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Bioherbicidal Activity of Endophytic *Aspergillus niger* Crude Extract Against Major Weeds of Rice Crops for Sustainable Agriculture

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ABSTRACT
Rice (*Oryza sativa*) is one of the most stable foods in Nigeria, but efficient production is limited by weeds attack. This study assessed the bioherbicidal activity of endophytic *Aspergillus niger* extract against *Echinochiola crus-galli*, *Cyperus iria,* and *I. rugosum* weeds. A fungus was isolated from healthy *Vitellaria paradoxa* leaves and molecularly identified as *Aspergillus niger* (MIK1) strain with accession number AM270408.1 recorded in the NCBI GenBank. PDA Pure mycelial culture of the fungus was grown in 250mLErlenmeyer flasks containing 100mL PDB medium on a shaker at 120 rpm and 28°C for 12days.The broth culture was extracted using ethyl acetate and yielded 4.57g of crude extract. GC–MS analysis detected 26 compounds in the extract. Bioherbicidal activity at varying concentration of 0.125, 0.25, 0.5 1.0µg/mL indicated that the extract inhibited seed germination of *E. crus-galli* (17.4 %), shoot length (6.3%), and radicle length (9.4 %) at the highest concentration(1.0 µg/mL). Screen house test showed a reduction of shoot fresh and dry weights of both weeds with over 80 % at 1.0 µg/mL. ANOVA test showed that germinations, seedling growths, and biomass reduction of both weeds were not significantly different (p> 0.05). These findings reveal that endophytic *Aspergillus niger* crude extract from Nigeria *V.paradoxa* possessed bioactive compounds with bioherbicidal activity against the test weeds. Further investigations for practical applications in the field to manage other weeds is suggested for sustainable agriculture.

Keywords: Endophytic Fungus, Extract, Bioherbicidal Activity, GC-MS, Weeds

INTRODUCTION

Weeds remain the major challenges detrimental to crop production that can lead to low yields and quality. Their control to provide food security for the large population is important, but critical to the agricultural sector. Synthetic herbicides had increased food production over the years, but the excessive usage had been reported to cause environmental degradation, adverse effects to human health, the emergence of resistance among weeds, and high cost of production (Torretta *et al*., 2018). Losses caused by weeds to major field crops are economically more significant than other crop pests (Gharde *et al*. 2018). Therefore, there is a great need to develop a cost-effective, efficient, and sustainable method for weed management with minimum environmental impacts (Gnanavel 2015).

The study on endophytic fungi is increasing for their potential in biological control to improve crop production (Strobel 2019). Therefore, exploring endophytic fungi in this field has been reported to reduce environmental impact with low toxicity, and potential for sustainable agricultural practices compared to synthetic agrochemicals (Sharma *et al*. 2017). Endophytic fungi are microbes that reside within plant tissues without causing any apparent disease symptoms to the host plant (Zhao *et al.* 201). They have been reported to produce wide range of bioactive compounds that can inhibit the growth and development of weed species (Nia *et al*. 2017; Rukachaisirikul 2012). The compounds produced by these organisms interfere with essential physiological processes in weeds, such as photosynthesis, cell division, or nutrient uptake. Lahlali and Hijri. (2010) reported that the global market for potential inoculants from fungal endophytes applied as biocontrol accounts for about 10% annually.

Medicinal plant plays a very important role in the search for new strains of endophytic fungi as a result of the bioactive compounds they produce (Kaul *et al*. 2012; Kusari *et al*. 2013). *Vitellaria paradoxa* (Shea tree) belongs to the family *Sapotaceae,* and grows well in the savannah region of West Africa, including Nigeria, particularly the Northern region, which supports the livelihoods of rural communities. The various organs of the plant (leaves, bark, and fruits) are used in traditional medicine to treat ailments like arthritis, gastrointestinal disorders, skin infections, and respiratory disorders. The seeds are essentially used in the food, cosmetic and pharmaceutical industries for the manufacture of soap, body cream (Talla *et al*., 2016; Maanikuu and Peker, 2017). *Echinochiola crus-galli*, *Cyperus iria,* and *I. rugosum* are annual weeds of tropical and subtropical crops with an extensive world distribution. They have become cosmopolitan weeds distributed in a wide range of soils and climates. The weeds compete with rice crops for nutrients, water, sunlight and space, causing about 5% global yield losses (Tilley 2019). This study aimed to assess the bioherbicidal activity of endophytic *Aspergillus niger* extract from *V.paradoxa* against major weeds of rice crops for sustainable agriculture.

MATERIALS AND METHODS

Isolation of Endophytic Fungus

The endophytic fungus used in this study was isolated from leaves of *V. paradoxa* ((BUKHAN 0489). Healthy leaves were collected from Bagaji village (8.9256°N, 7 8200°E), Nasarawa State, Nigeria. Small pieces of the samples were air-dried in the laboratory at room temperature and ground into powder using an electric blender. The powdered dry

sample was washed in tap water and sterilized in ethanol (70%) for 2-3 min, followed by soaking in 1% hypochlorite for 2-3 min, and finally rinsed three times in deionized water (Bisht *et al*. 2016). 2.0g of disinfected powder sample was blotted dried on sterile filter paper and evenly placed on PDA plates, and incubated for 7 days at 28˚C. The grown fungal colonies were sub-cultured onto fresh PDA plates. The pure culture was maintained on PDA slant at -4°C and preserved in the Forest Biotech Laboratory, Department of Forest Management, Universiti Putra Malaysia, for further use (Hamzah *et al*. 2018).

Morphological Identification

The endophytic fungus was codified as MIK1 and morphologically identified based on characters such as colony growth on PDA, spores, and pigmentation using the taxonomic key of Watanabe (2002). Microscopic examination was performed using slides mounted with a small piece of pure mycelium using a light microscope (Nikon Eclipse E200 and a Nikon Digital Sight DS-L1 camera, Co., LTD, Tokyo, Japan) at 40X magnification (Rana *et al*., 2019).

Production of Fungal Crude Extract

Five pieces of fresh of fungal culture (MIK1) were inoculated in to a 250 mL Erlenmeyer flask containing 100 mL of PDB medium (PDB; HiMedia, India), and incubated in a shaker (SK-300) at 120 rpm for 14 days at 28℃ (Prihanto et al. 2011; Uzma *et al*. 2016). After harvest, fermented broth culture was extracted three times with ethyl acetate (1:1, v/v). Organic phases were washed with 2M brine, and evaporated using a rotary evaporator at 40°C to obtain the crude extract of the fungus (Padhi *et al*. 2015; Bhardwaj *et al*. 2015). The crude extract was stored in labeled, sterilized vial at -4ºC in the Department of Management Laboratory, Universiti Putra Malaysia for GC-MS analysis.

Preparation of Test Materials

Five (5 ml) of ethyl acetate extract was dissolved in 10% (v/v) dimethyl sulfoxide (DMSO) to make 100 mg/ml. The test solution was stored in autoclaved labeled vials at –4^{O}C, and preserved in the Department of Forest Management Laboratory, Faculty of Forestry, Universiti Putra. (UPM), Serdang, Malaysia, for subsequent bioassays.

***In vitro* Test of Herbicidal Activity**

Herbicidal activity of the extract from *A. niger* (MIK1) was screened against seed germination and seedling growth of *E. crus-galli*, *Cyperus iria* and *I. rugosum* weeds using a Petri dish assay (Sun *et al*. 2020). Twenty (20) surface-sterilized seeds for each weed were evenly placed on Petri dishes (90 x 15mm) lined with cotton wool wetted with 4 mL sterile water. Test solutions at concentrations of 0.125. 0.25. 0.5 and 1.0 µg/ml mixed with a drop of 0.01% Tween-20 (v/v) were applied to each plate (Verdeguer *et al*, 2009). The control plate contains DSMO and PDA only. Plates were sealed with parafilm and incubated for 7 days at 28°C (Alshallash 2018). The experiment was replicated three times per treatment. The number of germinated seeds were counted after two weeks post-treatment. Shoots and radicle lengths were measured and compared to controls. Germination inhibition (GI %) was calculated using the formula.

$$GI(\%) = Cg - Tg/Cg \times 100 \quad (1)$$

Where:

Cg = Germination rate in the controls, and Tg = Germination rate in the treatments

Test of Herbicidal Activity in Screenhouse

Another test was further performed to assess the herbicidal activity of the fungal crude extract against *E. crus-galli*, *C. iria,* and *I. rugosum* seeds using the pot experiment and biomass reduction methods. Twenty (20) healthy seeds of each of the test weeds after surface sterilization in ethanol (70%) for 3min, followed by sodium hypochlorite (1%) for 3 min and finally rinsed in distilled water 2-3 times. The emerged seedlings were placed into plastic pots (9cm) filled with 100g of autoclaved soil and irrigated regularly with distilled water (0.02% of Triton-X 100). After 14 days of the seedling leaf stage, concentrations (0.125, 0.25, 0.5, and 1.0 µg/mL) with a drop of Tween-20 (0.01%) were added to the plants using a small hand sprayer. Control pots receive distilled water and DMSO only. Each treatment was replicated three times (n = 3 × 4 = 12). After 7 days post-treatment, the plants were gently uprooted, shoots cut with a sterile blade and wrapped in foil paper, oven-dried at 60°C for 30 minutes. The dried shoots were weighed, and percentage biomass reduction (R %) was determined using the following formula (Fawole and Yahaya, 2017).

$$R(\%) = W2 - W1/W2 \times 100 \quad (2)$$

Where:

R (%) = Reduction percentage, W_2 = Shoot fresh weight, and W_1 = dry shoot weight

DNA Extraction and PCR Amplification

Ten (10g) of genomic DNA fungus (MIK1strain) was extracted by mini Kit (Favor PrepTM Fungi/Yeast DNA Genomic DNA Extraction), following the manufacturer's protocols (Gul *et al*., 2017). PCR was performed using Maxima Hot Start PCR Master Mix at initial denaturation of 95 ˚C for 2 min, 35 cycles of denaturation at 95 ˚C for 1 min, annealing at 55 ˚C, extension at 72 ˚C for 1 min, and a final extension of 72 ˚C for 5 min (Thermo; K1051). Amplification of the ITS region was performed with primers ITS1-F (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4-R (5′-TCC TCC GCT TAT TGA TAT GC-3′), as previously described (White *et al*., 1990)**.** The PCR product was visualized on 1% agarose gel, and amplicons were sequenced by First Base Laboratory Sdn Bhd, Selangor, Malaysia. The identification of the species was determined based on the best sequence alignment score. A phylogenetic tree was constructed based on ITS sequenced data using the neighbor-joining method in MEGA 7.0 software (Kumar *et al.,* 2018).

GC- MS Analysis

The crude extract was partially purified by thin-layer chromatography (TLC), and subjected to quantitative GC–MS analysis. The analysis was performed in Agilent GC-19091S fitted with a 42an MS 433UI L mass detector (Palo Alto, CA, USA). The instrument was set to an initial temperature of 70°C and maintained at this temperature for 2 min. The oven temperature then rose to 280ºC, at the rate of an increase of 5ºC/min, and maintained for 9 min. The injection port temperature was ensured as 260ºC and the Helium flow rate as 1 mL/min. The ionization voltage was 70 eV. Samples were injected in split mode as 10:1. Mass spectral scan range was set at 45–450 (m/z). Identification of bioactive compounds present in the extract was performed by comparing the mass spectra (MS) with library data from NIST14.L (National Institute of Standards and Technology, US) (Hashem *et al*., 2022). The analysis was carried out at the Institute of Bioscience (IBS), Universiti Putra, Serdang, Malaysia.

Data Analysis

Data were analyzed using **o**ne-way ANOVA runs in MS Excel (Version 23). Means were separated using SigmaSTAT version 3.5 software (SatSoft Inc. 2024) and Spearman Rank Order Correlation test at P=0.05. Graphs were generated in MS Excel (Microsoft Corporation, Redmond, WA, USA).

RESULTS AND DISCUSSION

Identification of Endophytic Fungus

The identification of the fungal isolate has been previously reported by Mohammed *et.al*., (2025).

Extract and GC-MS Analysis

The average yield of crude extract obtained from the endophytic *Aspergillus niger* strain MIK1 was 4.57g. GC-MS analysis detected 26 compounds in the crude extract (Table 1), and the spectral output of the compounds (Figure.1). 9-Octadecenoic acid (Z)-, methyl ester (RT = 26.5404, Area% = 34.4626) was the major compound, constituting for 34.5% of the total compounds, followed by Pentadecanoic acid, 14-methyl-, methyl ester (RT = 23.1058, Area% = 20.7885), with about 20.8%. Other compounds found in the extract include heptyl propyl carbonate (RT = 5.1518, Area% = 0.0121), undec-10-ynoic acid tetradecyl ester (RT = 38.3498, Area% = 0.0139), Cyclohexasiloxane, dodecamethyl-(RT=8.7784, Area% = 0.1725), and Hexasiloxane, tetradecamethyl- (RT = 26.359, Area% = 4.3665) as the minor compounds.

Table 1: Qualitative *GC-MS* Analysis of *Aspergillus niger* (MIK1) Crude Extract (Mohammed *et.al*., 2025)

PK#	RT	Peak Area%	Compound Name	Ref#
1	5.1518	0.0121	Heptyl propyl carbonate	66456
2	8.7784	0.1725	Cyclohexasiloxane, dodecamethyl-	254923
3	12.956	0.7644	3,5-Dibutoxy-1,1,1,7,7,7-hexamethyl-3,5-bis(trimethylsiloxy)tetrasiloxane	272089
4	14.5563	7.0258	Phenol, 3,5-bis(1,1-dimethylethyl)-	70657
5	16.0311	2.8034	Diethyl Phthalate	84999
6	16.9082	2.3248	Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-	266854
7	17.3299	1.2502	2H-Pyran, 4-bromo-3,4-dihydro-	209406
8	18.7396	0.2661	Tetradecanoic acid, 12-methyl-, methyl ester, (S)-	117491
9	20.3816	2.9922	Malonic acid, bis(2-trimethylsilylethyl ester	162858
10	22.282	0.1996	1-Benzylbenzimidazole 3-oxide	87534
11	22.3419	0.208	Benzeneacetaldehyde	9551
12	23.1058	20.7885	Pentadecanoic acid, 14-methyl-, methyl ester	130841
13	23.4853	3.5132	2-(2',4',4',6',6',8',8'-Heptamethyltetrasiloxan-2'-yloxy)-2,4,4,6,6,8,8,10,10-nonamethylcyclopentasiloxane	274379
14	26.359	4.3665	Hexasiloxane, tetradecamethyl-	258609
15	26.5404	34.4626	9-Octadecenoic acid (Z)-, methyl ester	155751
16	27.0143	2.0618	Heptadecanoic acid, 16-methyl-, methyl ester	157955
17	28.95	2.2783	Hexasiloxane, tetradecamethyl-	258609
18	31.3573	2.3649	3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane	272163
19	33.6207	2.6309	Trisiloxane, 1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl)oxy]-	229156
20	34.3191	0.7025	Didodecyl phthalate	266710
21	35.7485	2.4376	Mercaptoacetic acid, 2TMS derivative	98864
22	37.2473	0.1789	Docosyl isobutyl ether	228716
23	37.7188	2.7501	Mandelic acid, 2TBDMS derivative	227090
24	38.0342	1.6981	n-Propyl heptyl ether	30655
25	38.0931	1.7575	2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-	85750
26	38.3498	S0.0139	Undec-10-ynoic acid, tetradecyl ester	226256

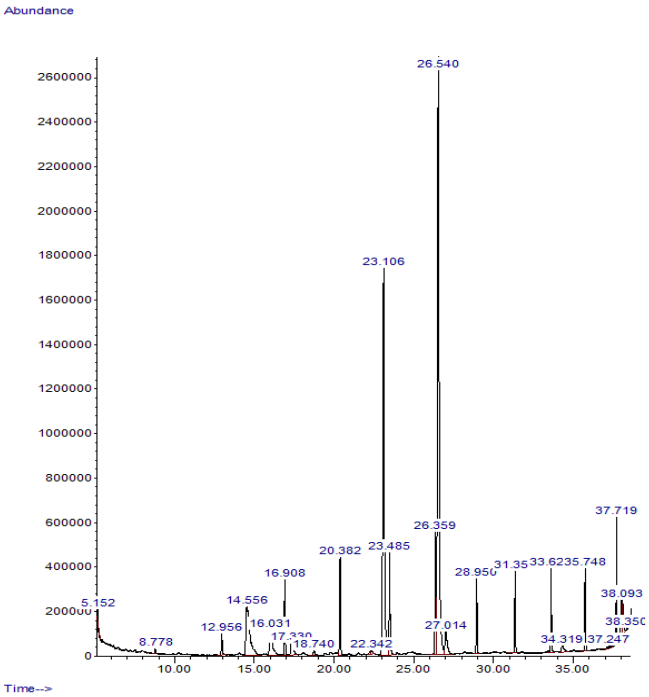

Figure 1: GC-MS chromatogram of *Aspergillus niger* (MIK1) Crude Extract

***In vitro* Herbicidal Activity of Extract**

Growth inhibition of *A. niger* (AM270408.1) extract against germination and seedling growth of *E. crusgalli* weed in the Petri dish assay is shown in Figure. 2. The results indicate that all the concentrations exhibited various levels of inhibition against seed germination, shoot, and radicle lengths of *E. crusgalli.* At concentrations 0.125µg/mL, the extract exhibited 19.4% reduction against germination of *E. crusgalli,* shoot length (20%), and radicle growth (17.2%). The inhibitory effect of the extract at concentration 0.25 µg/mL showed 15.4% reduction against germination of *E. crusgalli*\, shoot length (11.2%), and radicle elongation (10.4%). At 0.5 µg/mL, the extract inhibition value was 15.3% on the germination, while inhibition rates of 13% and 9.4% were observed against shoot and root lengths of *E. crusgalli,* respectively. The overall results showed that ethyl crude extract had demonstrated moderate inhibitions against *E. crusgalli* compared to the control. Therefore, germination rates, shoot, and root lengths differed significantly at $p < 0.05$.

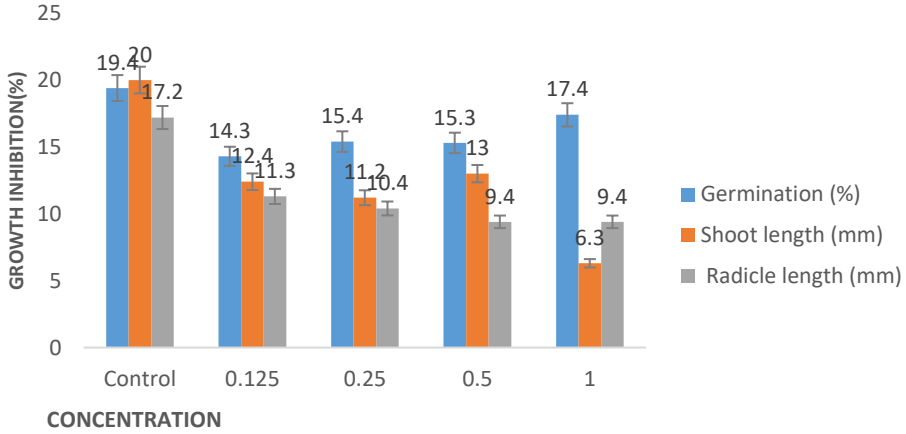

Figure 2: *In vitro* Inhibitory Activity of *A. niger* (MIK1) Crude Extract Against *E. crus-galli*

Herbicidal Activity of Extract in Screenhouse

The inhibitory effect of *A. niger* crude extract against the shoot fresh and dry weights of weed species in the screenhouse is shown in Figure.3. The results revealed that all the treatments caused a reduction in biomass of the tested weeds. At concentration 0.125µg/ml, *E. crus-galli* shoot biomass was reduced by 28.4% and 25.8%, *C. iria* (48.7% and 47.9%), *I. rugosum* (65.8% and 8.8%). At 0.25 µg/ml, the fresh and dry weights of *E. crus-galli* were inhibited by 28.8% and 29.7%, *C. iria* (72.1% and 56.4%), *I. rugosum* (0.7% and 52.1%). At 0.5 µg/ml, the biomass reduction was 39.2% and 31.5% against *E. crus-galli, C. iria* (81.5% and 57.5%), and *I. rugosum* (82.4% and 54.2%). At 1.0 µg/ml, the inhibition rates 81.8% and 51.2% were recorded against *E. crus-galli, C. iria* (82% and 59.1%), and *I. rugosum* (14.7% and 38.6%). ANOVA test shows that the extract had no significant effect on the shoot fresh and dry weights, biomass of the weeds tested at p>0.05.

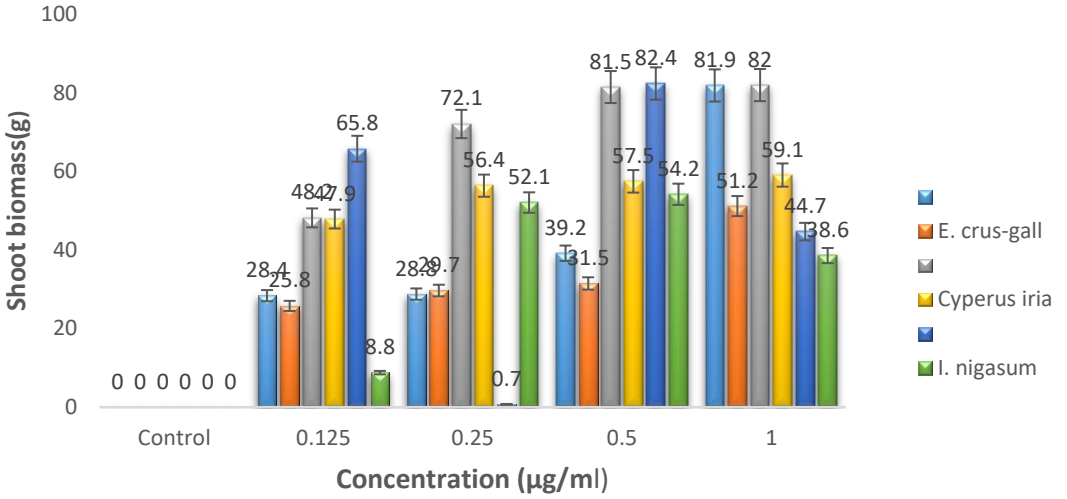

Figure 3: Inhibitory Activity of *A. niger* (MIK1) Crude Extract Against Shoot Fresh and Dry Weights of Three Weed Species in a Screen house

Discussion

In this study, the herbicidal activity of the extract from *A. niger* strain MIK1 was tested against three weeds in rice crops. The extract inhibited the growth of seed germination and seedling growth of both weeds at various concentrations. This corroborates with Marcias-Rubalcava *et al.,* (2014) work that an endophytic fungus, *Edeniagomez pompae* isolated from *Callicarpa acuminata* exhibited inhibitory activity against seed germination and root growth of *E. crus-galli in vitro.* Ahmad *et al*. (2020) reported that *Aspergillus spp* extracts reduced seed germination of *Chenopodium album, Avena fatua,* and *Convolvulus arvensis* at 65% rate. The inhibitory activities exhibited by the extract against the test weeds, both in laboratory and screen house experiments, signify their potential as a bio control agent. The herbicidal activity might be due to the effects on chlorophyll content, cellular respiration, regulation of shoot elongation, and cell division on both weeds (Kaur *et al*., 2010).

GC-MS analysis of the extract revealed the presence of different bioactive compounds mostly related to fatty acids, particularly acid esters. Similar compounds were extracted from the endophytic fungi residing in plants (Sharaf *et al*., 2021; Kaul *et al*., 2012), and showed different biological activity (Ganesh *et al*., 2017).

The molecular analysis of the fungus MIK1 through Internal Transcribed Spacer (ITS) region sequencing showed that it was *Aspergillus niger* with 98.50% similarity with the deposited sequences in NCBI GenBank via the BLAST program. Phylogenetic analysis using the Neighbor-Joining method showed that *A. niger* formed a distinct clade with a close match as the recorded sequence in the GenBank. Moreover, the top hit for *Aspergillus niger* contig An18c0160 with an E-value of 2e-127 id highly significant. The query covers (e.g., 99%, 97%) indicates that most of the sequence was matched to the reference sequences, showing high similarity (Figure 2). Percent identities are quite high, with some near or at 100%, indicating very close genetic relationships between the query and these sequences.

CONCLUSION

This study assessed the herbicidal activity of crude extract from *Aspergillus niger* (MIK1) isolated from powder dry leaves of *V. paradoxa* against three weeds in rice crops. GC–MS detected 26 main bioactive compounds in the fungal extract with varying degrees of inhibition against seed germination and seedling length of the tested weeds, both *in vitro* and screen house**,** indicating their herbicidal activity. Therefore, this could serve as a new novel alternative method other than synthetic chemicals for the management of tested weeds. Further research is suggested for practical applications in the field to control other weeds for sustainable agriculture.

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