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Identification of entomopathogenic fungi *Metarhizium anisopliae* and *Purpureocillium lilacinum* from oil palm plantation soils in Universiti Putra Malaysia

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ABSTRACT

Aims: Insect pests are one of the major constraints of oil palm production in Malaysia. However, synthetic chemical insecticides are the most common method for its control, despite their negative effects on non-target organisms and the development of resistance. Therefore, the present study is aimed to identify novel indigenous isolates of entomopathogenic fungi (EPF) in oil palm soil as part of integrated pest management (IPM) of oil palm insect pests.
Methodology and results: The potential of EPF were isolated from the soil collected from the oil palm plantation in UPM using a mealworm beetle larva (*Tenebrio molitor*) as an insect bait. Seven *Metarhizium anisopliae* and two *Purpureocillium lilacinum* isolates were identified by morphological characterization (macroscopic and microscopic observation) and molecular identification using internal transcribed spacer (ITS) region (ITS region amplification).
Conclusion, significance and impact of study: To our knowledge, this is the first time *P. lilacinum* has been found in the soil of an oil palm plantation or any other host in Malaysia. Furthermore, both of the isolates *M. anisopliae* and *P. lilacinum* may be potentially considered as biological control candidates for major insect pests in oil palm.

Keywords: Entomopathogenic fungi, insect baits, oil palm, soil, UPM

INTRODUCTION

Oil palm (*Elaeis guineensis* Jacquin) is an important economic tree that produces fruit all year and has an average economic life span of 25 to 30 years (Barcelos *et al.*, 2015). Malaysia and Indonesia are represent the two largest palm-producing countries, had a total of 4 and 5.3 million hectares grown by oil palm respectively (Boafo *et al.*, 2015; Jamian *et al.*, 2017; Ritchie and Roser, 2021). Together, these two countries produce 85% of all palm oil produced in the world (Ferdous Alam *et al.*, 2015; Ritchie and Roser, 2021). The oil palm is mainly planted in mineral soil in Malaysia, but it can also grow in peat soil in some areas (Kin *et al.*, 2017a).

Soil is a natural entomopathogenic fungi (EPF) habitat and most of the arthropods' threats to oil palm spend at least part of their developmental stage in the soil of oil palm plantations (Cheng *et al.*, 2008; Manjeri *et al.*, 2014; Kamarudin *et al.*, 2019). EPF are recognized to be natural enemies present in the insect population and have long been regarded as excellent microbial control agents in integrated pest management (IPM) system (Roy *et al.*, 2010; Jiang *et al.*, 2020; Halim *et al.*, 2021). Most species of EPF have been known to be isolated from soil, and infected insects or insect cadavers and grown in many artificial media (Rios-Velasco *et al.*, 2014; Abdullah *et al.*, 2015; Ibrahim *et al.*, 2016). It has been reported that native isolates or EPF strains from various hosts provide an open and unique control strategy to control several native pests because EPF are more suited to the environment (Sayed *et al.*, 2018).

In Malaysia, the EPF isolated from insect cadavers in oil palm soil has been extensively studied and explored, but only a few genera, such as *Beauveria*, *Metarhizium*

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Table 1: Coordinates and soil samples locations/geographical distribution of the fungal isolates.

S/No	Isolates	Location	Longitude	Latitude
1	Ma-01	Oil palm block A	2°58'47.0" N	101°43'44.41" E
2	Ma-02	Oil palm block A	2°58'51.99" N	101°43'41.89" E
3	Ma-03	Oil palm block A	2°58'44.50" N	101°43'42.09" E
4	Ma-04	Oil palm block B	2°59'6.86" N	101°43'42.96" E
5	Ma-05	Oil palm block C	2°59'18.90" N	101°43'28.07" E
6	Ma-09	Oil palm block B	2°58'57.47" N	101°43'17.93" E
7	Ma-15	Oil palm block B	2°59'1.74" N	101°43'17.67" E
8	Pl-01	Oil palm block A	2°58'47.0" N	101°43'44.41" E
9	Pl-05	Oil palm block C	2°59'18.90" N	101°43'28.07" E

and *Isaria* (formally known as *Paecilomyces* spp.) have been isolated and identified (Ramlah *et al.*, 1994; Moslim *et al.*, 1999; Bakeri *et al.*, 2009; Tajuddin *et al.*, 2010). There is very little information can be found on the discovery of indigenous EPF isolates from the soil of oil palm and their potentials as biological control agents to suppress major oil palm pests such as bagworm, rhinoceros beetles, bunch moths, nettle caterpillars and termites (Bakeri *et al.*, 2009; Lin *et al.*, 2017).

Identification of new isolates and species of EPF such as *Metarhizium anisopliae* and *Purpureocillium lilacinum* from the soil to control of these insect pests is one of the good steps towards attaining the target of producing sustainable biopesticides for pests and disease in the oil palm industry (OPI). *Metarhizium anisopliae* is one of the most important commercialized EPF and has long been used to control several oil palm insect pests in Malaysia and many other countries (Moslim *et al.*, 1999; Boafo *et al.*, 2015; Kin *et al.*, 2017b). *Purpureocillium* is a new divergent genus that is classified from *Paecilomyces lilacinus* and was established in 2011 by Luangsa-Ard *et al.* (2011). It is known to cause an epizootic on several insect pests and is also used as a biological control agent of certain fungal and nematodes phytopathogens (Baidoo *et al.*, 2017). Additionally, *P. lilacinum* has been found to colonize plants and grow endophytically, protecting the plant from pests and diseases and promoting plant growth (Deng *et al.*, 2012; Vega, 2018; Baron *et al.*, 2020).

Here, we surveyed UPM oil palm plantations to identify isolated EPF species using a combination of morphological and molecular techniques to find a native fungal biocontrol agent that could be used against oil palm insect pests. The present study is aimed to identify novel indigenous isolates of EPF from oil palm soil as part of integrated pest management of oil palm insect pests.

MATERIALS AND METHODS

Sampling sites and soil samples collection

Soil samples were collected from three different locations (oil palm block A, B and C) of UPM Serdang campus oil palm plantations in August 2020. At each location, soil samples were taken within 50-100 m of each other. A total of 30 soil samples (10 from each location) were collected and a total of 1 kg of soil was collected from the depth of 0-20 cm with the stainless-steel soil auger after removing litter and weeds on the sampling point and placed in appropriately labelled plastic bags. The global position of each sampling unit was recorded using Global Positioning System (GPS) (Table 1). The soil samples were then delivered to the Insect Biocontrol Laboratory, Faculty of Agriculture, UPM and allowed to air dry for 72 h before proceeding to the fungal isolation process.

Isolation of entomopathogenic fungi from soil samples

The wax moth, *Galleria mellonella* and mealworm, *Tenebrio molitor* beetle larvae *Tenebrio molitor* have been widely used as bait insects to isolate EPF from soils (Hernández-Domínguez *et al.*, 2016; Sharma *et al.*, 2018). In this study, a mealworm beetle, *T. molitor* was used as bait insects as described by Elham *et al.* (2018) with slight modification. The beetle larvae reared for culture collection at Insect Biocontrol Laboratory, Faculty of Agriculture, UPM were used in this study. Before baiting, about 300 larvae were placed in a 500 mL beaker and incubated at 65 °C in a water bath for 30 sec. From each soil sample, about 100 g were taken and transferred into a 200 mL transparent plastic container. Thereafter, five to ten last instars of larvae were carefully placed into the container with forceps and each sample was replicated five times. The cover of each container was sprayed with distilled water before closed. Mortality caused by EPF was checked daily and the dead larvae caused by bacteria or nematodes were removed and replaced with the new larvae in each container. On each date of observation, the cover of the container was maintained wet by spraying distilled water with spray bottle.

Dead larvae infected by EPF, covered with white, green and pink hypha around the integument were surfaces sterilized with 70% ethanol for 5 min, rinse 5 times with sterile distilled water and placed in a Potato Dextrose Agar (PDA) medium amended with antibiotic (100 mg of each streptomycin and tetracycline) to permit fungal growth. The plates were then incubated at 25 ± 2 °C and the presence or absence of EPF on the plates was observed daily for 10 days.

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Table 2: GenBank sequences data of fungal isolated.

Isolates	Species	GenBank Accession	Strain No.	Accession No.	Host	Location	Similarities (%)
Ma-1B	*M. anisopliae*	MW857162	FRIM885	MG020752.1	Soil	Malaysia	100
Ma-2B	*M. anisopliae*	MW857163	TCD 008	KX451122.1	Soil	Brazil	99.7
Ma-3E	*M. anisopliae*	MW857164	MaGX7002	MH483904.1	Soil	China	100
Ma-4G	*M. anisopliae*	MW857165	VKMA007	MH165400.1	Mosquito	India	100
Ma-5i	*M. anisopliae*	MW857166	MC 7	MN538360	Soil	India	100
Ma-9a	*M. anisopliae*	MW857167	LRC 148	EU307893.1	Peanut scarab	Australia	100
Ma-15	*M. anisopliae*	MW857168	MaGX1701	MH483858.1	Soil	China	100
Pl-01	*P. lelacinum*	MW857171	Plil_D92	MN808335.1	Soil	Portugal	100
Pl-05	*P. lelacinum*	MW857172	NRRL22958	GU980033.1	/	Thailand	100

Morphological identification of isolated entomopathogenic fungi

Pure cultures of the observed fungi were prepared on microscope glass slides. A sterile needle was used to pick a strand of mycelia and placed onto a single dropped of sterile distilled water and/or lactophenol blue mounted on a clean glass slide. Micro-morphological features were studied using a light microscope (Olympus CX31 series, England) and important morphological features were captured by microscope Dino-eye-piece camera (magnification 1000×). The macroscopic and microscopic characteristics of the isolated fungi were examined using references text from Watanabe (2010) and Humber (2012).

Molecular identification of isolated entomopathogenic fungi (EPF)

Direct PCR amplification was conducted according to the manufacturer's protocols for the KOD FX Neo PCR master mix (Toyobo Co. Ltd., Japan). Mycelium of 7 days fungal cultures were scraped and suspended in 100 µL TE buffer and vortexed for 30 sec. An approximately 2 µL mycelium suspension from each sample was mixed with other PCR mixture, which consist of 12.5 µL of 2× PCR buffer, 5 µL dNTP mixture, 1.5 µL of each forward and reverse primers, 0.5 µL of Kod FX Neo, primer pair of ITS1 (forward primer) (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (reverse primer) (5'-TCCTCCGCTTATTGATATGC-3') and 3 µL sterile water were used for ITS region identification (White *et al.*, 1990).

PCR was performed using Thermocycler Biometra (T-Persona, Germany). The PCR mixtures were amplified with following conditions: an initial denaturing temperature of 94 °C for 4 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 53.7 °C for 1 min and extension at 72 °C for 2 min, and a final elongation of 72 °C for 10 min. The PCR products were run on a 1.5% agarose gel in 1× TEA buffer for 30 min at 90 V and the bands were visualized under UV light. After that, sequencing was outsourced to the Apical Scientific Sdn. Bhd. Malaysia. DNA sequences were compared to other sequences available in the database from National Center for Biotechnological Information (NCBI) using the Basic Local Alignment Search Tool (BLAST). Phylogenetic trees for sequences of ITS region (ITS1/ITS4 amplification) were constructed by the maximum likelihood method with MEGA software version 7 (Figure 4) (Kumar *et al.*, 2016).

RESULTS

Isolation of entomopathogenic fungi

A total of 9 isolates of EPF were recovered from 8 out of the 30 soil samples collected from oil palm plantations (Table 2). At 14-21 days after insect baiting, several larvae from soil samples were infected with EPF. As can be seen from Figure 1A, the infection of larvae with both *M. anisopliae* and *P. lilacinum* started with the formation of a white hypha around the integument in the first 10 days after insect baiting. The entire body of larvae infected with *M. anisopliae* was covered with a mass of green spores during 10 to 20 days (Figure 1C). Infection of larvae by *P. lilacinum* was also at first start with the formation of white hyphae and progressed to the formation of smoky pink (Figures 1A and 1B).

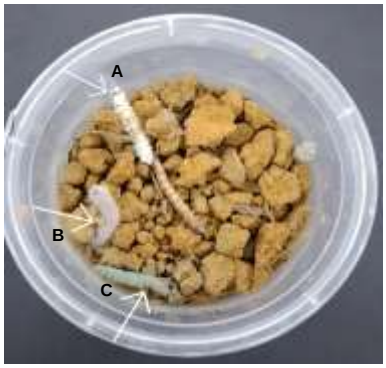

Figure 1: (A) Larvae infected by *M. anisopliae* and *P. lilacinum* started with white infection; (B) Larvae infected by *P. lilacinum* with a pink spore found on the entire body; (C) Larvae infected by *M. anisopliae* with a green spore found on the entire body.

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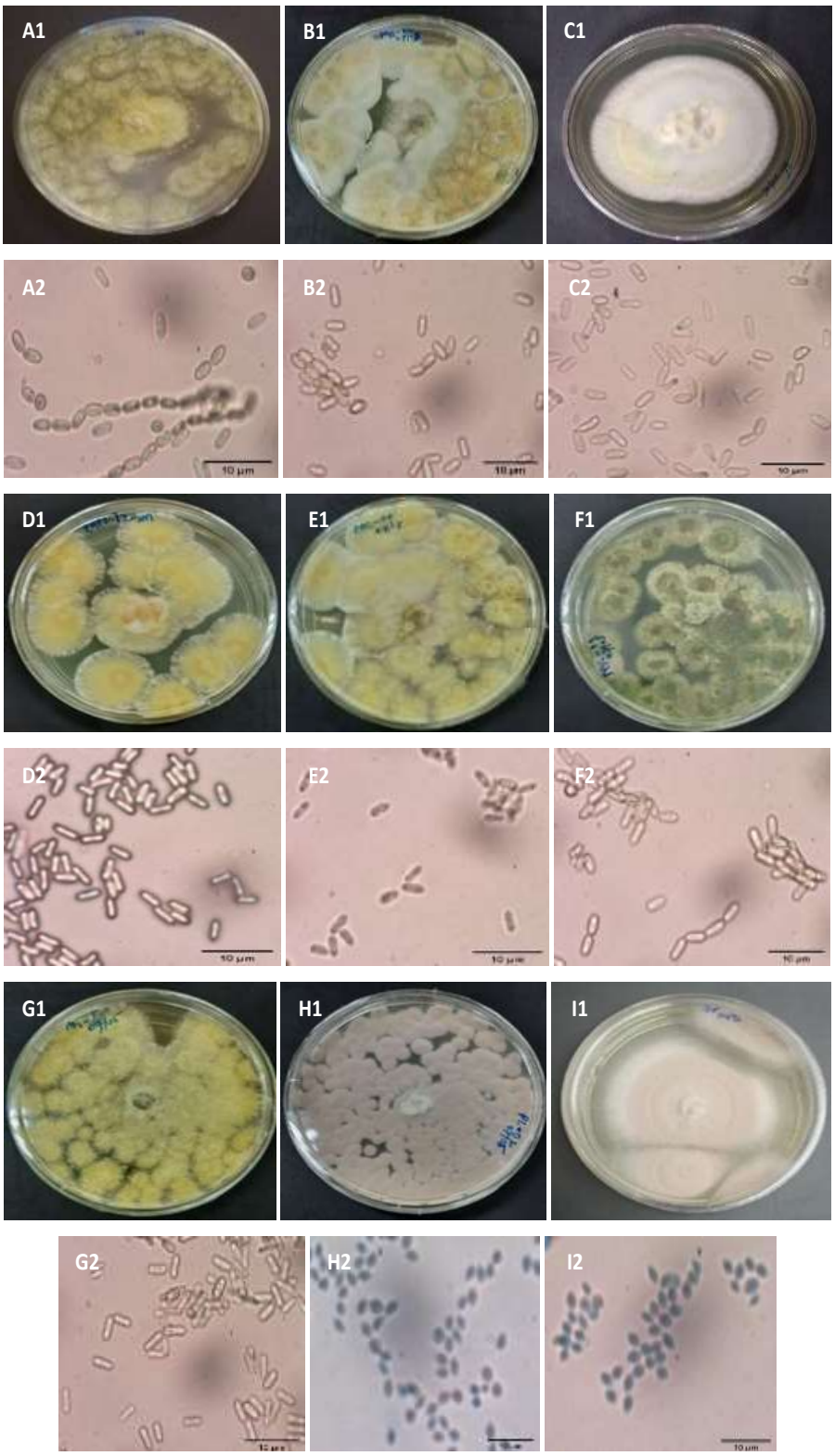

Figure 2: Pure culture and microscopic characteristics of ten days old of *M. anisopliae* (Ma) and *P. lilacinum* (Pl) isolated from soil of oil palm in UPM on PDA media. (A1, A2), Ma-01; (B1, B2), Ma-2b; (C1, C2), Ma-3E; (D1, D2), Ma-4G; (E1, E2), Ma-5i; (F1, F2), Ma-9a; (G1, G2), Ma-15; (H1, H2), Pl-01; (I1, I2), Pl-05 (magnification 1000×).

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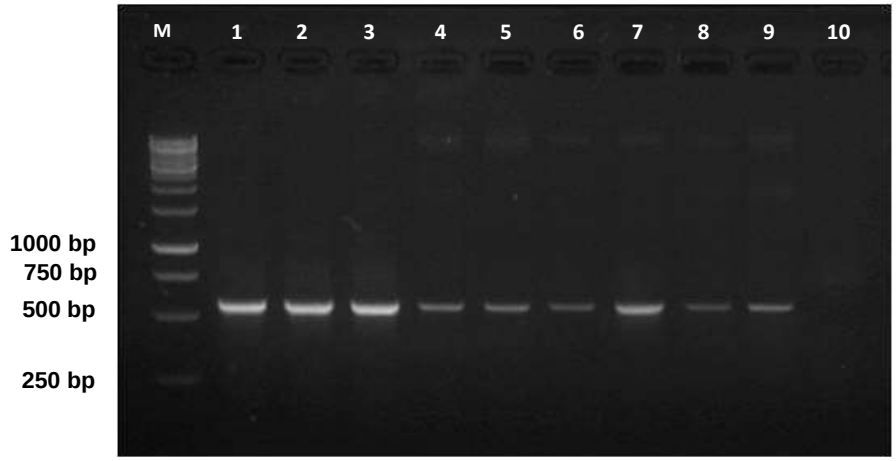

Figure 3: Agarose gel electrophoresis showing bands of PCR products from ITS region and amplification fragment showing approximately 570 bp. Lane M: 1 kb ladder; Lane 1-9: Ma-01, Ma-2b, Ma-3E, Ma-4G, Ma-5i, Ma-9a, Ma-15, Pl-01, Pl-05; Lane 10: Negative control (distilled water).

Morphological characterization and microscopic examination

The isolates were identified morphologically based on macro and micromorphological features such as conidial shape and size and colony morphology (Watanabe, 2010; Humber, 2012). Two species of EPF were successfully isolated and identified, namely *M. anisopliae* and *P. lilacinum*. The morphological identification was confirmed that seven isolates are *M. anisopliae* and two isolates are *P. lilacinum* (Figures 2A-2I). In addition, other opportunistic entomopathogens such as *Aspergillus*, *Trichoderma* and *Fusarium* species were morphologically identified, but special attention has been given to these two species due to their importance in the biological control of insect pests. The conidia of *M*. *anisopliae* isolates were cylindrical to ellipsoid with the length ranged from 6.8-7.6 × 2.4-2.8 µm (Figures 2A2-2G2). The colour of cultures varies from yellow green, olivaceous, to dark green (Figures 2A1-2G1). On the other hand, the conidia of *P. lilacinum* was a long ovoid, with the length 2.6-3.6 × 1.2-1.8 µm long (Figures 2H1 and 2I1). The colour of cultures varies from tan to smoky pink in mass (Figures 2H1 and 2I1).

Molecular characterization for entomopathogenic fungi

In characterizing fungal diversity, the Internal Transcribed Spacer (ITS) is considered as the official barcoding marker for species-level identification (D'Andreano *et al.*, 2020) and has been employed to classify diverse species of entomopathogenic fungi (Sayed *et al.*, 2018; Niu *et al.*, 2019). For all the fungal isolates, a 570 bp fragment of the ITS gene regions (ITS1 and ITS4) was successfully amplified and sequenced (Figure 3). The resulting sequence were compared with other strains of *M. anisopliae* and *P. lilacinum* in other studies. All the seven and two isolates were very similar to *M. anisopliae* var. and *P. lilacinum*, respectively. The nine isolates and their related sequences in GenBank had almost 100% sequence similarity, according to BLAST searches. Sequences from this study were deposited in the GenBank database and accession number as listed in Table 2. Figures 4A and 4B represent the phylogenetic relationship of all the isolates, and all the isolates were clustered in the same clade (clade 1) in respect of the species.

DISCUSSION

This study is aimed at investigating the isolates of EPF from the soil of oil palm at the UPM Serdang campus as part of the IPM of oil palm insect pests. EPF were isolated from 8 out of 30 soil samples collected from 3 different locations of oil palm plantations of UPM using insect bait methods. The insect bait method for isolation of EPF from the soil was first described by using *G. mellonella* during the last four decades (Sharma *et al.*, 2018). In recent years, insect baiting using *T. molitor* has been the most widely used to recover EPF from the soil (Steinwender *et al.*, 2014; Lin *et al.*, 2017; Elham *et al.*, 2018).

In Malaysia, EPF has been previously detected from the soil of oil palm plantations (Kin *et al.*, 2017a; 2017b). Two species of EPF, *M. anisopliae* and *P. lilacinum* were isolated from soil and *M. anisopliae* is the most encountered species. During many previous studies, *M. anisopliae* was found to be more commonly isolated from the soil than other insects' pathogenic fungi (Keller *et al.*, 2003; Lin *et al.*, 2017). This high occurrence of *M. anisopliae* could be related to its capacity to adapt to tropical environments compared to other EPF species such as *B. bassiana* that are more tolerant to the temperate environment (Vega *et al.*, 2012; Elham *et al.*, 2018). The finding of these two EPF species in contrast with the results obtained from the studies of Kin *et al.* (2017a; 2017b) who found that soil samples collected from oil palm plantations contain higher densities of *Isaria*

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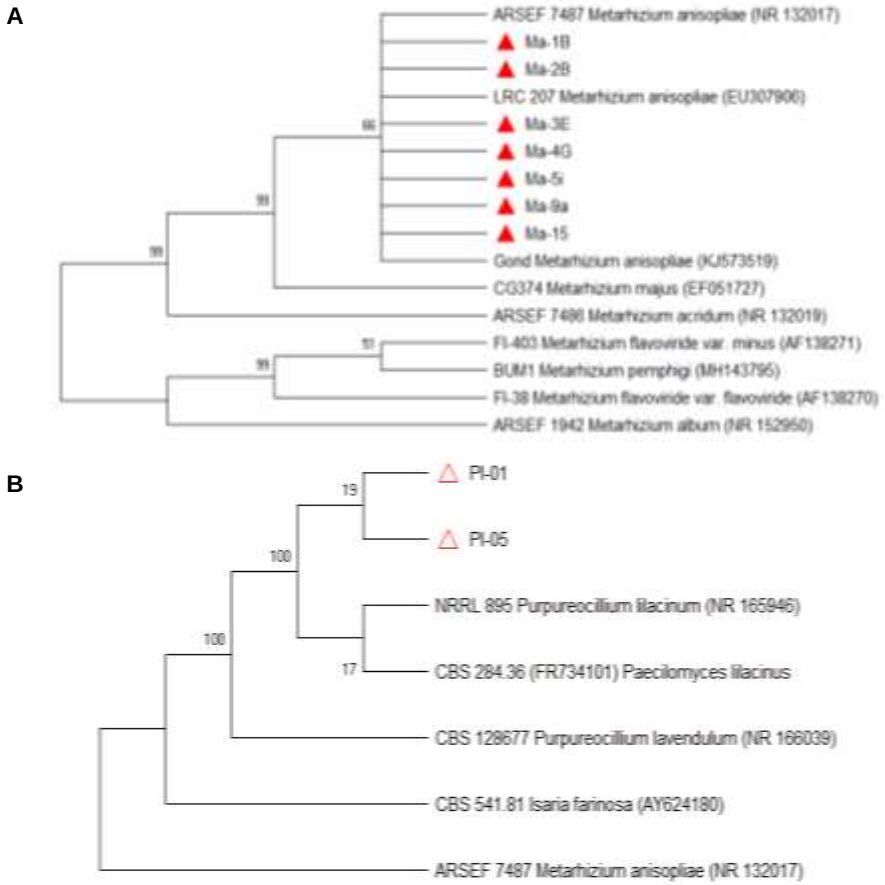

Figure 4: Molecular phylogenetic analysis based on sequence ITS regions: (A) *Metarhizium anisopliae* isolates; (B) *Purpureocillium lilacinum*. The tree was constructed by the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980).

species than *M. anisopliae*. However, most EPF-infected insects that live in oil palm soil spend at least part of their lifecycle in the soil. Hence, the soil becomes the main reservoir of EPF and is most likely to be isolated from the soil ecosystem (Cheng *et al.*, 2008; Manjeri *et al.*, 2014; Kamarudin *et al.*, 2019).

To our knowledge, this is the first study in Malaysia to discovered *P. lilacinum* from soil and identify it using both morphological and molecular approaches. But it has been isolated in some geographic areas close to Malaysia such as in Thailand and Japan (Hotaka *et al.*, 2015). *Purpureocillium* is a new divergent genus that is classified from *Paecilomyces lilacinum* and was established in 2011 by Luangsa-Ard *et al.* (2011). The species *P. lilacinum* is more frequent and commonly isolated from soil samples as has been observed in several previous studies (Demirci and Altuntaş, 2019; Niu *et al.*, 2019; Sun *et al.*, 2021).

Many isolates of *Purpureocillium* species have been used as biological control agents of nematodes phytopathogens such as root-knot nematode, *Meloidogyne* spp. and also to control insect pests such as fruit fly, *Anastrepha ludens* (Loew) (Baron *et al.*, 2019; Toledo-Hernández *et al.*, 2019; Du *et al.*, 2020). Moreover, the presence of *P. lilacinum* in the soil might be due to the intraspecific genetic diversity and occurrence of the nematodes phytopathogens in plant tissue (Baidoo *et al.*, 2017; Niu *et al.*, 2019). It is therefore believed that more species of *Purpureocillium* will be isolated from the Malaysian soil of oil palm in the future.

CONCLUSION

In this study, two species of EPF, *M. anisopliae* and *P. lilacinum* were isolated from the soil of oil palm using insect bait methods with *T. molitor*. The species *M. anisopliae* was discovered in higher density than the species *P. lilacinum.* The presence of these two EPF species in the soil indicates that insect pests of oil palm in UPM could be naturally regulated by EPF and these fungal isolates could be developed as mycopesticide for sustainable control of oil palm pests.

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