IDENTIFICATION OF *Cordyceps javanica* AND ITS EFFECTIVENESS IN CONTROLLING BAGWORM, *Pteroma pendula* JOANNIS (LEPIDOPTERA: PSYCHIDAE)

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ABSTRACT

The application of biological control agents as one of the control methods to suppress the infestation of bagworm in Malaysia has developed steadily with the inundation release of formulated entomopathogenic microbes to the outbreak area. In this study, we isolated and identified the entomopathogenic fungi (EPF) from the dead larvae of the bagworm, *Pteroma pendula*. Mycoses dead bodies of *P. pendula* were collected from two locations in Kemayan, Pahang, Malaysia. Morphological characterization of EPF was carried out by observing the macroscopic and microscopic growth on Potato Dextrose Agar (PDA) plates using a compound microscope. It was observed that white colonies on PDA changed into purple or pink shades after sporulation. The colony is slow-growing with floccose mycelium, which produces conidiophores with three to four phialides. The conidia were cylindrical to fusiform, smooth-walled and formed in chains on mononematous conidiophores. All the isolates were observed to be similar to *Cordyceps fumosorosea* features. Molecular identification using universal primer (ITS4 and ITS5) has identified that the isolates were *Cordyceps javanica* and *Parahevansia koratensis*. Bioassays of identified isolates were carried out against the second instar larvae of *Pteroma pendula* showed that *C. javanica* isolates BSB01 achieved LT_{50} - 6.76 days and recorded the shortest lethal time compared to the other isolates.

Keywords: Entomopathogenic fungi, biological control, bagworms, oil palm

ABSTRAK

Pengunaan kawalan biologi sebagai salah satu kaedah kawalan serangan ulat bungkus di Malaysia telah berkembang dengan baik menerusi formulasi mikroorganisma

entomopatogenik bagi mengawal kawasan letusan populasi ulat bungkus. Dalam kajian ini, kulat entomopatogen (EPF) telah dipencilkan dan dikenalpasti daripada larva ulat bungkus *Pteroma pendula* yang telah mati. *Pteroma pendula* yang telah mati dan mempunyai miselia telah dikumpul daripada dua lokasi di Kemayan, Pahang, Malaysia. Pengenalpastian ciri-ciri morfologi kulat entomopatogen telah dijalankan dengan melakukan pengecaman makroskopik terhadap miselia putih yang tumbuh di atas media Potato Dextrose menggunakan mikroskop Leica DM750. Pemerhatian koloni putih atas PDA berubah kepada warna ungu atau merah jambu selepas fasa sporulasi kulat. Koloni tersebut mengalami pertumbuhan yang perlahan dengan perkembangan miselia 'floccose' yang menghasilkan konidiofor dengan tiga atau empat 'phialides'. Konidia tersebut juga berbentuk silinder ke fusiform, berdinding halus dan membentuk rantaian di atas konidiofor yang 'mononematous'. Pengecaman molekul menggunakan pencetus umum (ITS4 dan ITS5) telah mengenalpasti kulat entomopatogenik yang diasingkan adalah *Cordyceps javanica* dan *Parahevansia koratensis*. Bioassai pencilan yang telah dikenalpasti dijalankan ke atas instar kedua larva *P. pendula* menunjukkan pencilan *C. javanica* BSB01 mencapai dos kematian LT_{50} dalam masa 6.76 hari dan telah merekodkan masa kematian terpendek berbanding pencilan lain.

Kata kunci: Kulat entomopathogen, kawalan biologi, ulat bungkus, kelapa sawit

INTRODUCTION

The bagworm infestations have seriously threatened the Malaysian oil palm industry. *Pteroma pendula, Metisa plana* and *Mahasena corbetti* are the three most destructive leaf defoliators in oil palm plantations (Wood & Kamarudin 2019). In Peninsular Malaysia, *M. plana* and *P. pendula* outbreaks have been the most reported cases in the oil palm planting areas. In central Johor, 32,475 ha of estate and 5,100 smallholder land in central Johor infested by bagworms (Mazmira et al. 2015). Moderate bagworm infestation notably *M. plana* and *P. pendula* causing 33-47% yield loss signifies the economic importance of this pest (Loong & Chong 2012). Another study strengthens the claim by proving that after 18 months of severe infestation by *P. pendula*, bunch production decreases to 36%, causing a disproportion in the sex ratio (Priwiratama et al. 2019). Various control methods have been implemented to lower the bagworm population under the economic threshold, but the outbreak persists. Bagworm outbreak recurrence in the oil palm industry has contributed to severe economic losses surpassing USD25 million annually, and immediate control measures should be undertaken (Mazmira et al. 2022). Chemical control has still the most preferred solution by plantation management to control these pests despite various health and environmental issues. Uncontrolled use of the broad-spectrum chemical has led to the recurrence of the bagworm outbreak on the plantation. Chemical pesticides overuse without organized approach would cause serious infestation over the years (Nur Robaatul Adawiyah et al. 2021). Wood & Kamarudin (2019) stated that the natural imbalance of pests and their natural enemies affected by this chemical pesticide is more prone to death than the target pest itself.

Cordyceps fumosorosea (Hypcerales: Clavicipitacae), formerly known as *Paecilomyces fumosoroseus* and *Isaria fumosorosea* is a renowned entomopathogenic fungus intensively studied for insect pest management (Du et al. 2021). Apart from its diversified host range and vast insecticidal effects, this fungus has the edge in its sustainable production cost and safety to human and non-target organisms. (Ali et al. 2017). This fungal species which is known as one of the common entomopathogenic fungi infecting *P. pendula*, contributes to 23.9% of the total bagworm mortality in oil palm plantations (Loong et al. 2010). It is known to affect the pupal population of the *P. pendula*, especially during wet seasons, where the pupae

hanging from branches collect raindrops with the conidia, causing the pupal population to be infected by the fungus (Lelana et al. 2022; Sajap & Siburat 1992).

Isolation, characterization, pathogenicity and mass production process are the dependent factors of microbial control successes in controlling insect pests (Sahayaraj & Namasivayam 2008). Selecting the best fungal isolate is the most fundamental process in developing mycoinsecticides. Critical parameters for selecting a fungal pathogen for use in inundation biocontrol include the cost-effective production of a stable, infective propagule that is suited for use in the environment where the insect must be controlled (Jackson et al. 2010). These factors influence the quality of the mycoinsecticide final product.

Pteroma pendula were also known as defoliators for various plants, shades, orchards, forest trees, or many ornamental shrubs in urban landscapes (Loong et al. 2010; Loong et al. 2013). One of the previously reported host plants in Malaysia was the exotic invasive tree, *Acacia mangium* (Loong et al. 2010; Nair 2001) which is also the host for at least 22 other pests (Nair 2001), could be the best focal source of a new outbreak due to its height, enabling bagworms to travel far by wind assistance. In this study, the *C. fumosorosea* isolates from the infected *P. pendula* collected from local oil palm plantations were tested for bioassay and its potential as a biocontrol agent was investigated.

MATERIALS AND METHODS

Isolation of Entomopathogenic Fungi from *Pteroma pendula* Samples

In this study, suspected *Cordyceps* fungi were isolated from *P. pendula* collected at oil palm plantation in Kemayan, (3°07’06.5”N,102°.43’71.3”E) and Bukit Senorang (3°09’95.6”N, 102°.42’82.9”E) of Pahang, and Rantau (2°54’90.6”N, 101°97’90.8”E), Negeri Sembilan, Malaysia. The mycelia that emerged from the dead *P. pendula* were picked with a sterilised needle. The mycelia were placed on PDA media and sealed with parafilm strip to prevent contamination. The plates were incubated at 27 °C with relative humidity 80% RH and were observed daily for the presence or absence of mycelia. The colonised culture on top of the media was observed daily. The colonies grown on the plate were sub-cultured to obtain pure cultures. Fungal conidia were examined under the compound microscope Leica DM750 at 400 times magnifications after the sporulation period for morphological identification. The general features of *C. fumosorosea* were determined according to Samson (1974b), Altre et al. (2001) and Domsch et al. (2007).

Table 1. List of fungal isolates name to be identified through molecular identification

Isolate Name	Origin
PF 02	Ladang Langkap, Hutan Melintang, Perak
PF 24	Ladang Langkap, Hutan Melintang, Perak
PF 39	Ladang Langkap, Hutan Melintang, Perak
PF 49	Ladang Langkap, Hutan Melintang, Perak
PF UPM	Universiti Putra Malaysia, Serdang, Selangor
PF 01	Ladang Langkap, Hutan Melintang, Perak
SEP 01	South East Pahang Kemayan, Pahang
BSB01	Bukit Senorang, Kemayan, Pahang
BSC01	Bukit Senorang, Kemayan, Pahang

Molecular Identification

DNA extraction

Fungal mycelia were scraped off from the culture grown on PDA media using sterilized sharp knife, weighed for 0.1g and ground with one mL Cetyl Trimethylammonium bromide (CTAB) buffer using mortar and pestle. The ground mixture was shaken thoroughly before adding three µL of 2-mercaptoethanol. The mixture was incubated in Eppendorf Thermomixer C at 65°C for one hour and inverted every 10 minutes. The mixture was centrifuged at 13 000 rpm for 5 minutes. The supernatant was then transferred into a new tube and mixed with 600 µL 24:1 chloroform: Isoamyl alcohol. The mixture was centrifuged again at 13 000 rpm for 5 minutes. The supernatant was again transferred into a new tube, and an equal volume of isopropanol was added to the supernatant. The supernatant mixture was placed in the freezer for half an hour before centrifuging at 13 000 rpm for 10 minutes. The supernatant was discarded, and the pellet was rinsed with 70% ethanol and air-dried before being suspended in 50µL Tris-EDTA (TE) Buffer. The method was conducted according to Lee et al. (1988).

PCR amplification

PCR amplifications were conducted according to White et al. (1990) which used universal primers targeting Internal Transcribe Spacer (ITS) region. The procedure was performed in a total volume of 25 µL reaction mixtures containing 12.5 µl PCR Mastermix, 1 µl DNA template, 1 µL forward primer ITS 4 and 1 µL reverse primer ITS 5 and 10.5 µL deionised water. The sequences of the ITS primers are shown in Table 2. DNA amplification was performed in a thermal cycler system (Bio-Rad) and underwent several steps started with pre-naturing process in 1 cycle of 95°C for 4 minutes, denaturing process 35 cycles of 95°C for 30 seconds, annealing process 55.2°C for 30 seconds, extention process 72°C for 1 minutes 7 seconds, and keeping it in 4°C. Quantification (µg/l) of amplified DNA after Polymerase Chain Reaction (PCR) was done using NanoDrop 2000.

Table 2. List of its primers and sequences (White et al. 1990) used in fungi identification

ITS Primers	Sequences
ITS 4	5'TCC TCC GCT TAT TGA TAT GC 3'
ITS 5	5'GGA AGT AAA AGT CGT AAC AAG G 3'

DNA sequencing

Sequence reactions were carried out in both directions for each purified double-stranded PCR product using dye, buffer and primers. Amplified samples were purified before being sent to Apical Scientific Sdn Bhd. for sequencing to obtain DNA sequences. Sequences obtained were aligned with BioEdit Sequence Alignment Editor Software to align forward sequence and reverse sequence (Hall 1999). The boundaries of the ITS region for all accession were determined by comparing the ITS sequences to those existing in BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Screening for Virulence of *C. javanica* Isolates

Preparation of fungal suspensions

Identified *C. javanica* isolates from the previous experiment were subjected to virulence screening against the bagworm, *P. pendula*. The sporulated *C. javanica* culture plates were flooded with 10 ml 0.05% Tween® 80 solution. The submerged cultures were stirred using an L-shaped glass rod to detach the conidia from conidiophores. The stirred suspensions were then filtered using a double-layer muslin cloth to filter out mycelia debris and large fungal fragments.

The conidia suspensions were collected and their concentrations were determined using a hemocytometer. The procedure was repeated using the rest of the isolates. All the suspensions were diluted until $1x10^7$ conidia mL^{-1} concentration and ready for treatment. The concentration was standardised to 1 x 10^7 conidia mL^{-1}as preliminary trial shows the concentration was adequate to cause mortality to the second instar *P. pendula*.

***Pteroma pendula* Rearing**

Female *P. pendula* bagworms were collected from the field. Each individual was placed inside a 250 mL plastic cup and monitored daily until the neonates hatched from the bag. The newly hatched neonates were transferred immediately from the plastic cups to young *Acacia mangium* leaves as the alternative host plant.

Bioassay

Ten second instar *P. pendula* larvae were dipped one after another into the prepared *C. javanica* suspension for a second. The spores treated bagworms were left dried on the fresh *A. mangium* leaf and left inside the plastic cup. The experiment was carried out in five replicates per *C. javanica* isolate suspensions. Larvae were dipped in 10 ml 0.05% Tween® 80 solution without fungal presence as control treatment. The procedure was repeated for all the isolate suspensions and left in the laboratory for daily observation for twelve days. The mortality of the bagworm was recorded daily. The isolate that achieved 25 bagworms mortality in the shortest period was considered the best strain to be formulated as a wettable powder in this study.

Corrected mortality was calculated as the formula:

$$\text{Corrected mortality } \% = \frac{\text{T\% } - \text{ C\%}}{100} - \text{C\% x } 100$$

T% = the percentage of dead test organisms
C% = the percentage of dead control organisms

Corrected mortality data at 3 DAT to 12 DAT were analysed using Polo Plus.

RESULTS AND DISCUSSION

Isolation and Identification of C*ordyceps* sp.

The collection and isolation process of entomopathogenic from infected *P. pendula* collected from United Malacca Berhad (UMB) Plantation in Negeri Sembilan and Pahang obtained 13 isolates. Three isolates showed similar morphological characteristics to *C. fumosorosea*. These isolates formed white colonies in the early growing days and turned into purple or pink shade after sporulation (Figure 1).

Figure 1. Pink shades colour of the sporulated *C. fumosorosea* BSB01 culture

Morphological observation under the microscope showed that those three fungal isolates fulfilled the characteristics of *C. fumosorosea* as described by Samson (1974b) and Domsch et al. (2007), where the conidia were cylindrical to fusiform, smooth-walled and formed in chains on mononematous conidiophores (Figure 2).

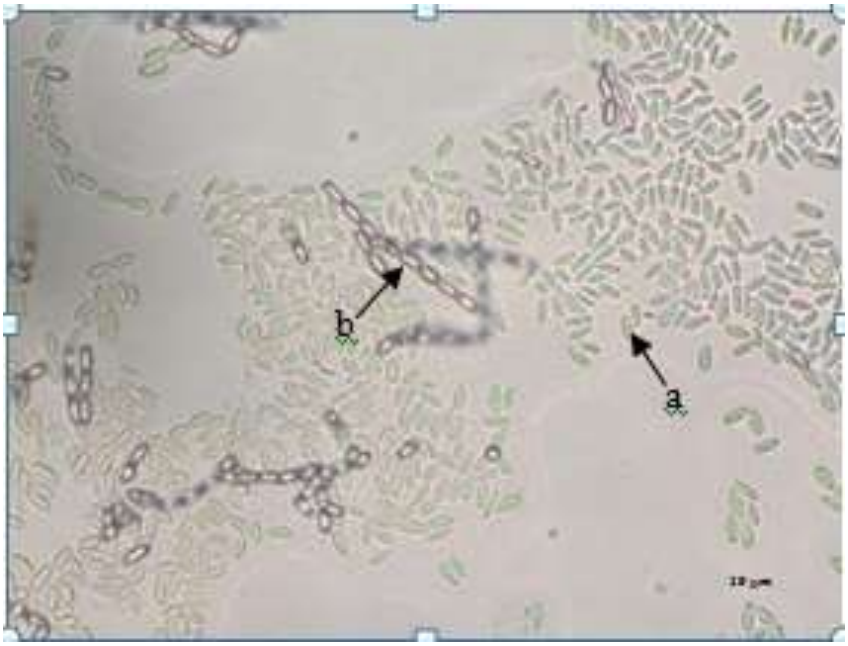

Figure 2. (a) Globose to ellipsoidal shape of *C. fumosorosea* BSB01 conidia under the microscope (400 times magnifications), (b) conidia formed in the chain

Based on taxonomic keys by Samson (1974b), there are two species of *Cordyceps* with pink colonies, namely *C. fumosorosea* and *C. amoenerosea*. This has been confirmed by Kin et al. (2017) who also described the shape with dimensions of *C. amenorosea* (2.5-3.5 µm long ×1.7-2.2 µm wide). The conidia of *C. amenerosea* were sub-globose or irregular shapes with dimensions between 2.0-3.0 µm long ×1.7-2.0 µm wide (Kin et al. 2017). *Cordyceps fumosorosea* conidia are longer than *C. amoenerosea* with the dimension between 3.0-4.0 µm

long × 1.0-2.0 µm wide. A study by Lei et al. (2021) found that both *C.fumosorosea* strain used in his study are 3.7 µm long x 1.2 µm wide.

All of the isolates were going for molecular identification. Six of nine isolates subjected to molecular identification were identified as *C. javanica* and the other three isolates belonged to *Parahevansia koratensis* (Table 3). The nuclear ribosomal ITS region has a long history of use as a molecular marker for species-level identification in ecological and taxonomic fungi studies (Hibbett et al. 2011). Molecular identification using ITS region identified isolates as *C. javanica* and *Pa. koratensis.*

Several studies have confirmed the existence of *C. javanica* in the oil palm ecology and are widely known as soilborne Ascomycetes. Previous studies have identified *Paecilomyces* sp. (synonym of *Cordyceps* sp.) as endophytes in oil palm (Pinruan et al. 2010). *Parahevansia* formerly known as *Akanthomyces* genus was also renowned to have an entomopathogenic history in insects and spiders (Hsieh et al. 1997), making this genus belong in the Cordycepticeae family.

Table 3. Fungal isolates name and molecular result after BLAST analysis

Isolate Name	**Species Name**	**Query Score (%)**	**Ident Score (%)**	**Accesssion No.**
PF 02	*Parahevansia koratensis*	93	97.8	GQ250010.1
PF 24	*Cordyceps javanica*	99	97.13	MH532892.1
PF 39	*Cordyceps javanica*	95	98.17	GQ250010.1
PF 49	*Cordyceps javanica*	96	97.54	MH532893.1
PF UPM	*Parahevansia koratensis*	94	98.32	GQ250010.1
PF 01	*Cordyceps javanica*	97	97.88	MG742216.1
SEP01	*Parahevansia koratensis*	95	98.99	GQ250010.1
BSB01	*Cordyceps javanica*	95	99.16	MG742216.1
BSC01	*Cordyceps javanica*	95	99.16	MG742216.1

This genus was characterised by producing white, cream or flesh-coloured cylindrical, attenuated synnemata covered with a hymenium of phialides (Hsieh et al. 1997). These conidiogenous cells are ellipsoidal, cylindrical, or narrowly cylindrical and gradually or abruptly tapering to a distinct neck. Its conidia are unicellular, hyaline, short, or long chains (Hsieh et al. 1997). This genus was separated from the *Paecilomyces* (*Cordyceps*) genus by the typical hymenium-like arrangement of the phialides as these structures were found to be loose and verticillate in the latter (Samson 1974a). The identical possession of entomopathogenic characteristics between these two fungi highlights the importance of extensive fungal identification. A recent study on the Cordycepticeae family has retained both genera in its family (Kepler et al. 2017). Although *Parahevansia* spp. was known to be pathogenic to spiders and Lepidiopteran pests, this study denotes first report of *Pa. koratensis* infested *P. pendula* bagworm in Malaysia.

Screening of *Cordyceps javanica* Virulence Against *Pteroma pendula*

The screening of identified isolates against *P. pendula* was conducted with the exclusion of *Pa. koratensis* isolates after preliminary tests (unpublished data). The test conducted by dipping the bagworms into spore suspension shows all the identified *Pa. koratensis* (PF02, PFUPM and SEP01) did not cause any mortality to the bagworms, which is against the Koch Postulates.

Since the fungus is slow growing and does not show clear sign of sporulation (Hywell-Jones 1996), the concentration of spore suspension might not be sufficient or the fungus need an ideal climate to cause infection. There were not many studies regarding the *Akanthomyces* species found by infesting Salticid Spider in Korat, Thailand (Hywell-Jones 1996), but the isolation of this species from oil palm major pest shows that there is some potential of this species to be developed into biopesticide.

The screening process proceeded with the other identified isolates, which could give mortality to bagworms. The efficacy of *C. javanica* against *P. pendula* was shown in Figure 3. In this experiment, 50 larvae were used to establish the LT_{50}. Isolate BSB01 was the first isolate to reach 50% mortality in the screening against *P. pendula* bagworm, followed by PF49 with 7 days This value might be slightly longer than LT_{50} achieved on *P. pendula* in another study which was LT_{50}-5.72 days, and EPF, *Metarhizium anisopliae* with LT_{50}- 5.40 days at concentration of 2 x 10^9 conidia ml^{-1} (Loong et al. 2013). The number of LT_{50} could be shortened by increasing the concentration of the suspension. The strain could be more lethal than what has been used by Loong et al. (2010). Another factor could be the host plant itself since the bagworm used leaf fragments to be used as the bag. However, *P. pendula* living on both host plants perform relatively similar in terms of growth and build-up performance (Loong et al. 2010).

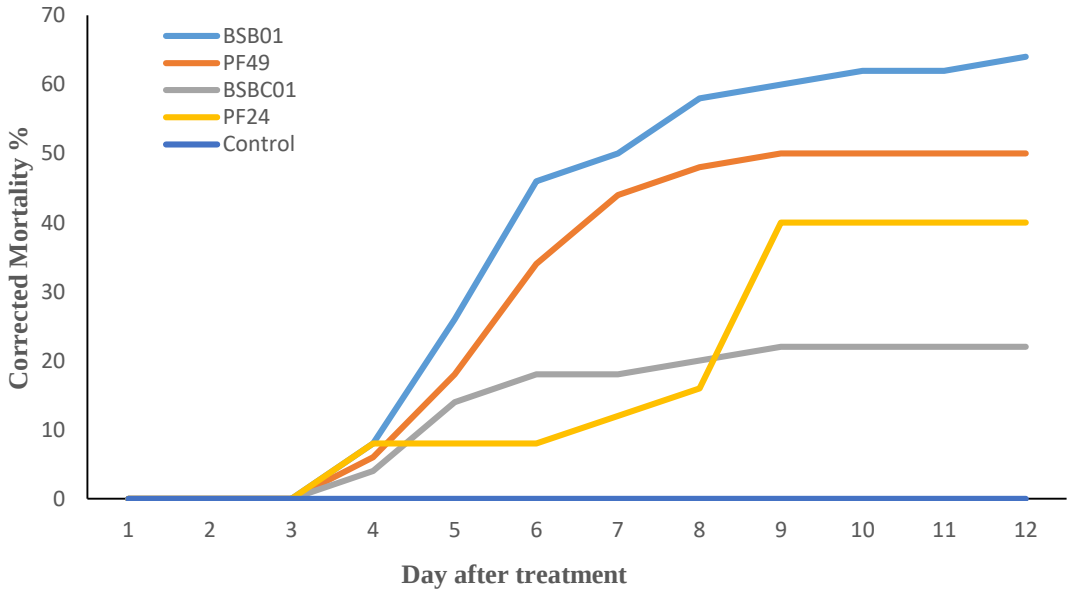

Figure 3. Bagworm corrected mortality percentage against different *C. javanica* isolates 50% *P. pendula* mortality

The percentage mortality obtained for the BSB01 isolate failed to reach 80% at the end of the bioassay. Bakeri et al. (2009) showed LT_{80} of 11 days using *I. farinos*a (*C. farinosa*) and *I. carneus* (*Keithomyces carneus*). Different species of entomopathogenic fungi exhibit variability of virulence, which may be attributed to the genomic variability of the fungi (Bidochka et al. 1994; Kumar et al. 2021). Both BSC01 and PF24 isolates which were identified as the same species could not reach 50% mortality. In this study, BSB01 achieved the fastest and highest mortality. Usually, the inoculum produced on naturally infected arthropods is highly infective to susceptible hosts, whereas when produced on artificial media

can lose virulence (Butt 2002). Apart from virulence, attenuation of other fungal original features such as growth and sporulation were also affected by a repeated subculture of an isolate in the laboratory (Hayden et al. 1992; Khachatourians 1991; Sönmez et al. 2022). In previous studies, the use of EPF to control oil palm insect pests was focused on EPF isolated from infected insects (Moslim et al. 1999; Tajuddin et al. 2010). The fungi isolated from soil need to infect the insect before being used as biological control agents. The virulence of the fungus and pathogenicity of isolates towards any insect differs and are influenced by the unclear definition of virulence for entomopathogenic fungi (in relation to the specified gene-for-gene relationship between phytopathogenic fungi and their hosts). This situation justified the need of 'improved' strain selection to be used as active ingredient in mycoinsecticides (Feng et al. 1994).

Wraight et al. (1998) find out that *C. fumosorosea* could colonize several millimeters across the leaf surface and infect aleyrodids. This ability could increase the possibility of the fungus to be consumed by the insect and cause mortality subsequently. Conidia often relies to the direct contact to the bare body of the pest. Bagworms are protected with their bag and infection could only occur during foraging when the leaf chewed along with the conidia adhered on it. Since conidia have the hydrophobic surface, conidia could better stick on the leaf with hydrophobic surface. This is the reason why the fungal propagules must have the ability to adhere on the leaf surface.

The limited body area exposed by bagworm has shift the need of a formulation with better distribution to the target area and be palatable one as the only area exposed is the bagworm's head. Holder et al. (2007) already expected that hydrophilic propagules like blastospores able to disperse well in water but bind poorly to hydrophobic surfaces. If the target surface is the cadaver of the insect skeleton or other hydrophobic surfaces, conidia suspension might give the better performance. He added that hydrophobic cell walls of aerial conidia is said able to adhere rapidly to both hydrophobic and hydrophilic surfaces, but the possibility of aggregation in aqueous suspensions might reduce the efficiency of formulation towards the target host.

CONCLUSION

Cordyceps fumosorosea morphological features like pinkish colour at maturing sporulating stage make it easy to be distinguished. The fungus function as the natural control of oil palm bagworms was successfully proved by the isolation of the fungus from the mycosed dead bagworms collected from the field. Universal primers targeting ITS region of the DNA confirmed that the all of the suspected *C. fumosorosea* were indeed *C. javanica*. The effectiveness of this species under laboratory test has shown that the strain collected from the field was more virulent than the other strains collected from *Cordycep* species collection in UPM forestry laboratory. The strain lethalness could only be optimized if the screened strains were freshly isolated from the insect host. The inundation of this fungal species to control bagworms probably needs to be highlighted given that the fungus is dominantly found infecting bagworms in oil palm compared to other entomopathogenic fungi species.

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The authors declare that they have no conflict of interest.

Ethics Declarations
No ethical issue required for this research.

Data Availability Statement
The author confirm that the data supporting the findings are available within the article. Raw data available from the corresponding author upon reasonable request.

Authors' Contributions
MNY and DO conceived this research and designed experiments; MNY and DO participated inthe design and interpretation of the data; MNY performed experiments and analysis; MNY, SNA and SAB wrote the paper and MMMM, NA, and DO participated in the revisions of it. All authors read and approved the final manuscript.

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