rt) had significant changes between the treatments. Unlike the A1 treatment because the application is carried out continuously so that the residue in the soil accumulates. The final value of the A1 treatment was 22.91 mg kg^{-1}. This contrasts with the A2, A3, and A4 treatments, where entomopathogenic fungi were applied. In Table 5, the final data treatment column decreased residue levels, which decreased the value of the column (Rt). In the A2 treatment (*B. bassiana*), the residue value dropped from 1.12 mg kg^{-1} to 0.59 mg kg^{-1}. The A3 treatment, from a value of 2.78 mg kg^{-1}, dropped to 0.39 mg kg^{-1}. Meanwhile, for the consortium, 1.79 mg kg^{-1} only decreased by 1.60 mg kg^{-1}. Based on these results, the consortium treatment does not have much more potential to degrade pesticide residues. However, the single treatment, in this case, the fungi *M. anisopliae*, has more potential to degrade pesticide residues in the soil.

Discussion

Entomopathogenic fungi have a different mode of entry from other microorganisms, which can penetrate the cuticle layer of insects (Ortiz-Urquiza & Keyhani 2013, Singh et al. 2017). Entomopathogenic fungi can penetrate and enter the host. The fungus has the speed of damaging the host cell so that it can enter and release enzymes as a destroyer. After successfully penetrating the cuticle, the spores can then develop almost completely, covering the target insect. Briefly, the stages for infection begin with spores that stick and enter the insect surface, and after a certain time, hyphae are formed to penetrate, the infection causes the insects to lyse and die, and colonization. On the other hand, this entomopathogenic fungus has volatile compounds known as several volatile organic compounds (VOCs) which attack the insect's immunity physiologically without direct contact (Rana et al. 2024).

In this study, the treatment was carried out with a consortium test between entomopathogenic fungi. Several studies have reported that there is synergism and compatibility between *B. bassiana* and *M. anisopliae* (Nawaz et al. 2022, Rice & Furlong 2023, Rodrigues et al. 2017). It has been reported to be effective in controlling several House Fly (*Diptera: Muscidae*) pests (White et al. 2021). The effect of the combined application of *B. bassiana* and *M. anisopliae* has also been reported for the development of Cucumber Mosaic Virus (CMV) disease in cucumber plants (Shaalan et al. 2022). Even in Australia, it has been reported that the compatibility between *B. bassiana* and *M. anisopliae* was tested on fungicides and insecticides. The test results are found in the concentration indicator on macadamia plants (Khun et al. 2020). In this study, bioassay testing (Fig. 3) produced information that there were significant results with a = 5% at 24 hours and 48 hours of observation. The highest mortality is certainly in the A1 treatment, namely with the active ingredient pesticide Profenofos. The mortality rate reached 100% at 48 hours. While in

single and combination treatments, the highest mortality rate reached 70%. In this study, the consortium and single fungal application were significantly different in terms of mortality rate. It needs to be proven in the consortium treatment of *B. bassiana* and *M. anisipliae* in terms of the speed of infecting *S. litura.*

Biodegradation is a process for overhauling harmful compounds or residues using beneficial microbes. The entomopathogenic fungi *B. bassiana* and *M. anisopliae* have been reported to have the potential to degrade pesticide residues. The results of research from Karaghool & Hashim (2021) entomopathogenic fungi *B. bassiana* can remove colouring compounds, namely Methyl Orange, as mycoremediation. The toxin possessed by *B. bassiana* is able to have biodegradation properties using enzymatic compounds (Wang et al. 2021). *Metarhizium anisopliae* has also been reported to degrade residual concentrations of the active ingredients Chlorpyrifos and Cypermethrin in soil (Ong et al. 2019). In this study, both single and consortia of *B. bassiana* and *M. anisopliae* can biodegrade insecticide residues in soil.

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