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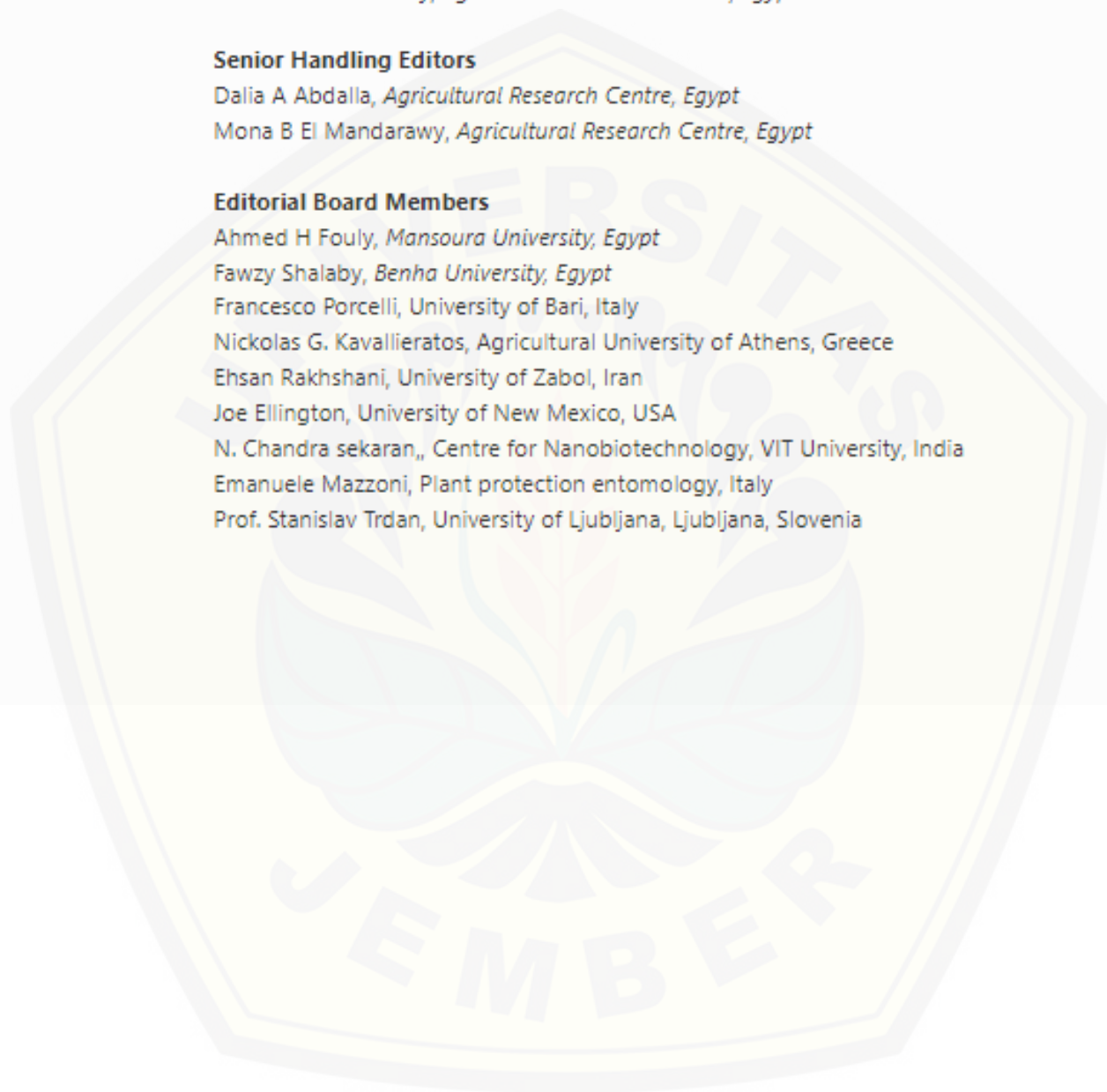
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Virulence of *Metarhizium* isolated from infected *Oryctes rhinoceros* L. (Coleoptera: Scarabaeidae) larvae around coconut plantations in East Java, Indonesia

Metarhizium is one of the entomopathogenic fungi (EPF) that has been widely reported as a useful agent for controlling the coconut rhinoceros beetle (CRB), *Oryctes rhinoceros* L. (Coleoptera: Scarabaeidae). Applic...

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Nematicidal potential of plant growth-promoting rhizobacteria against *Meloidogyne incognita* infesting tomato under protected cultivation

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The flower thrips, *Frankliniella schultzei* Trybom (Thysanoptera: Thripidae) is a notorious pest that attacks the plants of economic importance. The anthocorid bug *Blaptostethus pallescens* Poppius (Heteroptera: An...

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The fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), is a notorious polyphagous pest that has recently invaded India in 2018. Within a short period, this pest has spread throughout the...

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Antagonistic potential of an Egyptian entomopathogenic nematode, compost and two native endophytic bacteria isolates against the root-knot nematode (*Meloidogyne incognita*) infecting potato under field conditions

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Biocontrol potential of endosymbiotic bacteria of entomopathogenic nematodes against the tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae)

The tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is a major pest of tomato plants threatening global tomato production. The control of the pest is becoming increasingly difficult due to t...

Ebubekir Yüksel

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Biology, predatory potential and growth parameters of the syrphid fly, *Scaeva pyrastris* (L.) (Diptera: Syrphidae) feeding on the cabbage aphid, *Brevicoryne brassicae* (L.)

Scaeva pyrastris (L.) (Diptera: Syrphidae) is a large-size Palaearctic region syrphid fly. The larval stage of *S. pyrastris* is aphidophagous in nature, while the adults are efficient pollinators of various crops. T...

Shivani Paliyal, S. C. Verma, P. L. Sharma, R. S. Chandel, Rakesh Kumar, Meenu Gupta, Nidhi Sharma and Priyanka Sharma

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RESEARCH

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Virulence of *Metarhizium* isolated from infected *Oryctes rhinoceros* L. (Coleoptera: Scarabaeidae) larvae around coconut plantations in East Java, Indonesia

Sigit Prastowo^{1,3*} , Soeharto², Hardian Susilo Addy^{2,3}  and Tri Handoyo⁴ 

Abstract

Background: *Metarhizium* is one of the entomopathogenic fungi (EPF) that has been widely reported as a useful agent for controlling the coconut rhinoceros beetle (CRB), *Oryctes rhinoceros* L. (Coleoptera: Scarabaeidae). Application of this fungus as a biopesticide is influenced by regional environmental conditions that affect the pathogenic activity against the targeted pest. Several studies have proven that the native fungal isolates have strong pathogenic activity than EPF introduced from other regions. The identification of local EPF species is a strategic approach to develop potential biopesticides with standard properties including host specificity, climate suitability, and significantly suppressing the target pest population. This study aimed to identify *Metarhizium* species isolated from infected CRB larvae with high pathogenicity to host pests in East Java, Indonesia.

Results: Thirteen isolates were obtained which were divided into 4 clades based on phylogenetic analysis by the ITS rDNA region, namely *M. anisopliae* var. *lepidiotae*, *M. anisopliae* var. *anisopliae*, *M. brunneum*, and *M. majus*. Identified *Metarhizium* species exhibited varied sizes of conidia, but fell within the size ranges reported in previous studies. Interestingly, the isolate MaSi produced conidial lengths well above the range of conidial sizes recorded and placed these isolates (*M. anisopliae*) in the MGT clade, known as MALC (*M. anisopliae* s.l. with large conidia). The present investigation reported that isolates MaLe, MaMa, MaWa, and MaSi produced higher mortality values than other isolates, supporting that *M. majus* and MALC had higher pathogenicity against *O. rhinoceros* larvae than other *Metarhizium* species. Neighbor-joining analysis showed a close resemblance between the isolate MaMa and the strain ARSEF 1946, which was isolated from the CRB. In addition, the isolate MaMa had the highest virulence against *O. rhinoceros* larval cadaver with a faster lethal time (for 50% mortality). This result indicated a possible relationship between phylogenetic status or DNA sequence polymorphisms with *Metarhizium* pathogenicity and host specificity.

Conclusions: The local species of *Metarhizium* have been isolated from infected CRB larvae, and *M. majus* isolates exhibited high pathogenicity against *O. rhinoceros* larvae. The close similarity of *M. majus* isolates and CRB-isolated strains suggests a possible relationship between pathogenicity and host specificity with phylogenetic status or DNA sequence polymorphisms.

Keywords: *Metarhizium*, Local entomopathogenic, Coconut rhinoceros beetle, *Oryctes rhinoceros*, Phylogenetic, Pathogenicity

*Correspondence: prastowo_hpt.faperta@unej.ac.id

¹ Study Program of Plant Protection, Faculty of Agriculture, Universitas Jember, Jl. Kalimantan Tegalboto No.37, East Java 68121, Indonesia
Full list of author information is available at the end of the article

Background

Oryctes rhinoceros L. (Coleoptera: Scarabaeidae) is one of the important pests on coconut and oil palm that causes production losses and young plant mortality reaching 60% and 25%, respectively (Fauzana et al., 2018). Severe attack rates by this pest affects tree damage ranging from 50 to 100% (Manjeri et al. 2014). The two life stages of *O. rhinoceros* can attack different parts of the coconut trees. The second- and third-instar larvae can be growing rapidly in the root area of plants and feed on the rootstock near the soil surface and plant roots. Early detection of larval attack is difficult and controlling this pest at advanced levels of crop damage is inefficient (Hosang et al. 2022). Therefore, direct treatment of breeding sites such as decomposing trunks or compost heaps using biological control agents is considered as an efficient strategy against *O. rhinoceros* in the field (Gopal et al. 2006). On the other hand, pests that are in the adult stage are strong fliers, thus their control at this stage is an effective procedure because infected beetles may quickly spread and distribute their conidia to other breeding and foraging sites (Paudel et al. 2021). The adult beetle attacks the young leaf midrib by broaching and entering the young tissue which results in plant death in high-level attacks (Alouw et al. 2020).

The coconut rhinoceros beetle (CRB) can be controlled using entomopathogenic fungi (EPF) as biological control agents. *Metarhizium* is one of the EPF that have a broad host distribution and has been reported to be able to control many crop pests. Moreover, *Metarhizium* has long been known as a pathogen that infects all life stages of CRB, and the third-instar larval stage is the most frequently infected (Paudel et al. 2021). In India, the application of *M. majus* spores at vermicompost sites is succeed in reducing about 70% of the CRB larval population (Gopal et al. 2006). In the same country, fresh conidial suspension of *M. majus* killed about 85–90% of third instar CRB larvae within 2 weeks (Velavan et al. 2018). The treatment of rotting oil palm debris with *M. majus* could reduce larval populations by 80% in Malaysia (Moslim et al. 2006). *Metarhizium* controls its hosts by attaching conidia to the larval integument and further infecting through three phases of adhesion, germination, and adsorption (Sharma and Sharma 2021). After penetrating the host, the fungus produces toxins that interfere with and damage the host's immune system (Gabarty et al. 2014). Conidia reproduce by contact with moist soil substrate, actively absorb groundwater, and begin to germinate (Indriyanti et al. 2017a).

Several countries in Asia have developed biopesticides commercially, and Indonesia is one of the providers that produce entomopathogenic fungi-based biopesticides, which utilize the *M. anisopliae* as a biological control

agent of CRB on oil palm plantation (Paudel et al. 2021). Optimization of temperature, pH, and relative humidity are required to produce a high fungal pathogenicity against target pests. The effectiveness of *Metarhizium* strains is certainly different when treated in tropical and subtropical regions, and geographical factors are also involved. The use of EPF is ineffective in countries with relatively less rainfall, and conversely, the application in countries with heavy rainfall reduces the dissemination of the fungus (Indriyanti et al. 2017b). Therefore, the identification of local EPF species is a strategic approach of develop potential biopesticides with standard properties that should be possessed as biological control agents including host specificity, and climate matching, and could significantly suppress the target pest population. The development of knowledge and modern technology makes it possible to isolate and identify new pathogens from infected host pests, which is a proposed step to provide a high opportunity to obtain biological control agent with standard properties. This study aimed to identify the species of *Metarhizium* isolated from larval CRB cadavers and to determine isolates with high pathogenicity as candidates for the development of EPF-based biopesticides to control CRB attacks in East Java, Indonesia.

Methods

Source of fungal isolates and *O. rhinoceros* larval collections

The experiment was conducted in the Entomology and Plant Pathology Laboratory, Faculty of Agriculture, University of Jember, Indonesia. The fungi used in this study were isolated from *O. rhinoceros* cadavers explored from a coconut plantation area in East Java, and the four already cultured isolates of fungi were obtained from Balai Besar Pengkajian Penelitian Teknologi Pertanian (BBPPTP), Indonesia (Table 1). The fungi isolation using the method described previously (Shah et al. 2022). The dead larvae was sterilized by submerging in alcohol (70%) for 1 min and was rinsed using sterile distilled water, dried on filter paper then transferred to potato dextrose agar (PDA) (Merck, Germany, supplemented with 0.1% chloramphenicol) and incubated at 25 ± 1 °C and 65 ± 5 % relative humidity for 14 days. The fungal colonies formed were then purified by subculturing on PDA medium with the addition of 0.5% thiamycin. The purification was performed twice to enhance the performance of isolates and to confirm that the culture is free from contaminants. The *O. rhinoceros* 3rd larval instar was obtained from sawdust piles in Condong village, Gading district, Probolinggo, Indonesia ($7^{\circ}51'45.0''$ S $113^{\circ}22'28.1''$ E). The larvae were incubated for 5 days to ensure that they were not infected by other fungi originating from the field.

Table 1 Isolates of *Metarhizium* collected from infected *Oryctes rhinoceros*

Isolate code	Location	Site coordinates and note
MaBe	Bedewang, Banyuwangi, East Java	8°16'22.1" S 114°13'13.4" E
MaBl	Blater, Jember, East Java	8°21'34.2" S 113°40'19.6" E
MaBt	Bluto, Sumenep, East Java	7°05'57.6" S 113°43'46.7" E
MaBo	Bondowoso, East Java	BBPPTP collection
MaJe	Jenggawah, Jember, East Java	8°17'08.6" S 113°38'47.1" E
MaKa	Kalianget, Situbondo, East Java	7°43'41.1" S 113°38'52.4" E
MaLe	Legung, Sumenep, East Java	6°54'45.8" S 114°02'18.7" E
MaMa	Madiun, East Java	BBPPTP collection
MaWa	Wates, Kediri, East Java	BBPPTP collection
MaWp	Wringin Putih, Banyuwangi, East Java	8°28'34.0" S 114°21'05.7" E
MaSi	Selomukti, Situbondo, East Java	7°44'58.8" S 113°45'39.3" E
MaSu	Sukorejo, Situbondo, East Java	7°45'04.8" S 114°16'50.0" E
MaSm	Sumbermulyo, East Java	BBPPTP collection

Morphological identification of *Metarhizium* isolates

The mycelium disk (4 mm) from the second purification of isolates at 7 days was inoculated on a plate containing growth medium and incubated at 25 ± 1 °C and $65 \pm 5\%$ RH for 14 days. The radial growth of the colonies was measured at two different points and recorded daily. Fungal characteristics including a color of mycelium, and a texture of hyphae, were observed at 14 days after inoculation. The size of conidia was determined on the mature colonies (14 days after inoculation) by picking up spores ($n = 100$) and placed on a glass slide with a coverslip, then width and length were both measured, using an Eclipse E100 LED Binocular microscope equipped with a Miconos camera and image raster software 3 (Optilab, Indonesia).

Molecular Identification of *Metarhizium* isolates

Fungal DNA isolation was conducted by extracting mature conidia, using the Zymo Research Quick-DNA fungal/bacterial miniprep kit (California, USA), according to the manufacturer's instructions. Molecular identification of *Metarhizium* isolates used the internal transcribed spacer (ITS) region with primer pairs, ITS1 (5'-TCGGTAGGTGAACCTGCGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') (Tangthirasunan et al. 2010). DNA amplification was performed in a T100 thermal cycler (Bio-Rad, Irvine, CA, USA) with a final volume of 25 μ l suspension containing 2 μ l template DNA, 2 μ l primer pairs, 10 μ l master mix, and 9 μ l nuclease-free water. The PCR thermocycler conditions used were: 95 °C for 5 min; 35 cycles at 94 °C for 90 s, 55 °C for 2 min, and 72 °C for 3 min, as well as a final step at 72 °C for 5 min. The PCR products were visualized on a 0.8%

agarose gel electrophoresis, using ethidium bromide, documented with GelDoc (Major Science, Saratoga, California, USA), then purified and sequenced by Genetica Science (Banten, Indonesia). The Sanger sequencing results were edited, using the Bioedit program, then aligned with the published full-length sequences in the Basic Local Alignment Search Tool (BLAST) databases on NCBI for collecting fungal species references (percent identity close to 99%). Phylogenetic analysis based on ITS-5.8S rDNA sequence was performed with Clustal-W function and neighbor-joining method using MEGA software version 7.

Bioassays of *Metarhizium* isolates on *O. rhinoceros* larvae

To evaluate the pathogenicity of *Metarhizium* isolate against *O. rhinoceros* larvae, the fungus was cultured on PDA media with corn material for 14 days at 25 ± 1 °C. Conidia were harvested with a sterile spatula and suspended in sterile distilled water with Tween 80 (0.02% v/v), then the concentration was calculated using a hemocytometer (Sondheim, Germany). The concentration (1.0×10^7 conidia/ml) of *Metarhizium* conidial suspension was determined for pathogenic test based on preliminary test by preparing a dilution series of aqueous conidial suspension (1.0×10^4 , 1.0×10^5 , 1.0×10^6 , 1.0×10^7 , and 1.0×10^8 conidia/ml) from four isolates randomly. Bioassays test was conducted by immersing the larvae in a fungal suspension for 20 s and drying, then transferring the larvae in a plastic container filled with soil and high organic matter. The experiment was carried out in a completely randomized design (CRD) with five replications and each container containing 10 larvae. The larval mortality was recorded daily for 15 days, starting on day 3 after inoculation. Larval cadavers of *O.*

rhinoceros were transferred to a dry container to confirm the presence of *Metarhizium* by observing the sporulation on the surface of the larvae.

Statistical analysis

The quantitative data included the mean radial growth and mortality rates were performed in an analysis of variance (ANOVA), followed by Tukey's test ($P \leq 0.05$). The mortality data were arcsine transformed before analysis. Comparison of virulence among *Metarhizium* isolates was determined by probit analysis to calculate the lethal time 50% (LT_{50}) using SPSS program version 12.

Results

Characterization of colony morphology and conidia isolates of *Metarhizium* spp.

The identified isolates of *Metarhizium* species were cultured on PDA media for 14 days. Colony morphology observations showed that the most of *Metarhizium* the isolates produced circular mycelium with fibrous hyphae and mat-like mass of conidia. Flat mycelium with fine fibrous hyphae was produced by the isolate MaKa, while

ununiform circular mycelium was formed in the isolate MaSi (Table 2). Dark green mycelia mats with various colors of circular rings were exhibited in the upper part of colonies with a predominance of black and brown in the lowest part of colonies (Fig. 1, Table 2).

Radial colonies of *Metarhizium* isolates were measured daily for 14 days after inoculation. Early growth was observed in the radial colonies varying from 3.75 to 5.83 mm on day 1. All isolates of *Metarhizium* had almost the similar radial growth from day 1 to day 5 after inoculation. For the isolate MaBt, a high radial growth was observed from day 4 to day 8 after inoculation. The mean radial growth of the several of isolates was significantly different. The isolates MaWa and MaSi showed the lowest mean radial growths, were significantly different than the isolates MaWp, MaBo, MaKa, which resulted in the high mean radial growth (Fig. 2; Tukey's test, $P \leq 0.05$). One week after inoculation, several isolates showed an elevated colony growth rate including isolates MaKa, MaBt, MaBo, MaWp, varying from 55.25 to 60.83 mm (on the last day of evaluation) with a mean radial growth of 3.58–4.07 mm day⁻¹. Intermediate radial growth

Table 2 Cultural characteristics of 13 *Metarhizium* isolates cultured in potato dextrose agar

Isolate	Mycelium		Color	
	Top	Bottom	Top	Bottom
MaBe	Sparse circular mycelium with thick fibrous hyphae	Sparse Circular mycelium with glassy hyphae and wavy rings	Dark green mycelia mat with a white of circular rings	Brownish green mycelia mat with a light green circular rings
MaBl	Circular hyphae with fibrous edges	Sparse circular hyphae with fibrous edges	Dark green mycelia mat with a brownish green of circular rings	Brown mycelia mat with a yellowish green of circular rings
MaBt	Circular mycelium with thick fibrous hyphae	Circular mycelium with fibrous hyphae	Black mycelia mat with a yellowish white of circular rings	Black mycelia mat with a yellowish white of circular rings
MaBo	Circular mycelium with a compacted center and thick fibrous hyphae	Circular mycelium with fibrous hyphae	Dark green mycelia mat with a yellowish white of circular rings	Black mycelia mat with a brownish yellow of circular rings
MaJe	Circular mycelium with thick fibrous hyphae	Wavy circular mycelium with glassy hyphae	Dark green mycelia covering whole colony	Brownish yellow mycelia covering whole colony
MaKa	Flat mycelium with smooth fibrous hyphae	Flat mycelium with glassy hyphae	White mycelia mat with a dark green of circular rings	Black mycelia mat with a dark yellow of circular rings
MaLe	Flat mycelium, wavy and tight, with densely fibrous hyphae	Circular mycelium, wavy and tight, with densely fibrous hyphae	Dark green mycelia mat with a gray of circular rings	Black mycelia mat with a yellowish brown of circular rings
MaMa	Circular mycelium with thick fibrous hyphae	Circular mycelium with fibrous hyphae	Dark green mycelia covering whole colony	Black mycelia mat with a yellowish brown of circular rings
MaWa	Circular mycelium with thick fibrous hyphae	Circular mycelium with fibrous hyphae	Dark green mycelia covering whole colony	Black mycelia mat with a yellowish brown of circular rings
MaWp	Circular mycelium with smooth fibrous hyphae	Circular mycelium with smooth fibrous hyphae	Dark green mycelia mat with a white and green of circular rings	Brownish yellow mycelia mat with a white circular rings
MaSi	Ununiforms circular mycelium with fibrous hyphae	Ununiforms circular mycelium with fibrous hyphae	Dark green mycelia covering whole colony	Black mycelia mat with a yellowish brown of circular rings
MaSu	Circular mycelium with fibrous hyphae	Circular mycelium with fibrous hyphae	White mycelia covering whole colony	Brownish yellow mycelia mat with a white circular rings
MaSm	Circular mycelium with smooth fibrous hyphae	Circular mycelium with smooth fibrous hyphae	Dark green mycelia covering whole colony	Black mycelia mat with a light yellow of circular rings

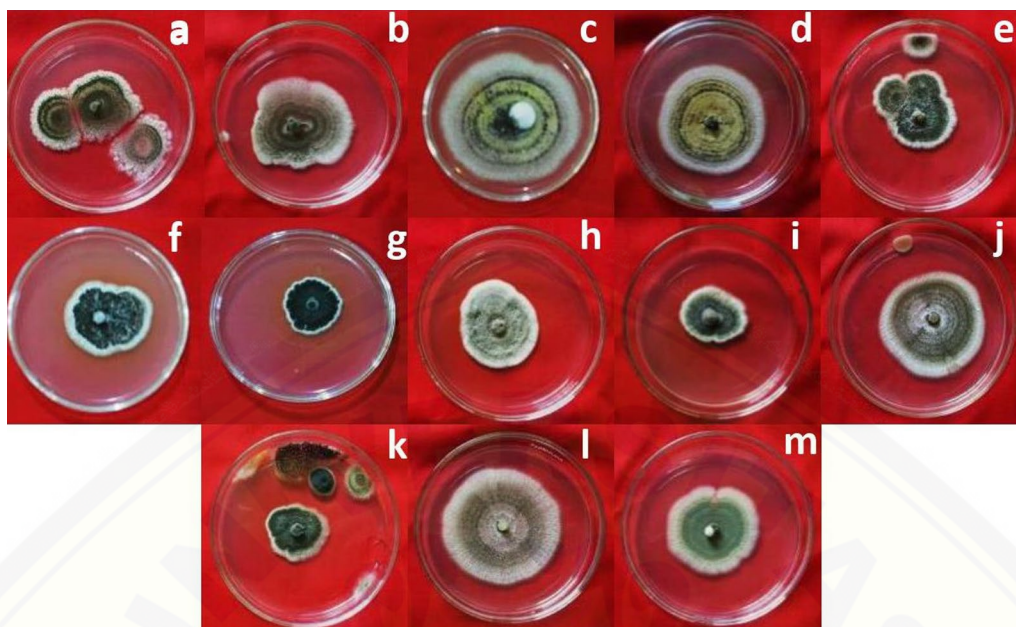


Fig. 1 Colonial morphology of *Metarhizium* isolates cultured in potato dextrose agar. **a** MaBe, **b** MaBl, **c** MaBt, **d** MaBo, **e** MaJe, **f** MaKa, **g** MaLe, **h** MaMa, **i** MaWa, **j** MaWp, **k** MaSi, **l** MaSu, **m** MaSm

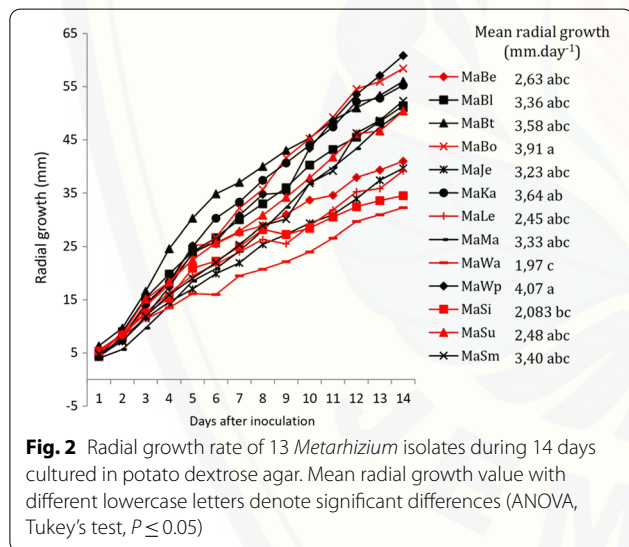


Fig. 2 Radial growth rate of 13 *Metarhizium* isolates during 14 days cultured in potato dextrose agar. Mean radial growth value with different lowercase letters denote significant differences (ANOVA, Tukey's test, $P \leq 0.05$)

produced by isolates MaSu, MaMa, MABl, MaSm, varied from 50.41 to 52.33 mm with a mean radial growth of 2.48–3.40 mm day⁻¹. The growth of *Metarhizium* colony sloped in several isolates at one week after inoculation including MaWa, MaSi, MaLe, MaBe, and MaJe, and they were resulted in the lower mean radial growth (Fig. 2).

Conidial length of isolates varied in size from 7.56 to 21.69 μm, with the longest conidia belonging to the isolates MaWa, MaSi, MaLe, MaJe, MaSu, and MaMa (Table 3), and also had highest the width conidia values

Table 3 The conidia size of 13 *Metarhizium* isolates cultured in potato dextrose agar after 14 days after inoculation

Isolates	Length (μm)	Width	Length/width ratio
MaBe	7.81	3.34	2.40
MaBl	7.66	3.33	2.30
MaBt	7.68	3.45	2.30
MaBo	7.56	3.11	2.49
MaJe	10.87	3.83	2.88
MaKa	8.93	3.96	2.29
MaLe	17.89	4.30	4.25
MaMa	10.13	3.83	2.70
MaWa	21.69	5.24	4.25
MaWp	9.81	3.62	2.75
MaSi	18.65	4.30	4.40
MaSu	10.37	3.94	2.69
MaSm	8.22	3.22	2.62

were 5.24 μm, 4.30 μm, 4.30 μm, 3.83 μm, 3.94 μm, and 3.83 μm, respectively. The conidial width of isolates varied in size from 3.11 to 5.24 μm, resulting in a length/width ratio in the range of 2.30 to 4.25 μm. Based on the value of length/width ratio of conidia, *Metarhizium* isolates were divided into two groups: the high ratio group with length/width ratio ≥ 3.00 μm, were the isolates MaWa, MaSi, and MaLe, while other isolates included in

the medium ratio group with length/width ratio of 2.29 to 2.88 μm .

Molecular characterization of *Metarhizium* isolates based on the ITS rDNA region

Molecular identifications of 13 isolates of *Metarhizium* using the ITS rDNA region were intended to determine the identity of the investigated fungi clustered in certain clades. The ITS region is one of the common markers used to identify a broad group of fungi (Raja et al. 2017). PCR product of rDNA region amplification using ITS1 and ITS4 primer was purified and subsequently analyzed to determine the nucleotide sequence. Analysis of phylogenetic was carried out to confirm the taxonomic identity of isolates involving 8 reference fungal species listed in the GenBank. The amplification of the ITS region resulted in a single product with a size of ± 540 bp in all isolates (Fig. 3). Phylogenetic analysis classified fungal isolates into 4 clades of *Metarhizium*. The isolates MaSu, MaWp, MaBe, MaJe, MaSi, MaBl, and MaKa were clustered in the clade *Metarhizium anisopliae* var. *lepidiotae*, with isolates MaBl and MaKa exhibiting a very close similarity to strains ARSEF4587, M32, ACCC30104. In addition, the isolate MaWp belonged to the clade *M. anisopliae* var. *lepidiotae*, but the long genetic distance allows it to be suggested as a new subtype. The isolates MaBo and MaBt were shown to be very close and they were clustered in the *M. anisopliae* var. *anisopliae* clade, while the isolate of MaSm was separately classified in *M. brunneum*. Isolate MaMa proved to be very close to the strain ARSEF 1946, adjacent to isolates of MaWa and MaLe, were clustered in the *M. majus* clade (Fig. 4).

Bioassays test of *Metarhizium* spp. isolates against the *O. rhinoceros* larvae

The pathogenicity evaluation of 13 *Metarhizium* isolates determined by observing *O. rhinoceros* mortality for 15 days after inoculation. The stages of infection of *O. rhinoceros* larvae by *Metarhizium* are shown in (Fig. 5),

starting with the formation of whitish conidia on the surface of the larvae, then the green conidia were growth to covering the whole of larval cadavers after 7 days of dead. The best conidia concentration was determined based on a laboratory bioassay by preparing a dilution series of aqueous conidial suspension (1.0×10^4 , 1.0×10^5 , 1.0×10^6 , 2.0×10^7 , 1.0×10^8 conidia/ml) from four isolates randomly, then sprayed on *O. rhinoceros* larvae. The result showed that the concentration (1.0×10^7 conidia/ml) of *Metarhizium* conidial suspension killed 51% of the test specimens 8 days after inoculation (Fig. 6), the highest larval mortality rate and significantly different than other concentration levels. These concentrations were used in the pathogenicity test of 13 *Metarhizium* isolates.

Mortality percentage of *O. rhinoceros* larvae was significantly different 9 days after inoculation, and more than 50% mortality rate was observed 10 days after inoculation in most of the *Metarhizium* isolates (Table 3). Interestingly, the isolates MaMa and MaLe resulted in a high larval mortality rates of *O. rhinoceros* of 93.33 and 83.33%, respectively. The observation at 11 days after inoculation, the average mortality rate of larvae reached $>60\%$, and a high increased in mortality occurred in MaWa isolates, which reached 96.67%. Out of all tested isolates, the isolate MaWa showed the highest mortality rate in the shortest time, reaching 100% at 13 days after inoculation, followed by the isolates of MaMa, Male, MaSi, with mortality rates of 93.33, 96.67, and 93.33%, respectively. The other isolates achieved a 90% mortality rate at 15 days after inoculation (Table 4).

Metarhizium virulence is determined by the ability of the isolates to kill the test specimens in a short time. The mortality value of infected *O. rhinoceros* larvae was performed by probit analysis to determine the lethal time at 50 percent mortality (LT_{50}) at the concentration of 10^7 conidia/ml. The results showed that the LT_{50} values for *Metarhizium* isolates varied from 8.21 to 9.96 days (Table 5). The isolate MaMa showed a high virulence against infected *O. rhinoceros* larvae with LT_{50}

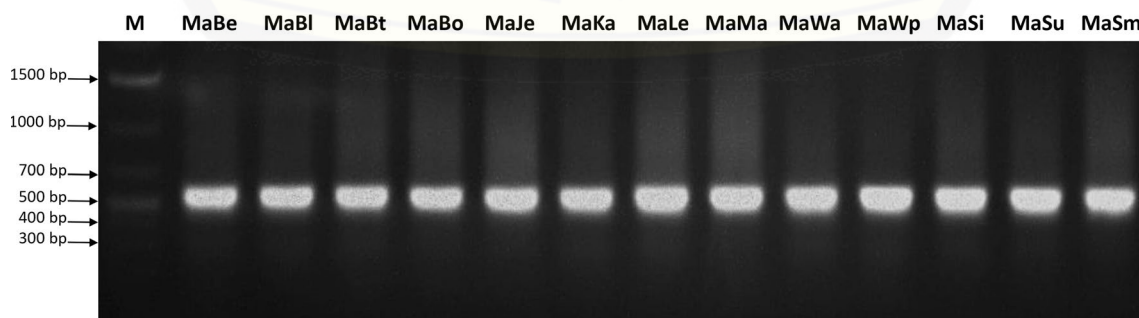
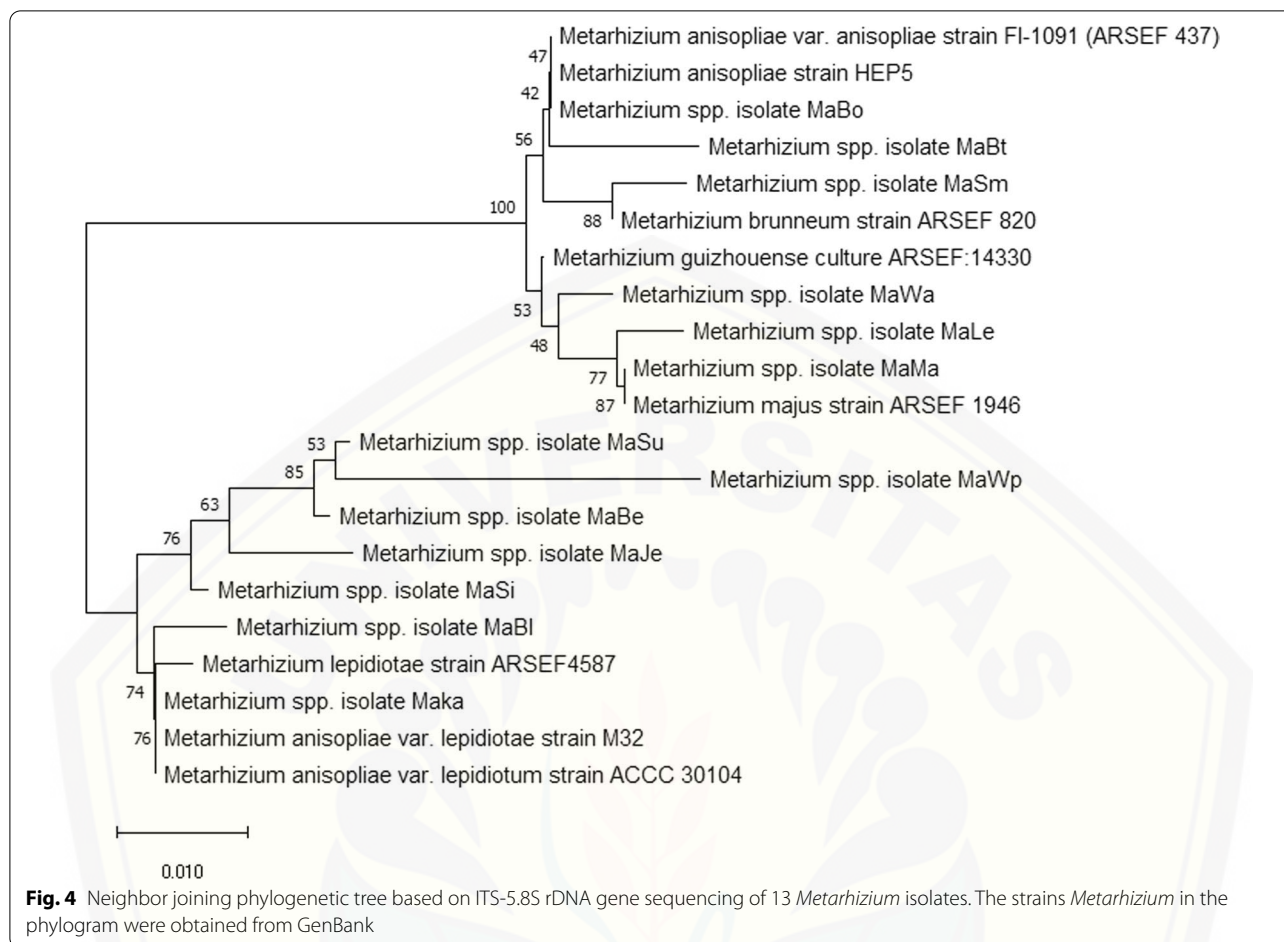


Fig. 3 PCR products amplified with ITS primers from 13 isolates of *Metarhizium*



values ranging from 4.47 to 9.64 days and an average of 8.21 days. The adjacent LT_{50} values shown by isolates MaWa and MaSm were 8.44 and 8.41 days, respectively. They were proposed as candidate isolates with a high virulence to kill *O. rhinoceros* larvae in a short time.

Discussion

Exploration and identification of EPF as a biological agent to control pests have been widely reported, especially *Metarhizium* isolates from infected insect cadavers with various degrees of pathogenicity (Visalakshi et al. 2020). The characterizations of these fungi can be partially demonstrated based on morphological traits, as well as size and shape of the conidia (Ayele et al. 2020). A circular form, thick or smooth hyphae, a mat-like mass of conidia, and greenish color on the surface are common features shown in the morphology of *Metarhizium* colonies on culture media and the surface of infected insect cadavers (Mathulwe et al. 2021). A similar morphological appearance was also produced by *Metarhizium* colonies on both PDA medium and infected *O. rhinoceros* larvae in this study. The formation of white conidia on the

surface of the larvae at the early stage of infection, then the green conidia spread to cover the whole larval cadavers (Sutanto et al. 2021).

The taxonomic identity of *Metarhizium* isolates was divided into 4 clades, namely *M. anisopliae* var. *lepidiotae*, *M. anisopliae* var. *anisopliae*, *M. brunneum*, and *M. majus*. The phylogenetic analysis showed that about 76% of the *Metarhizium* isolates were clustered into the PARB clades, and 69% of them belonged to the *M. anisopliae* clade. The outperforming percentage isolates prove that *M. anisopliae* is the most abundant species, widely distributed in soil with various environments, and has broad host ranges (St. Leger et al. 2020). The isolates Male, MaMa, and MaWa, which belonged to the *M. majus* clade, had the largest conidia size than other isolates, ranging from 10.13 to 21.69 μm in length and from 3.83 to 5.24 μm in width. These results supported that the three isolates were classified as *M. majus*, which morphologically had the largest conidia size of all *Metarhizium* species, namely $>10 \mu\text{m}$ in length and $>4 \mu\text{m}$ in width (Mathulwe et al. 2021). In addition, Nishi et al. (2015) reported that the conidial size of the

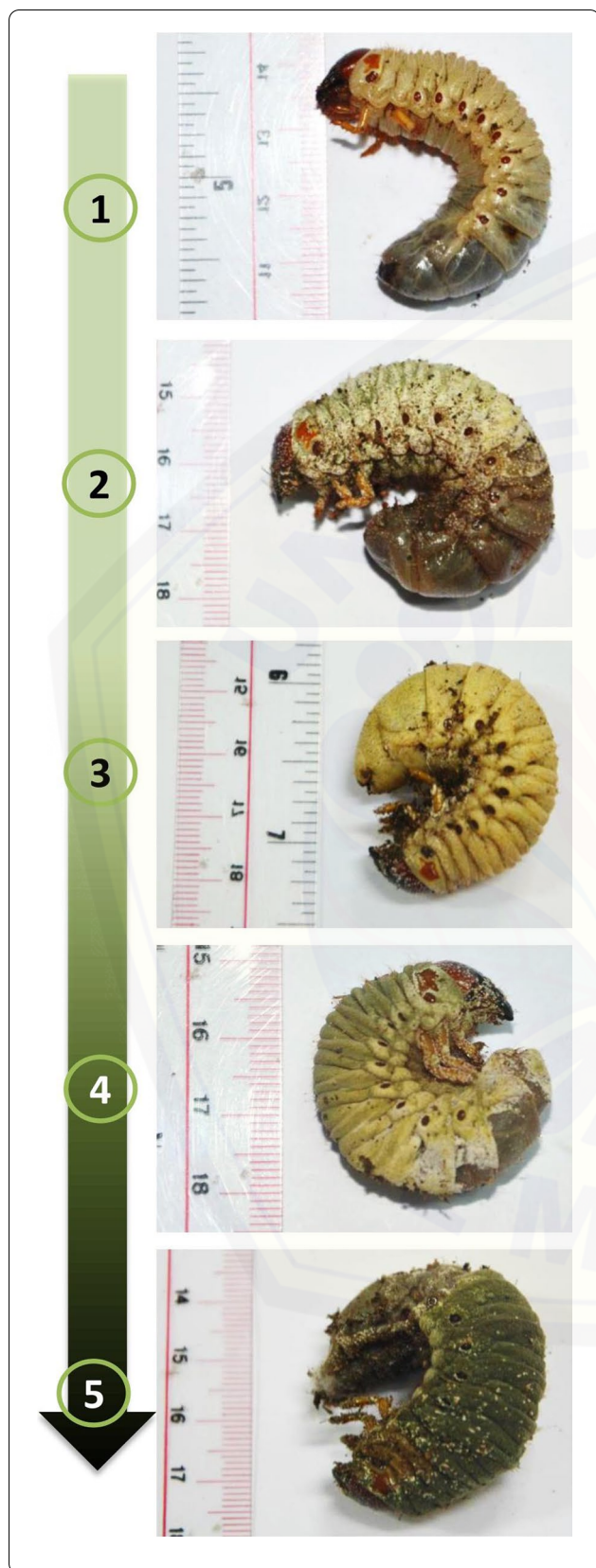
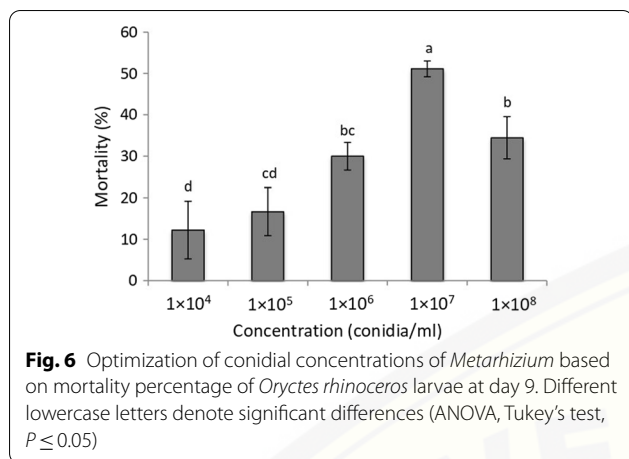


Fig. 5 Stages of *Oryctes rhinoceros* larvae infected by *Metarhizium*. (1) Healthy larvae, (2) 2 days after dead, whitish growth of conidia on larvae, (3) conidia sporulation were covering the whole of larvae after 4 days of dead, (4) in the next stage, the appearance of conidia becomes greenish after 5 days of dead, (5) the green conidia were covering the whole of larval cadavers after 7 days of dead

isolates of *M. majus* varied from 5.1 to 13 μm in length and from 2.9 to 3.2 μm in width, and the largest conidia exhibited by the ARSEF 1946 isolate. In this study, phylogenetic analysis showed that MaWa and MaLe isolates, adjacent to MaMa isolate were very similar to the ARSEF 1946 strain, but they displayed larger conidia sizes. These results indicated that the size of the *Metarhizium* conidia varies in each species, influenced by factors such as strains and culture media nutrition (Oetari et al. 2020). For example, the *M. anisopliae* var. *anisopliae* had conidial lengths, were 5.0–7.5 \times 2.0–3.5 μm (Mongkolsamrit et al. 2020), and consistently also exhibited by isolates MaBt and MaBo. In another case, Sepulveda et al. (2016) reported that five strains of *M. anisopliae* grown on PDA medium had a low conidia length and width values, ranging from 4.8–5.5 \times 1.6–2.2 μm . A wider range of medium-length of conidia was produced by *M. brunneum* of 4.5–9 \times 2–3 μm (Mongkolsamrit et al. 2020), and isolate MaSm was in this range. The isolates of *M. anisopliae* var. *lepidiotae* (MaSu, MaWp, MaBe, MaJe, MaSi, MaBl, and MaKa) showed conidial sizes in the range of 7.0–10 \times 3.0–4.0 μm , which is in accordance with previous studies (Navarro-Barranco et al. 2019). On the other hand, the isolate MaSi produced conidial lengths of 18.65 \times 4.30 μm , well above the range of conidial sizes recorded in the *M. Anisopliae* var. *lepidiotae*. The large conidia exhibited by isolate MaSi placed the *M. anisopliae* in the MGT clade, commonly referred to as *M. anisopliae* var. *majus* or known as MALC (*M. anisopliae* s.l. with large conidia) (Tulloch 1976). Several studies reported that isolates of *M. majus* and MALC are biological control agent for the coconut rhinoceros beetle (Moslim et al. 2006), and *O. rhinoceros* is known to be a host of MALC (Nishi et al. 2015).

Bioassays have been conducted on *O. rhinoceros* larvae with TCS (Topical conidial suspension) formulation containing the *Metarhizium* isolates (identified in the clades of *M. robertsii*, *M. anisopliae*, and *M. majus*), and the highest mortality was produced by *M. majus* (90% at 7 days after inoculation) with the highest virulence (low median Lethal concentration value) (Velavan et al. 2018). In the present investigation, the isolates MaLe, MaMa, MaWa, and MaSi, resulted in higher mortality value than other isolates (at 13 days after inoculation), supporting



that *M. majus* dan MALC had the highest pathogenicity against *O. rhinoceros* larvae than of *M. anisopliae* and *M. brunneum*. Furthermore, the highest larval mortality of *O. rhinoceros* was observed in MaMa isolate (93%) at 10 days after inoculation, significantly different than the other isolates. Neighbor-joining analysis showed a close resemblance between the MaMa isolate and the strain ARSEF 1946, which was known to be isolated from the coconut *rhinoceros* beetle. The highest mortalities of fruit beetle larvae were produced by Isolates Fkk38-1 and Hn1, which identified as *M. majus* and MALC based on their placement in the phylogeny of the DNA sequence of 5'-EF1- α and were very closely related to ARSEF 1914

and ARSEF 1946 strains (Nishi et al. 2015). This investigation indicated a possible relationship between phylogenetic status or DNA sequence polymorphisms with metarhizium pathogenicity and host specificity.

The *M. majus* and MALC isolates do not always infect or have a high virulence to their original hosts. *Metarhizium* distinction virulence against insect host is influenced by many factors including environment such as temperature and pH, strains, and culture media nutrition (Oetari et al. 2020). In the case of culture media, for example, decreasing the C/N ratio in the medium resulted in lowers radial growth, protease activity, and virulence of *Metarhizium* (Safavi et al. 2007). The mean radial growth among *Metarhizium* isolates also varied when grown on different culture media (PDA/potato dextrose agar or SDAY/Sabouraud dextrose agar) (Hoe et al. 2009). In the present investigation, isolates of *Metarhizium* were cultured on PDA media for 14 days, and the isolates MaWa and MaSi produced lower mean radial growth than the other isolates. On the other hand, the high mortality and virulence (lower LT_{50} value) was observed in both isolates, and it was concluded that colonial growth influenced by culture media was not related to the pathogenicity and virulence of the *M. majus* isolates. These results are supported by a previous study (Oetari et al. 2020), which reported that *M. majus* was consistently virulent on *O. rhinoceros* larvae, regardless of the different substrates in the media.

Variation in the virulence of the tested isolates of *Metarhizium* was closely related to the capacity of the

Table 4 Mortality percentage of *Oryctes rhinoceros* larvae treated with 10^7 conidia/ml of *Metarhizium* isolates

Isolates	Day after inoculation								
	3	6	8	9	10	11	13	14	15
MaBe	0.00	23.33	46.67	56.67 a	63.33 cde	70.00 c	73.33 d	76.67 c	86.67 b
MaBl	0.00	20.00	30.00	36.67 b	50.00 de	70.00 c	80.00 cd	86.67 bc	90.00 ab
MaBo	0.00	6.67	20.00	36.67 b	46.67 e	63.33 c	80.00 cd	86.67 bc	96.67 a
MaJe	0.00	13.33	26.67	56.67 a	76.67 bc	83.33 bc	90.00 bcd	90.00 abc	93.33 ab
MaKa	0.00	26.67	36.67	56.67 a	60.00 cde	63.33 c	73.33 d	83.33 bc	86.67 b
MaLe	0.00	6.67	23.33	63.33 a	83.33 b	86.67 ab	96.67 ab	96.67 ab	96.67 a
MaMa	0.00	13.33	26.67	66.67 a	93.33 a	93.33 a	93.33 abc	96.67 ab	96.67 a
MaWa	0.00	20.00	36.67	53.33 ab	70.00 bc	96.67 a	100.00 a	100.00 a	100.00 a
MaWp	0.00	20.00	33.33	53.33 ab	66.67 cd	70.00 c	80.00 cd	86.67 bc	86.67 b
MaSi	0.00	16.67	36.67	46.67 ab	66.67 cd	80.00 bc	93.33 abc	93.33 abc	93.33 ab
MaSu	0.00	10.00	36.67	60.00 a	73.33 bc	83.33 bc	90.00 bcd	93.33 abc	96.67 a
MaSm	0.00	26.67	40.00	53.33 ab	70.00 bc	76.67 bc	83.33 bcd	86.67 bc	90.00 a
F value		1.95 ns	1.11 ns	5.18**	24.74**	12.24**	8.39**	4.78**	2.46*
HSD value				6.91	6.29	14.85	17.82	19.23	23.08

Different lowercase letters denote significant differences (ANOVA, Tukey's test, $P \leq 0.05$)

ns, not significantly different

*significantly different ($P \leq 0.05$); **significantly different ($P \leq 0.01$)

Table 5 LT₅₀ with 95% confidence limits following infection of *Oryctes rhinoceros* with *Metarhizium* isolates at a concentration of 10⁷ conidia/ml

Isolates	LT ₅₀ (day)	Slope (b) ± SE
MaBe	8.72 (6.72–10.42)	− 5.29 ± 0.79
MaBl	9.42 (3.32–11.32)	− 2.30 ± 2.49
MaBo	9.96 (8.23–10.84)	− 3.86 ± 2.04
MaJe	8.69 (7.12–9.73)	− 4.89 ± 1.39
MaKa	8.89 (6.64–10.64)	− 4.71 ± 0.93
MaLe	8.63 (7.08–9.51)	− 5.37 ± 1.71
MaMa	8.21 (4.47–9.64)	− 4.73 ± 1.72
MaWa	8.44 (6.51–9.89)	− 1.25 ± 3.29
MaWp	8.89 (6.87–10.33)	− 4.74 ± 1.08
MaSi	8.69 (6.25–9.97)	− 3.65 ± 1.82
MaSu	8.58 (7.25–9.51)	− 5.36 ± 1.35
MaSm	8.41 (6.44–9.87)	− 4.88 ± 0.99

fungus to secrete insect cuticle degrading enzymes (proteases and chitinases), which is also correlated with pathogenic conidia that easily penetrate the host, rapidity of penetration and colonization, and mortality of target pests (Santos et al. 2018). Moreover, this fungus also releases toxins that are correlated with their EPF, called destruxin (Petlamul and Prasertsan 2012). In vivo virulence test by measuring the activity of extracellular cuticle-hydrolyzing enzymes and toxins content will be the focus of further research, which is needed to select the most virulent *Metarhizium* strain against the target pests.

Conclusions

The local species of *Metarhizium* were isolated from infected CRB larvae, and *M. majus* isolates exhibited a high pathogenicity against *O. rhinoceros* larvae. The close similarity of *M. majus* isolates and CRB-isolated strains suggests a possible relationship between pathogenicity and host specificity with phylogenetic status or DNA sequence polymorphisms. The identification of new local species from infected host pests provides a great opportunity to obtain biological control agents with standard properties for development of biopesticides.

Abbreviations

EPF: Entomopathogenic fungi; CRB: Coconut rhinoceros beetle; ITS: Internal transcribed spacer; MALC: *M. anisopliae* S.l. with large conidia; MGT: *majus guizhouense taii*; PARB: *pinghaense anisopliae robertsii brunneum*; PDA: Potato dextrose agar.

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Author contributions

SP and S conceived and designed this study. SP conduct the experiments, recorded data, and interpreted the results. SP, S, and TH provide validation and data curation. SP, S, HSA wrote the manuscript, editing, and review. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this manuscript.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹Study Program of Plant Protection, Faculty of Agriculture, Universitas Jember, Jl. Kalimantan Tegalboto No.37, East Java 68121, Indonesia. ²Study Program of Plant Protection, Faculty of Agriculture, Universitas Jember, Jl. Kalimantan Tegalboto No.37, East Java 68121, Indonesia. ³Applied Molecular and Microbial Biotechnology (AM2B) Research Group, University of Jember, Jl. Kalimantan Tegalboto No.37, East Java 68121, Indonesia. ⁴Department of Agronomy, Faculty of Agriculture, University of Jember, Jl. Kalimantan Tegalboto No.37, East Java 68121, Indonesia.

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