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Endophytic fungi confirmed as entomopathogen of a new invasive pest, Spodoptera frugiperda infesting maize in South Sumatra, Indonesia

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Abstract:	Background: Spodoptera frugiperda is a new invasive pest in Indonesia and caused financial losses. S. frugiperda larvae hide in the leaf midrib all day. To overcome the hidden larvae, it needs pathogenic endophytic fungi. The objective of this research was to molecularly identify the endophytic fungal species from South Sumatra and determine the most pathogenic species against S. frugiperda larvae. Molecular identification was based on morphological characteristics and molecular- based gene sequential analysis of Intergenic Transcribed Spacer (ITS) 1 and ITS 4. The bioassay of 20 isolates of endophytic fungi was used the second larvae instar of S. frugiperda . Result: The results of molecular identification were 20 isolates of the endophytic fungi consisted of 7 species, namely Chaetomium sp. (GaTpeOi, JgTjPr, PiCrPga, and CaCjPga isolates), Aspergillus niger (PsgTjPr, JgByU, and JaBuBys), Beauveria bassiana (JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, dan JaTpOi (1) isolates), Curvularia lunata (JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr isolates), Aspergillus flavus (JgPWSR isolate), Penicillium citrinum (JaTpOi(2) isolate), and Metarhizium anisopliae (CaTpPGA isolate). All isolates have been deposited in the GenBank. The endophytic fungi species of B. bassiana (JgSPK, JaGiP, JgCrJr, JaTpOi1 isolates) causing up to 22.67% mortality, C. lunata (JaSpkPga(3) isolate) causing 17.33% mortality, and M. anisopliae (CaTpPga isolate) causing 8% mortality were pathogenic against S. frugiperda larvae. First report of B. bassiana and C. lunata isolated from maize, and M. anisopliae isolated from red chili in Indonesia were found as entomopathogenic endophytic fungi against S. frugiperda larvae. Conclusion: The endophytic fungi species of B. bassiana , C. lunata , and M. anisopliae had high potential as entomopathogens of S. frugiperda .
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Endophytic fungi confirmed as entomopathogen of a new invasive pest, *Spodoptera frugiperda* infesting maize in South Sumatra, Indonesia

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Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data are available in the article and the materials used in this work are of high quality and grade.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

SH performed research concept and design, writing the article, and final approval of article. MG performed collection and assembly of data. SS prepared and performed morphological identification and critical revision of the article. RS performed and performed molecular identification and data analysis and interpretation. JMPS performed collection and assembly of data. RPL performed collection and assembly of data. All the authors read and approved the manuscript.

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Abstract

Background: *Spodoptera frugiperda* is a new invasive pest in Indonesia and caused financial losses. *S. frugiperda* larvae hide in the leaf midrib all day. To overcome the hidden larvae, it needs pathogenic endophytic fungi. The objective of this research was to molecularly identify the endophytic fungal species from South Sumatra and determine the most pathogenic species against *S. frugiperda* larvae. Molecular identification was based on morphological characteristics and molecular- based gene sequential analysis of Intergenic Transcribed Spacer (ITS) 1 and ITS 4. The bioassay of 20 isolates of endophytic fungi was used the second larvae instar of *S. frugiperda.*

Result: The results of molecular identification were 20 isolates of the endophytic fungi consisted of 7 species, namely *Chaetomium* sp. (GaTpeOi, JgTjPr, PiCrPga, and CaCjPga isolates), *Aspergillus niger* (PsgTjPr, JgByU, and JaBuBys), *Beauveria bassiana* (JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, dan JaTpOi (1) isolates), *Curvularia lunata* (JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr isolates), *Aspergillus flavus* (JgPWSR isolate), *Penicillium citrinum* (JaTpOi(2) isolate), and *Metarhizium anisopliae* (CaTpPGA isolate). All isolates have been deposited in the GenBank. The endophytic fungi species of *B. bassiana* (JgSPK, JaGiP, JgCrJr, JaTpOi1 isolates) causing up to 22.67% mortality, *C. lunata* (JaSpkPga(3) isolate) causing 17.33% mortality, and *M. anisopliae* (CaTpPga isolate) causing 8% mortality were pathogenic against *S. frugiperda* larvae. First report of *B. bassiana* and *C. lunata* isolated from maize, and *M. anisopliae* isolated from red chili in Indonesia were found as entomopathogenic endophytic fungi against *S. frugiperda* larvae.

Conclusion:

The endophytic fungi species of *B. bassiana*, *C. lunata*, and *M. anisopliae* had high potential as entomopathogens of *S. frugiperda*.

Keywords: *Aspergillus flavus*, *Aspergillus niger*, *Beauveria bassiana*, *Chaetomium* sp., *Curvularia lunata*, fall armyworm, *Metarhizium anisopliae*, *Penicillium citrinum*

Background

Fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae) is a new invasive pest originating from South America (Otim et al. 2018). Currently, the fall armyworm has spread throughout the world and first entered Indonesia on March 2019 in West Sumatra (Sartiami et al. 2020; Nelly et al. 2021) and July 2019 in South Sumatra (Hutasoit et al. 2020) and now has been spreading throughout Indonesia, such as Bengkulu (Mukkun et al. 2021), Lampung (Trisyono et al. 2019; Lestari et al.

2020), West Java (Maharani et al. 2019), East Nusa Tenggara (Mukkun et al. 2021), Bali (Supartha et al. 2021). The pest destroys maize and various other plant species (Montezano et al. 2018). The financial losses caused by this pest are up to 250-630 million US dollars per year in Africa (Bateman et al. 2018). This pest destroys maize by means of its larvae stage eating leaves, stems, flowers, fruits, growing points, and the whole maize until it is bare (Montezano et al. 2018; Ginting et al. 2020).

Larvae which are the destructive stage of *S. frugiperda* can be found on the surface of leaves or maize stalks in the morning around 6.30 to 8.00 a.m. and after that the larvae hide in the leaf midrib (Gustianingtyas et al. 2021). It is difficult to control these pests because they hide all day in the leaf midrib. However, the results of experiments in the laboratory showed that the entomopathogenic fungi, *Metarhizium* sp. can kill *S. frugiperda* larvae up to 78% (Herlinda et al. 2020) and *Beauveria bassiana, Metarhizium anisopliae*, and *Metarhizium rileyi* also kill *S. frugiperda* larvae more than 80% (Ramanujam et al. 2020), but these treatments were treated topically (contact) with the larvae exposed. Yet, in the field the presence of larvae hiding all day in the midrib of maize leaves causes the entomopathogenic fungi whose mode of action is contact to be less effective (Gustianingtyas et al. 2021).

To overcome the hidden larvae of *S. frugiperda*, it needs entomopathogenic fungi living in plant tissues or known as endophytic fungi (Ramos et al. 2020; Gustianingtyas et al. 2021). The endophytic fungi systemically colonize plant tissues and associate mutually with their host plants (Lira et al. 2020). The results of previous studies have proven that eight isolates of endophytic entomopathogenic fungi could topically kill *S. frugiperda* larvae (Gustianingtyas et al. 2021), however, the endophytic fungi in this study were only isolated from roots and the molecular identification had not yet been carried out. In this study, the fungi were isolated from leaves, shoots, and roots and identified molecularly. In addition, the application method in this study was not topical but by mouth by means of the larvae eating plants that had been colonized by the fungus. This study aimed to molecularly identify the endophytic fungal species from South Sumatra (Indonesia) and determine the most pathogenic fungal species against *S. frugiperda* larvae.

Methods

The fungal exploration was carried out in South Sumatra. The purification and identification, and bioassay of fungi were conducted from January to March 2021. The endophytic fungal species were identified based on the molecular analysis carried out at a laboratory accredited according to the ISO 17025 standard. Bioassay of the fungi was carried out in an incubator at controlled and constant temperature and the relative humidity, respectively 25 ºC and 97%.

Exploration, isolation, and purification of fungi

The exploration of endophytic fungi was carried out by taking the leaves, shoots, and roots of maize (*Zea mays*) and other crop plants around it, such as bananas (*Musa*

sp.), ridged gourd (*Luffa acutangula*), and red chilies (*Capsicum annuum*). The selection of individual crop plant samples to obtain the endophytic fungi followed the method of Kasambala et al. (2018), which was the healthiest plant. The taken plant parts were put into an ice box, then taken to the laboratory. The exploration of endophytic fungi was carried out from the lowlands to the highlands of South Sumatra, Indonesia namely in Banyuasin District (2.8833°S 104.3831°E), Ogan Ilir District (3.43186°S 104.62727°E), Prabumulih City (3.4328°S 104.2356°E), Pagar Alam City (3°52′43.8″S 103°21′30″E) (Fig. 1 and Table 1).

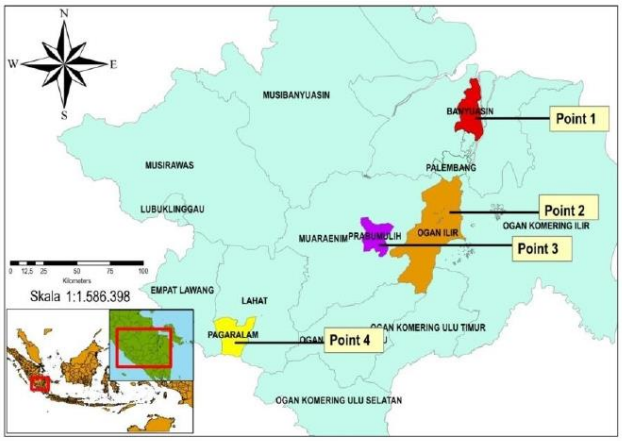

Fig. 1 Surveys locations for endophytic fungi in South Sumatra, Indonesia: Point 1 = Banyuasin District, Point 2 = Ogan Ilir District, Point 3 = Prabumulih City, and Point 3 = Pagar Alam City

The leaves, shoots, and roots of the sample plants were cleaned and washed aseptically with running tap water. Then, the plant parts were surface sterilized using method of Elfita et al. (2019) with 70% EtOH (Ethyl alcohol) and 1% NaOCl (Sodium hypochlorite), then rinsed three times. The last rinse water was used to check whether the microorganisms causing surface contamination were still present and carried out by growing them onto Sabouraud Dextrose Agar (SDA) (Russo et al. 2020).

To obtain endophytic fungi isolates, it started by growing in the media the plant parts that have been sterilized earlier. The roots were grown on a specific medium, the malt extract agar (MEA) following the method of Silva et al. (2018). The shoots and roots of the sample plants were grown on SDA media. The plant parts were grown as many as five pieces per petri dish with a length of 5 mm and a diameter of 1-5 mm (depending on the diameter of the roots and shoots). Fungi growing from the leaves, shoots, and roots were purified to be one isolate per sample. The isolates

formed were then observed for their macroscopic and microscopic characteristics and continued with the fungal colonization test into the maize seed tissue.

Table 1 The origin of isolates of endophytic fungi from South Sumatra, Indonesia

Location (village, district/city)	Isolate origin	Altitude (m)	Fungal species	Fungal isolate code	GenBank Acc. No.
Tanjung Pering. Ogan Ilir	Ridged gourd	36.0	*Chaetomium* sp.	GaTpeOi	MZ359734
Tanjung Pering. Ogan Ilir	Bananas	36.0	*Aspergillus niger*	PsgTjPr	MZ242060
Simpang Padang Karet. Pagar Alam	Maize	797.7	*Beauveria bassiana*	JgSPK	MZ356494
Tanjung Pering. Ogan Ilir	Maize	36.0	*Chaetomium* sp.	JgTjPr	MZ359736
Gunung Ibul. Prabumulih	Maize	53	*Beauveria bassiana*	JaGiP	MZ356495
Curup Jare. Pagar Alam	Bananas	806	*Chaetomium* sp.	PiCrPga	MZ359735
Gunung Ibul. Prabumulih	Maize	53.0	*Curvularia lunata*	JaGiPRB	MZ359815
Tanjung Payang. Pagar Alam	Red chilies	689,6	*Curvularia lunata*	CMTJP	MZ359816
Mulia Sari. Banyuasin	Maize	19.0	*Curvularia lunata*	JaMsBys	MZ359819
Simpang Padang Karet. Pagar Alam	Maize	789.5	*Beauveria bassiana*	JaSpkPGA(2)	MZ356496
Curup Jare. Pagar Alam	Maize	806.7	*Beauveria bassiana*	JgCrJr	MZ356497
Tanjung Pering. Ogan Ilir	Maize	36.0	*Beauveria bassiana*	JaTpOi (1)	MZ356498
Simpang Padang Karet. Pagar Alam	Maize	797.7	*Curvularia lunata*	JaSpkPga(3)	MZ359818
Curup Jare. Pagar Alam	Red chilies	806.0	*Chaetomium* sp.	CaCjPga	MZ359737
Banyuurip. Banyuasin	Maize	13.0	*Aspergillus niger*	JgByU	MZ242059
Telang Sari. Banyuasin	Maize	15.0	*Curvularia lunata*	JgTgSr	MZ359817
Banyuurip. Banyuasin	Maize	20.0	*Aspergillus niger*	JaBuBys	MZ242058
Purwosari. Banyuasin	Maize	15.0	*Aspergillus flavus*	JgPWSR	MZ359829
Tanjung Pering. Ogan Ilir	Maize	36.0	*Penicillium citrinum*	JaTpOi(2)	MZ359812
Tanjung Payang. Pagar Alam	Red chilies	689.6	*Metarhizium anisopliae*	CaTpPga	MZ242073

Assess endophytic fungal colonization

Fungal inoculation on maize seeds was carried out to observe the colonization of fungi into the maize seed tissue and to further ensure that the isolates found were truly endophytic. The 15 seeds of maize were surface sterilized using 70% EtOH (Ethyl alcohol) and 1% NaOCl (Sodium hypochlorite) (Elfita et al. 2019). The seeds were immersed in 10 mL of fungal suspension with a concentration of 1 x 10^6 conidia mL^{-1} for 6 hours, while for the control the sterilized seeds were only immersed in 10 mL of destilled water. Then, 5 seeds were grown in a sterile glass bottle (250 mL volume) with a sterile filter paper (whatman no. 42) at the bottom which was moistened with 1 mL destilled water and incubated for 10 days. All the treatments in this experiment were repeated three times. After the maize seedlings were 10 days

old, the stems and leaves were sliced crosswise and longitudinally with a thickness of 0.02 mm each. The slices were observed with a light microscope at 40 x magnification with 0.05% lactofenol trypan blue dye. Mycelia endophytic fungi found in the maize seed tissue were documented. The fungal isolates that were proven to be endophytic were used for bioassays. Prior to the bioassay, the isolates were observed for macroscopic and microscopic characteristics for morphological identification and followed by molecular identification.

Molecular identification of fungal species

Macroscopic and microscopic observation

Morphological observations were carried out based on the microscopic and macroscopic characteristics of endophytic fungi. Macroscopic observations of the fungi were observed, such as the colony color and shape, while the microscopic observations were the shape and size of conidia and conidiophores following the method of Herlinda et al. (2020).

DNA extraction and PCR amplification

The used endophytic fungal DNA extraction method refers to the Swibawa et al. (2020) and Lestari et al. (2020) method. DNA extraction was carried out on fungal conidia. The conidia were harvested from pure cultures of 7 days old fungus which was cultured in sterile petri dishes with a diameter of 9 cm. As much as 10 mL of sterile water was put into a petri dish containing fungal cultures and slowly harvested using a drigalski. The obtained conidia suspension was transferred to a centrifuge tube (volume 30 mL) and centrifuged using CF15RXII (Hitachi, Japan) for 10 minutes at a speed of 14,000 rpm. After being centrifuged, 1 mL of 70% ethanol was added to the centrifuge tube and then centrifuged again for 10 minutes. After that, the supernatant was removed and 1 mL of extraction buffer was added with a composition of 0.5 mL Tris HCl, 1 mL SDS 1% + 2.8 mL NaCl, 0.2 mL Mercaptho Ethanol, 2 mL EDTA, 3.5 mL sterile water and then homogenized. The suspension was transferred to a sterile mortal and incubated at -40 ºC for 24 hours. After that, the frozen suspension was ground until it was smooth and melted. A total of 500 µL of suspension was then transferred into a 1.5 mL tube. A total of 400 µL of 2% cetyltrimethylammonium bromide (CTAB) was then added to the tube, homogenized and then incubated at 65 ºC for 1 hour using a water bath (Brookfield TC 550 MX-230, USA). After the incubation, 500 µL of Phenol Chloroform Isoamyl alcohol (PCI) (25:24:1) was added, homogenized and centrifuged (Microspin12; Biosan, Latvia) at 14,000 rpm for 10 minutes. A total of 600 µL of supernatant was taken and transferred to a new 1.5 mL tube. A total of 600 µL Chloroform Isoamyl Alcohol (CI) (24:1) was added to the tube, homogenized and centrifuged (Microspin12; Biosan, Latvia) at 14,000 rpm for 10 minutes. A total of 400 µL of supernatant was then transferred to a new 1.5 mL tube and 400 µL of cold isopropanol was added, homogenized, and incubated at -40 ºC for 20 minutes. After the incubation, it was centrifuged (Microspin12; Biosan, Latvia) at 14,000 rpm for 15 minutes. The

supernatant was then discarded. After the supernatant was removed, 500 µL of 70% cold ethanol was added and centrifuged (Microspin12; Biosan, Latvia) at 14,000 rpm for 5 minutes. The supernatant was then discarded and the pellets obtained were dried at room temperature for 24 hours. After drying, the tube was then added as much as 50 µL 1x Tris-HCL EDTA (TE) pH 8.0 (1st Base Malaysia).

PCR amplification was carried out using the Sensoquest Thermal Cycler (Germany) PCR machine on the Internal Transcribed Spacer (ITS) using ITS1 and ITS4 primers (White et al. 1990). The PCR was carried out with a total volume of 25 µL consisting of a mixture of Master Mix (Red Mix) (bioline) as much as 12.5 µL, 10 µM of primer ITS 1 (5'TCC GTA GGT GAA CCT TGC GG 3 ') and ITS 4 (5'TCC TCC GCT TAT TGA TAT GC 3') 1 µL each, 1 L of template DNA and 9.5 µL of sterile water. The DNA amplification stage consisted of 1 initiation cycle at 95 ºC for 5 minutes, followed by 30 cycles consisting of denaturation at 95 ºC for 1 minute, primer attachment at 52 ºC for 1 minute, primer extension at 72 ºC for 1 minute, and 1 elongation cycle at 72 ºC for 5 minutes. The PCR results were then electrophoresed using 0.5% agarose in 20 mL of 1x Tris-Boric Acid-EDTA (TBE) buffer (1st Base Malaysia) and added 1 µL of Ethidium Bromide (EtBr 10 mg/mL). The electrophoresis was carried out in 1x TBE buffer solution at 50 volts for 70 minutes. Electrophoresis results were visualized using a DigiDoc UV transilluminator (UVP, USA).

Sequencing and analysis of the results

The PCR results were then sent to 1st Base Malaysia for the sequencing process. The results of the sequencing were analyzed using Bio Edit ver. 7.2.6 for windows. Then, the results were submitted to the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to find out the genus or species that had the greatest homology or similarity and molecularly. The phylogeny tree was created using the Mega 7 for Windows program (Kumar *et al.* 2016) using the UPGMA (jukes and cantor model) method. The ITS region sequences for several strains used as a reference in this study were obtained from NCBI (https://www.ncbi.nlm.nih.gov/).

Calculation of conidial density and viability

All the isolates of endophytic fungi were counted for their conidial density and viability. The conidial density of the 7 old days fungi was calculated according to the method of Sumikarsih et al. (2019) using a haemocytometer and observed with a light microscope at 40 x magnification. The viability was observed by growing 1 mL of conidia fungal suspension (1 x 10^6 conidia mL^{-1}) onto 2% agar-water medium, then the culture was incubated for 1 x 24 hours and 2 x 24 hours under a light microscope at 40 x magnification.

The mass rearing of *Spodoptera frugiperda*

The mass-rearing of *S. frugiperda* was carried out following the method of Herlinda et al. (2020). The larvae were collected from the maize plants grown around the campus, then they were reared in the laboratory for more than five generations to obtain the larvae with relatively homogeneous genetics and to eliminate the effects of exposure to synthetic insecticides from the field. In the laboratory, the larvae of *S. frugiperda* were kept individually in a porous plastic cup (Ø 6.5 cm, height 4.6 cm) because the larvae were cannibals. The larvae were fed daily with the maize leaves measuring 2 cm x 5 cm. During the prepupae stage, all the prepupae were transferred to a plastic container (Ø 15 cm, height 25 cm) on which its bottom had been given sterile soil (5 cm thick). The plastic container containing the prepupae was placed in a wire mesh cage (30 x 30 x 30 cm^3) and inside this cage were also put fresh maize leaves which were changed daily for the adults to lay eggs. The one day old second instar larvae were used for the bioassay.

The bioassay of endophytic fungi against larvae of *Spodoptera frugiperda*

The bioassay of endophytic fungi followed the method of Zea et al. (2019). The young maize (seedling) already inoculated with the endophytic fungi and not-inoculated (control) aged 10 days old were given to the second instar larvae of *S. frugiperda*. Each individual larvae was given one maize seed whose cotyledons were already removed. The larvae were allowed to eat the leaves and stems of the young maize for 6 hours in a sterile room (a laminar air flow cabinet) and each replication was tested for 25 individuals of *S. frugiperda* larvae. Then, the larvae were transferred to a porous plastic cup (Ø 6.5 cm, height 4.6 cm) and fed maize leaves measuring 2 x 5 cm^2 per day per larvae. This experiment was designed using completely randomized designs with treatments of 20 isolates, three replications per treatment. The dead larvae were recorded daily for 12 days following the method of Herlinda et al. (2020). The dead larvae were grown in the agar-water medium to confirm whether the infection was caused by the endophytic fungi or not. The number of dead larvae were used to calculate the mortality.

Data Analysis

The differences in the mortality data of *S. frugiperda* larvae were analyzed using analysis of variance (ANOVA). Tukey's Honestly Significant Difference (HSD) test (Tukey's test) was employed to test for the significant differences among the treatments (isolates) at P = 0.05. All data were calculated using software of SAS University Edition 2.7 9.4 M5.

Results

Colonization of endophytic fungi on maize tissues

All fungal isolates isolated from the leaves, shoots, and roots of the maize, bananas, ridged gourd, and red chilies were found to be 124 isolates. However, after assessing for the fungal colonization in young maize (seedlings) tissue, there were only 20 isolates of the fungi confirmed as endophytic fungi (Fig. 2). Evidence of

fungal colonization that entered the maize seedlings tissue was that mycelia fungi were seen entering and growing spread throughout the stem and leaf tissue. Mycelia endophytic fungi were able to invade all parts of the maize tissue, the mycelia appeared to be branched. The size of mycelia varied day by day from 13.51 µm to 34.25 µm. In the maize tissue which their seeds were not inoculated with the endophytic fungi (control) there was no mycelia of endophytic fungus and the cells in the maize tissue were clean without fungal colonization.

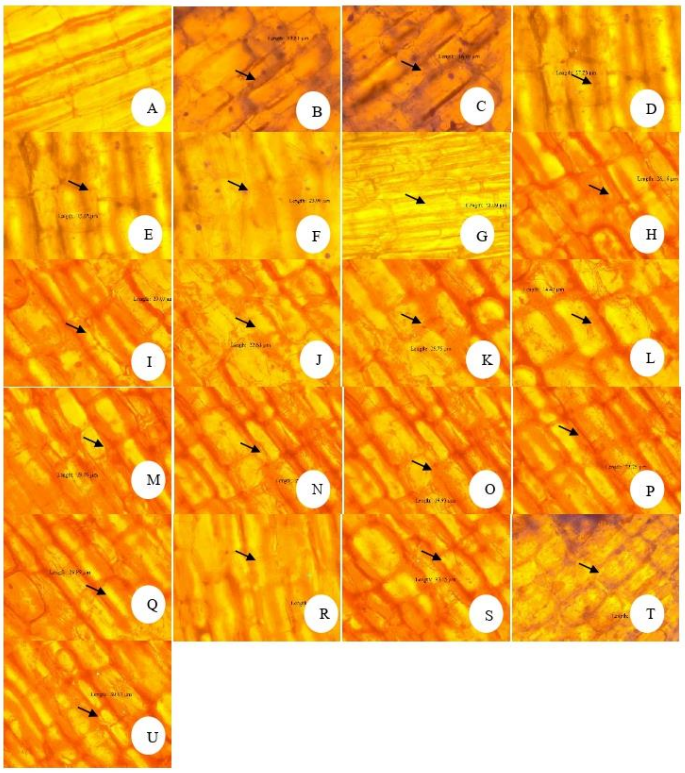

Fig 2 Young maize tissues colonized by endophytic fungi: Control (A), GaTpeOi (B), PsgTjPr (C), JgSPK (D), JgTjPr (E), JaGiP (F), PiCrPga (G), JaGiPRB (H), CMTjP (I), JaMsBys (J), JaSpkPGA(2) (K), JgCrJr (L), JaTpOi(1) (M), JaSpkPga(3) (N), CaCjPga (O), JgByU (P), JgTgSr (Q), JaBuBys (R), JgPWSR (S), JaTpOi(2) (T), CaTpPga (U)

The growth of maize seedlings colonized by the endophytic fungi showed differences contrasted to the control maize seedlings without the fungal colonization

(Fig. 3). The inoculated young maize tended to be taller and had longer roots and many branches than those that were not inoculated with the fungi (control). However, this experiment only observed morphological differences and did not measure agronomic characters.

Identification results of the endophytic fungal isolates

The colony form of 20 isolates of the endophytic fungi showed different colors (Fig. 4) and likewise the shapes of hyphae and conidia of each isolate showed their own characteristics (Fig. 5). The colony isolates of GaTpeOi, JgTjPr, PiCrPga, and CaCjPga grown on SDA media showed similarities, namely the colony was pale gray, hyphae and mycelia were septate, the conidia were in the shape of letter D (asymmetric/elliptical) and non-septation. Based on the phylogenetic tree, these 4 isolates were placed within the group of *Chaetomium* sp. (Fig. 6). The isolates have been deposited in the GenBank with the accession number MZ359734 (GaTpeOi), MZ359736 (JgTjPr), MZ359735 (PiCrPga), MZ359737 (CaCjPga) (Table 1).

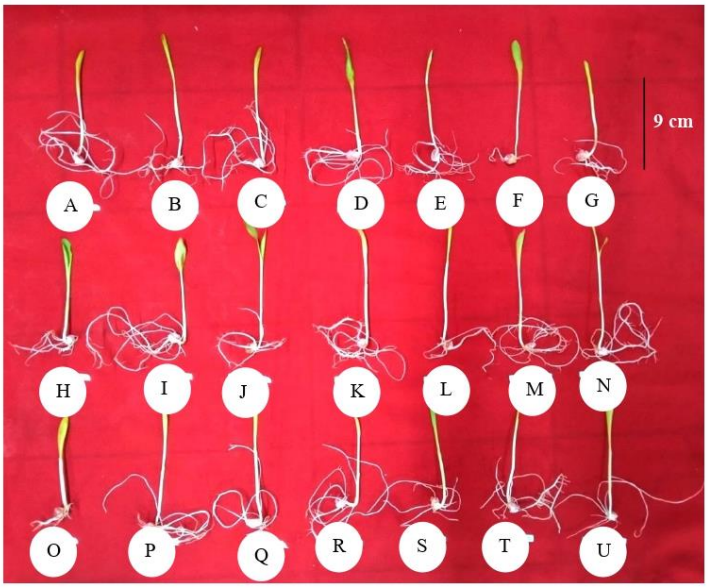

Fig 3 Young maize plant colonized by endophytic fungi: Control (A), GaTpeOi (B), PsgTjPr (C), JgSPK (D), JgTjPr (E), JaGiP (F), PiCrPga (G), JaGiPRB (H), CMTjP (I), JaMsBys (J), JaSpkPGA(2) (K), JgCrJr (L), JaTpOi(1) (M), JaSpkPga(3) (N), CaCjPga (O), JgByU (P), JgTgSr (Q), JaBuBys (R), JgPWSR (S), JaTpOi(2) (T), CaTpPga (U)

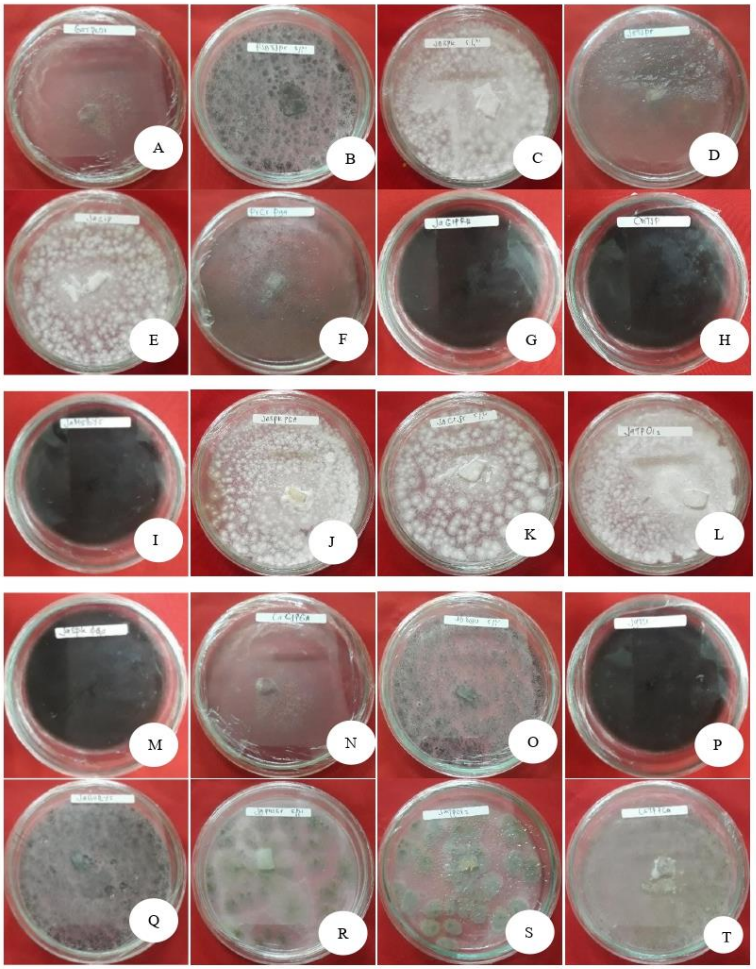

Fig. 4 Colony morphology of endophytic fungal isolate: GaTpeOi (A), PsgTjPr (B), JgSPK (C), JgTjPr (D), JaGiP (E), PiCrPga (F), JaGiPRB (G), CMTjP (H), JaMsBys (I), JaSpkPGA(2) (J), JgCrJr (K), JaTpOi(1) (L), JaSpkPga(3) (M), CaCjPga (N), JgByU (O), JgTgSr (P), JaBuBys (Q), JgPWSR (R), JaTpOi(2) (S), CaTpPga (T)

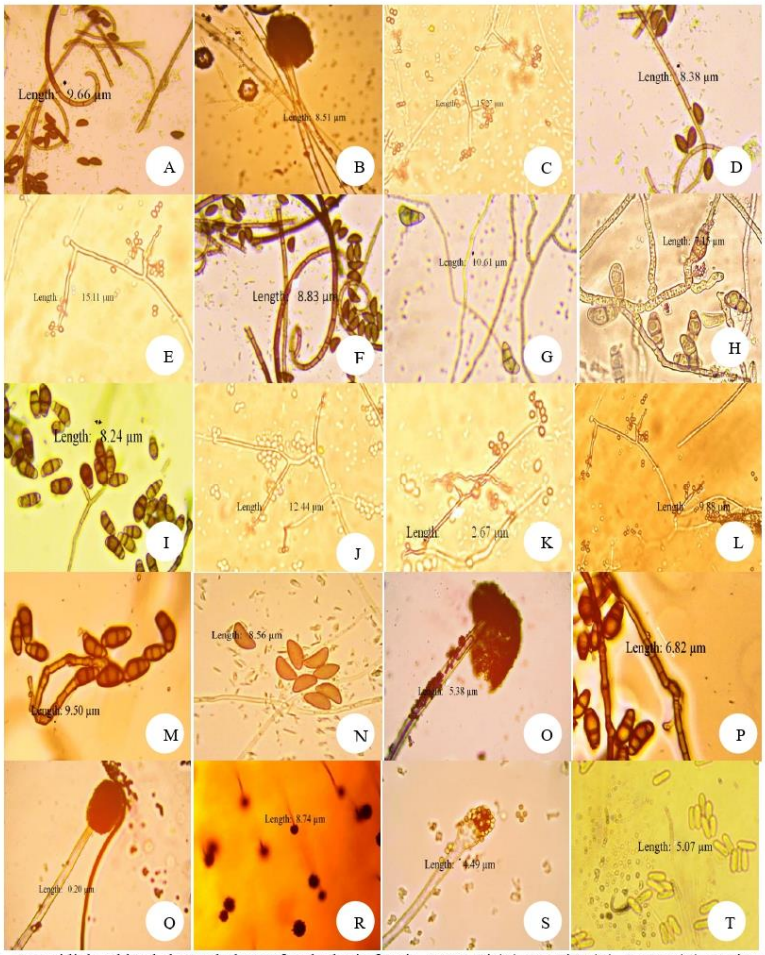

Fig. 5 Conidial and hyphal morphology of endophytic fungi: GaTpeOi (A), PsgTjPr (B), JgSPK (C), JgTjPr (D), JaGiP (E), PiCrPga (F), JaGiPRB (G), CMTjP (H), JaMsBys (I), JaSpkPGA(2) (J), JgCrJr (K), JaTpOi(1) (L), JaSpkPga(3) (M), CaCjPga (N), JgByU (O), JgTgSr (P), JaBuBys (Q), JgPWSR (R), JaTpOi(2) (S), CaTpPga (T)

The colony isolates of PsgTjPr, JgByU, and JaBuBys showed similarities, their colonies were black, the hyphae and mycelia were also black, the conidia were non-septate, globose in shape. The three isolates were placed within the group of *Aspergillus niger* (Fig. 6). The isolates have been deposited in the GenBank with the

accession number MZ242060 (PsgTjPr), MZ242059 (JgByu), and MZ242058 (JaBUBys) (Table 1).

The isolates of JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, and JaTpOi (1) had white colony, white hyphae and mycelia, and the globose conidia which were non-septate conidia. The isolates were placed within the group of *B. bassiana* (Fig. 6). The isolates have been deposited in the GenBank with the accession number MZ356494 (JgSPK), MZ356495 (JaGiP), MZ356496 (JaSpkPGA(2)), MZ356497 (JgCrJr), and MZ356498 (JaTpOi1) (Table 1).

The isolates of JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr had black colony, black hyphae and mycelia, two-septate boomerang-shaped conidia. All the isolates were placed within the group of *Curvularia lunata* (Fig. 6). The isolates heve been deposited in the GenBank with the accession number MZ359815 (JaGiPRB), MZ359816 (CMTJP), MZ359819 (JaMsBys), MZ359818 (JaSpkPga(3)), and MZ359817 (JgTgSr) (Table 1).

The isolate of JgPWSR had green colony, green hyphae and mycelia, non-septate globose conidia. The JgPwSr isolate was placed within the group of *Aspergillus flavus* (Fig. 6). The isolate has been deposited in the GenBank with the accession number MZ359829 (JgPWSR) (Table 1).

The color of the colony JaTpOi(2) isolate was initially white, as the fungus got older it turned bluish green with white margin, rounded conidia. The isolate was placed within the group of *Penicillium citrinum* (Fig. 6). The isolate has been deposited in the GenBank with the accession number MZ359812 (JaTpOi(2)) (Table 1).

The isolate of CaTpPGA had a white colony and as the fungus got older it turned greenish white to dark green, green hyphae and mycelia, the conidia are clear, cylindrical and non-septation. The isolate was placed within the group of *M. anisopliae* (Fig. 6). The isolate has been deposited in the GenBank with the accession number MZ242073 (CaTpPGA) (Table 1).

Pathogenicity of the endophytic fungi against *Spodoptera frugiperda* larvae

Of the 20 isolates of endophytic fungi found, there were 6 isolates that were the most pathogenic against *S. frugiperda* larvae (Table 2). The four isolate (JgSPK, JaGiP, JgCrJr, and JaTpOi1) species with the resulting mortality of 22.67%, 21.33%, 21.33%, and 18.67% consecutively was *B. bassiana*. In addition, the isolate JaSpkPga(3) isolate of *C. lunata* could cause a fairly high mortality (18.67%) and was not significantly different from the mortality caused by the isolates of JgSPK, JaGiP, JgCrJr, JaTpOi1. The CaTpPga isolate of *M. anisopliae* caused 8% larvae mortality and was not significantly different from the mortality caused JaSpkPga(3) isolate. The *B. bassiana* and *C. lunata* isolates were isolated from maize, and the *M. anisopliae* isolate was isolated from red chili. The JaSpkPGA(2) isolate of *B. bassiana* resulting mortality was only 2.67%. Likewise, *P. citrinum*, *Chaetomium* sp., *A. niger, A. flavus* caused the lowest larvae mortality of less than 8%.

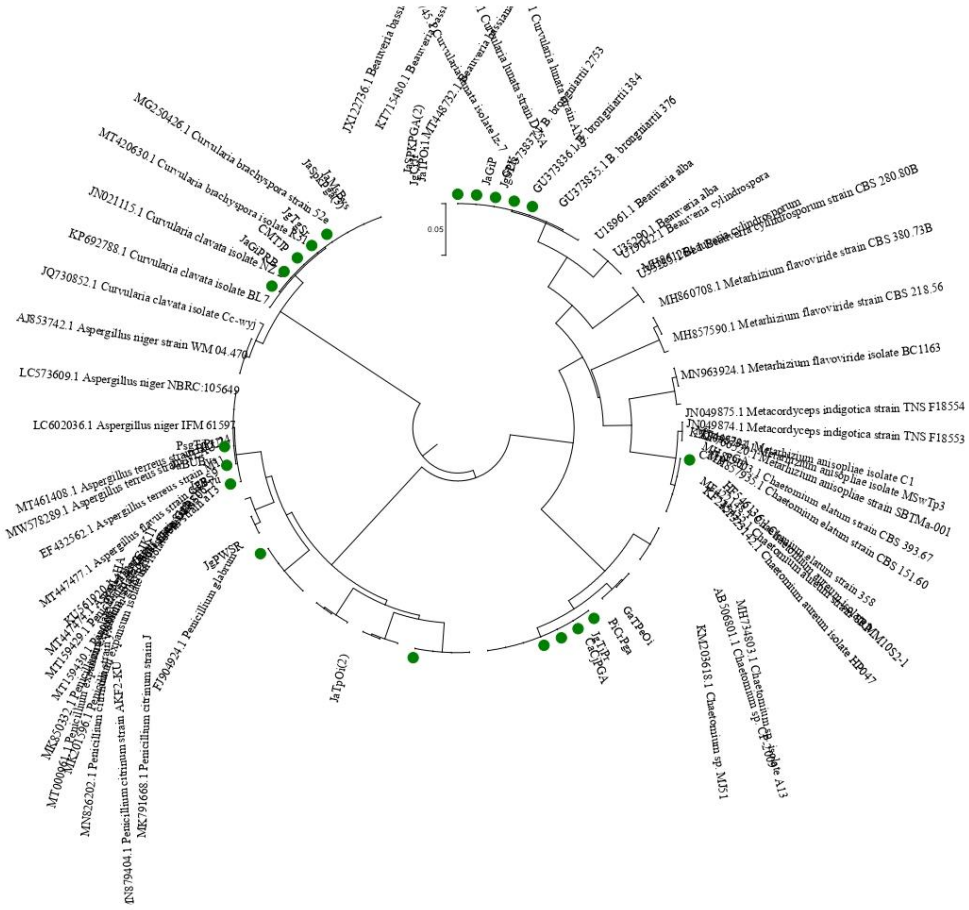

Fig. 6 Phylogenetic tree based on the sequencing of Internal Transcribed Spacer (ITS) region using UPGMA method (jukes and cantor model) performed with Mega 7 program for windows (Kumar et al 2016). This research found 20 isolates of entomopathogenic fungi: 4 isolates placed within the group of *Chaetomium* sp., 3 isolates placed within the group of *Aspergillus niger*, 5 isolates placed within the group of *Beauveria bassiana*, 5 isolates placed within the group of *Curvularia lunata*, 1 isolates placed within the group of *Aspergillus flavus*, 1 isolates placed within the group of *Penicillium citrinum*, and 1 isolate placed within the group of *Metrhizium anisopliae*. ● = fungal isolates obtained from this research

Table 2 Mean mortality of *Spodoptera frugiperda* larvae treated with endophytic fungi (1 x 10^6 conidia mL^{-1}), and conidial density and viability of endophytic fungi

Fungal isolate code	Fungal species	Mortality ± SE (%)	Conidial viability ± SE (%) for 1 x 24 hours	Conidial viability ± SE (%) for 2 x 24 hours
Control	-	0.00 ± 0.00a	-	-
GaTpeOi	*Chaetomium* sp.	4.00 ± 1.89bcd	45.17±1.51bcd	47.00±0.34a
PsgTjPr	*Aspergillus niger*	2.67 ± 2.18abc	38.20±0.79ab	51.63±0.31abcd
JgSPK	*Beauveria bassiana*	22.67 ± 2.88f	47.59±1.33efgh	55.23±2.12cde
JgTjPr	*Chaetomium* sp.	2.67 ± 1.08abcd	35.74±2.36a	55.76±1.90de
JaGiP	*Beauveria bassiana*	21.33 ± 2.18f	42.86±3.39abc	52.91±1.03abcd
PiCrPga	*Chaetomium* sp.	5.33 ± 1.09cd	44.59±1.37cde	47.99±2.87abc
JaGiPRB	*Curvularia lunata*	4.00 ± 1.89bcd	45.18±1.76bcd	52.75±2.51abcd
CMTJP	*Curvularia lunata*	2.67 ± 1.09abcd	45.25±2.65efg	54.94±2.62bcde
JaMsBys	*Curvularia lunata*	6.67 ± 1.09d	49.98±1.64gh	58.60±3.00de
JaSpkPGA(2)	*Beauveria bassiana*	2.67 ± 1.09abcd	47.20±1.77cde	48.15±1.95abc
JgCrJr	*Beauveria bassiana*	21.33 ± 3.93f	46.50±1.50cd	52.63±2.31abcd
JaTpOi (1)	*Beauveria bassiana*	18.67 ± 1.09f	54.02±1.28efg	56.55±2.03de
JaSpkPga(3)	*Curvularia lunata*	17.33 ± 2.18ef	50.85±3.75def	56.93±1.59de
CaCjPga	*Chaetomium* sp.	2.67 ± 1.09abcd	37.24±3.40a	47.77±1.68ab
JgByU	*Aspergillus niger*	1.33 ± 1.09ab	58.84±1.72gh	69.57±3.81g
JgTgSr	*Curvularia lunata*	4.00 ± 0.00bcd	55.33±1.75efgh	61.24±1.12ef
JaBuBys	*Aspergillus niger*	2.67 ± 1.09abcd	58.45±0.20gh	76.50±2.08h
JgPWSR	*Aspergillus flavus*	4.00 ± 0.00bcd	62.54±2.08h	68.39±0.34g
JaTpOi(2)	*Penicillium citrinum*	6.67 ± 2.17cd	57.44±0.27fgh	65.98±1.35fg
CaTpPga	*Metarhizium anisophiae*	8.00 ± 1.88de	50.76±0.81def	68.75±3.29f
F-value		7.27*	9.05*	15.67*
P-value		0.00	0.00	0.00
HSD value		8.8	4.27	4.07

Note: * = significantly different; values within a column followed by the same letters were not significantly different at $P < 0.05$ according to Tukey's HSD test

The larvae of *S. frugiperda* that died by eating the leaves colonized by the endophytic fungi had characteristics, i.e. dull and dark integument, shriveled body, dry, and odorless. The feces excreted by sick larvae was wetter than the those of untreated larvae (control). The larvae that died as a result of treatment by the endophytic fungi when grown in SDA media, their cadavers were not overgrown with mycelia fungi.

Discussion

The result of BLAST search revealed that the isolates of GaTpeOi, JgTjPr, PiCrPga, and CaCjPga had a similarity (100%) with the *Chaetomium* sp. isolate A13 (Acc. No. MH34803.1), isolate MJ51 (Acc No. KM203618.1), and isolate CP-2009 (Acc. No. AB50680.1). The isolates of PsgTjPr, JgByU, and JaBuBys showed 100% of similarity with *A. niger* NBRC:105649 (Acc. No LC573609.1), strain WM04.470 (Acc No. AJ853742.1) and isolate IFM61597 (Acc. No. LC602036.1). The isolates of JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, and JaTpOi (1) showed (100%) similarity to the *B. bassiana* isolate GZMS-28 (Acc. No. KT715480.1), strain TF6-1B (Acc No. JX122736.1), and isolate BSwTd4 (Acc. No. MT4487322.1). The isolates of JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr showed 99.81% of similarity to *C. lunata* strain D25A (Acc. No MH010917.1), strain AN 2 (Acc No. KY859790.1), and isolate 1z -7 (Ac. No. MN213745.1). The isolate of JgPWSR showed 100% of similarity to *A. flavus* strain GFRS9 (Acc. No MT447477.1), strain GFRS06 (Acc. No MT447474.1), and strain aT3 (Acc. No KU561920.1). The isolate of JaTpOi(2) showed 100% of similarity to *P. citrinum* strain yx-001 (Acc. No. MN826202.1), strain AKF2-KU (Acc No. MN879404.1), and strain J (Acc. No. MK791668.1). The isolate of CaTpPGA had a similarity (99.29%) to *M. anisopliae* isolate MSwTp3 (Acc. No MT448733.1), strain STBMa-001 (Acc No. KF766520.1), and isolate C1 (Acc. No. KX809520.1) .

The isolates of the endophytic fungi found in this study had an rDNA sequence similarity value of more than 99% to the reference species (BLAST), meaning that the isolates had a high phylogenetic relationship and were in the same species. Bich et al. (2021) state that ribosomal DNA sequences are used to determine the phylogenetic relationships of organisms to taxa species. Based on the similarity value, it is stated that if the similarity value is 100%, it means that the isolates are the same strain, whereas if a similarity value of 99% indicates the isolates are the same species, and if the similarity value of the isolates is 89-99%, it means they are the same genus (Henry et al. 2000). An organism is declared the same species when the difference in DNA sequences is between 0.2–1% (Shenoy et al. 2007).

The results of identification based on the morphological characters of several species of fungi showed the similarities, for example the morphology of the colony and the shape of the conidia isolates of *B. bassiana* were similar to the morphology of *P. citrinum*, but after the molecular identification, they showed different species. According to Minarni et al. (2021) proper species identification is very important in determining a species as an entomopathogen.

Of the 20 isolates of the endophytic fungi found in this study, there were 7 species of fungi. The four isolates (GaTpeOi, JgTjPr, PiCrPga, and CaCjPga) were in the group of *Chaetomium* sp., the three isolates (PsgTjPr, JgByU, and JaBuBys) were in the group of *Aspergillus niger*, the five isolates (JgSPK, JaGiP, JaSpkPGA(2) , JgCrJr, and JaTpOi (1)) were placed within the group of *B. bassiana*, the five isolates (JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr) were in the group of *C.*

lunata, an isolate (JgPWSR) was in the group of *A. flavus*, an isolate (JaTpOi(2)) was in the group of *P. citrinum*, and an isolate (CaTpPGA) was in the group of *M. anisopliae*. The fungi proved to be endophytic based on the characteristics of maize stalks and leaves colonized by the fungal mycelia. According to Barra-Bucarei et al. (2020) endophytic fungal isolates have a systemic mode of action ability to colonize their host plants. The endophytic fungi can still be found on roots, stems, and leaves up to 30 days after inoculation (Carolina et al. 2020). The research results of Shikano (2018) showed the endophytic fungi were able to live in the tissue for several months depending on the age of the host plant.

Of the 20 isolates of the endophytic fungi found in this study, there were only 6 isolates the most pathogenic against the larvae of *S. frugiperda*. The isolates JgSPK, JaGiP, JgCrJr, JaTpOi1 belong to *B. bassiana*, the JaSpkPga(3) and CaTpPga isolates belong to *C. lunata* and *M. anisopliae*, respectively. First report of *B. bassiana* and *C. lunata* isolated from maize, and *M. anisopliae* isolated from red chili in Indonesia were found as entomopathogenic endophytic fungi against *S. frugiperda*. The endophytic *B. bassiana* has been reported to be effective in killing several species of the insect pests, such as *S. frugiperda* (Ramos et al. 2020), *Diaphorina citri* (Bamisile et al. 2019), and *Trialeurodes vaporariorum* (Barra-Bucarei et al. 2020). *Curvularia* spp. also proves to suppress insect populations (Assaf et al. 2011), but Gao et al. (2014) stated that *C. lunata* is an important maize foliar fungal pathogen. The endophytic *M. anisopliae* effectively killed the larvae of *S. frugiperda* (Ramos et al. 2020) and *Agrotis ipsilon* (Ahmad et al. 2020).

The endophytic fungi species of *B. bassiana* (JgSPK, JaGiP, JgCrJr, JaTpOi1 isolates), *C. lunata* (JaSpkPga(3) isolate), and *M. anisopliae* (CaTpPga isolate) had high potential as entomopathogens of *S. frugiperda* larvae. *S. frugiperda* larvae that died from consuming plants colonized by the endophytic fungi showed that the fungi could be applied through seed treatment, especially for maize seeds. The seed treatment through seeds causes the endophytic fungi to colonize plants (Gustianingtyas et al. 2021) and if *S. frugiperda* larvae eat them it can cause them die (Ramos et al. 2020). The young maize inoculated with the endophytic fungi showed higher body morphology and larger roots, but this study did not measure agronomic characters, but only observed the plant morphological differences. These plant morphology are in line with the results of research conducted by Gustianingtyas et al. (2021).

The JaSpkPGA(2) isolate of *B. bassiana*, and all isolates of *P. citrinum, Chaetomium* sp., *A. niger*, and *A. flavus* showed no potential as entomopathogens of *S. frugiperda* with their resulting mortality of less than 8%. In contrast to the research results of Gustianingtyas et al. (2021) which showed the endophytic fungi species (*Aspergillus* sp. and *Chaetomium* sp.) applied topically (contact) caused mortality of *S. frugiperda* larvae as many as 18.67% and 14.67%, respectively and the mortality caused by *Beauveria* sp. reached 29%. This difference in mortality was due to different ways of application of fungi, in this study the larvae of *S. frugiperda*

consumed the plants that had been colonized by the endophytic fungi which were not applied topically.

The larvae of *S. frugiperda* that were sick and died by eating leaves colonized by the endophytic fungus had different characteristics from the larvae that were sick and died due to the topical suspension of the endophytic fungus. The sick larvae of *S. frugiperda* in this study had wetter feces and the cadavers were not overgrown and covered by the fungal mycelia, while the sick larvae caused by contact with suspension of the endophytic fungi according to Gustianingtyas et al. (2021) had dry feces and the cadavers were covered with the fungal mycelia. The differences in the characteristics of sick and dead insects are new information for the development of further research.

Conclusions

Finally, the results of molecular identification were found 20 isolates of the endophytic fungi consisted of 7 species, namely *Chaetomium* sp. (GaTpeOi, JgTjPr, PiCrPga, and CaCjPga isolates), *Aspergillus niger* (PsgTjPr, JgByU, and JaBuBys), *Beauveria bassiana* (JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, dan JaTpOi (1) isolates), *Curvularia lunata* (JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr isolates), *Aspergillus flavus* (JgPWSR isolate), *Penicillium citrinum* (JaTpOi(2) isolate), and *Metarhizium anisopliae* (CaTpPGA isolate). The endophytic fungi species of *B. bassiana* (JgSPK, JaGiP, JgCrJr, JaTpOi1 isolates), *C. lunata* (JaSpkPga(3) isolate), and *M. anisopliae* (CaTpPga isolate) were pathogenic against *S. frugiperda* larvae. First report of *B. bassiana* and *C. lunata* isolated from maize, and *M. anisopliae* isolated from red chili in Indonesia were found as entomopathogenic endophytic fungi against *S. frugiperda*. The endophytic fungi species of *B. bassiana*, *C. lunata*, and *M. anisopliae* had high potential as entomopathogens of *S. frugiperda* larvae.

List of abbreviations

ANOVA: analysis of variance; BLAST: Basic Local Alignment Search Tool; CTAB: cetyltrimethylammonium bromide; DNA: Deoxyribonucleic acid; EtOH: Ethyl alcohol; HSD: Tukey's Honestly Significant Difference; ITS: Internal Transcribed Spacer; MEA: the malt extract agar; NaOCl: Sodium hypochlorite; SDA: Sabouraud Dextrose Agar; TBE: Tris-Boric Acid-EDTA.

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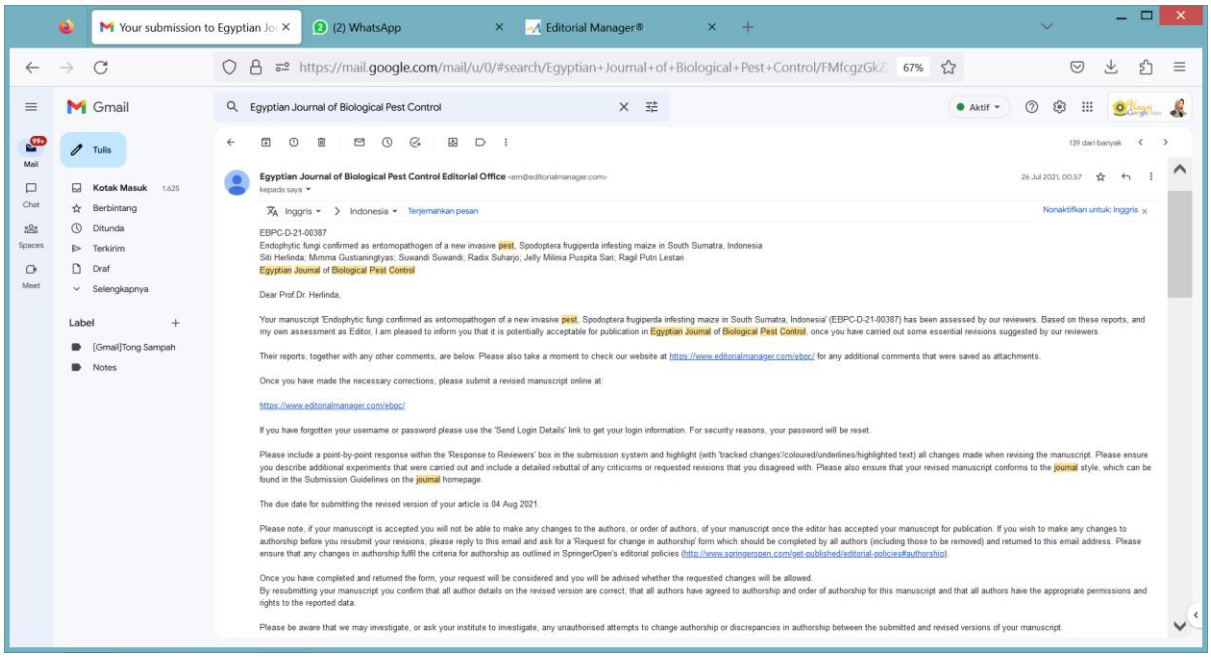

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Endophytic fungi confirmed as entomopathogen of a new invasive pest, Spodoptera frugiperda infesting maize in South Sumatra, Indonesia
Siti Herlinda; Mimma Gustianingtyas; Suwandi Suwandi; Radix Suharjo; Jelly Milinia Puspita Sari; Ragil Putri Lestari
Egyptian Journal of Biological Pest Control

Dear Prof.Dr. Herlinda,

Your manuscript 'Endophytic fungi confirmed as entomopathogen of a new invasive pest, Spodoptera frugiperda infesting maize in South Sumatra, Indonesia' (EBPC-D-21-00387) has been assessed by our reviewers. Based on these reports, and my own assessment as Editor, I am pleased to inform you that it is potentially acceptable for publication in Egyptian Journal of Biological Pest Control, once you have carried out some essential revisions suggested by our reviewers.

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Endophytic fungi confirmed as entomopathogen of a new invasive pest, Spodoptera frugiperda infesting maize in South Sumatra, Indonesia

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Abstract:	Background: Spodoptera frugiperda (JE Smith) is a new invasive pest in Indonesia. It has caused financial losses. S. frugiperda larvae hide in the leaf midrib all day. To overcome the hidden larvae, it needs pathogenic endophytic fungi. The objective of this research was to molecularly identify the endophytic fungal species from South Sumatra and determine the most pathogenic species against S. frugiperda larvae. Endophytic fungal identification was based on morphological and molecular characteristics. The molecular identification was based on gene sequential analysis of Intergenic Transcribed Spacer (ITS) 1 and ITS 4. Bioassay of the endophytic fungal species was treated against the second larvae instar of S. frugiperda. Result: The results of molecular identification were the endophytic fungal species consisted of Chaetomium sp. (GaTpeOi, JgTjPr, PiCrPga, and CaCjPga isolates), Aspergillus niger (L.) Van Tieghem (PsgTjPr, JgByU, and JaBuBys), Beauveria bassiana (Balsamo) Vuillemin (JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, and JaTpOi (1) isolates), Curvularia lunata (Wakker) Boed. (JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr isolates), Aspergillus flavus Link. (JgPWSR isolate), Penicillium citrinum Thom F 1539 (JaTpOi(2) isolate), and Metarhizium anisopliae (Metschn.) Sorokin (CaTpPGA isolate). All fungal isolates have been deposited in the GenBank. The endophytic fungal species of B. bassiana (JgSPK isolate) causing up to 22.67% mortality, C. lunata (JaSpkPga(3) isolate) causing 17.33% mortality, and M. anisopliae (CaTpPga isolate) causing 8% mortality were pathogenic against S. frugiperda larvae. First report of B. bassiana and C. lunata isolated from maize (Zea mays L.), and M. anisopliae isolated from red chili (Capsicum annuum L.) in Indonesia were found as entomopathogenic endophytic fungi against S. frugiperda larvae. Conclusion: The endophytic fungi species of B. bassiana, C. lunata, and M. anisopliae had potential as entomopathogens of S. frugiperda.
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Response to Reviewers:	Response to Reviewers #1: 1.All the scientific names have been written in full with authority and systematics and the italics have been used only for genus and species. The revisions in the manuscript have been highlighted with green colour. 2.The English in some parts have been revised and highlighted in the manuscript with green colour. 3.Fig. 6 has been changed with more understandable graph. 4.The conclusion has been improved and highlighted in the manuscript with green colour. Response to Reviewers #2: 1.Page 1: - line 16: the revision for citing the losses is the present perfect and highlighted with yellow colour in manuscript. - line 25: the sentence has been revised (highlighted with yellow colour) - line 30: the revision in the manuscript was highlighted with yellow colour - line 35: the revision in the manuscript was highlighted with yellow colour - line 35: the revision in the manuscript was highlighted with yellow colour - line 43: We are referring to the species and the revision in the manuscript was highlighted with yellow colour. 2.Page 2: - line 11: the revision was highlighted with yellow colour - line 15: We have deleted it and the revision was highlighted with yellow colour 3.Page 3: - line 40: the revision in the manuscript was highlighted with yellow colour - line 55: the revision in the manuscript was highlighted with yellow colour 4.Page 7: - lines 9-10: We have inserted the geographical coordinates and the revision was highlighted with yellow colour - line 30: the revision in the manuscript was highlighted with yellow colour - line 35: the revision in the manuscript was highlighted with yellow colour 5.Page 8: - line 9: the revision in the manuscript was highlighted with yellow colour 6.Page 8-9: - lines 57-58 and 9-12: We have deleted these sentences and also deleted Fig. 3. The revisions in the manuscript were highlighted with yellow colour 7.Page 10-11: Fig. 3 and Fig 4: We have cited the species of fungi and created a link between them and the codes. The revisions in the manuscript were highlighted with yellow colour 8.Page 12: - line 46: the revision in the manuscript was highlighted with yellow colour - line 49: the revision in the manuscript was highlighted with yellow colour 5.Page 13: Fig. 6 has been changed with more understandable graph. Lines 56-57: the revision in the manuscript was highlighted with yellow colour Line 57: the revision in the manuscript was highlighted with yellow colour 9.Page 14: - lines 54-56: the revisions in the manuscript were highlighted with yellow colour 10.Page 15: - lines 9-31: The first paragraph of a discussion has been moved to the results chapter. The revisions in the manuscript were highlighted with yellow colour. 11.Pages 15-16: - lines 52-58 and 8-10: The sentences have been moved to the results chapter. The revisions in the manuscript were highlighted with yellow colour. 12.Page 16: - lines 11-18: The information on the host plants and the pests have been provided and discussed in the manuscript studied by those authors. The revisions in the manuscript were highlighted with yellow colour.

	- lines 36-37: The revisions in the manuscript were highlighted with yellow colour. - lines 44-46: We have deleted this part. 13.Page 17: - line 27: The revision in the manuscript was highlighted with yellow colour. - line 38: The revisions in the manuscript were highlighted with yellow colour. About there are two references with the same citation (Herlinda et al. 2020), we have differentiated by using Herlinda et al. 2020a and Herlinda et al. 2020b. The revisions in the manuscript were highlighted with yellow colour.
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Endophytic fungi confirmed as entomopathogen of a new invasive pest, *Spodoptera frugiperda* infesting maize in South Sumatra, Indonesia

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Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data are available in the article and the materials used in this work are of high quality and grade.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

SH performed research concept and design, writing the article, and final approval of article. MG performed collection and assembly of data. SS prepared and performed morphological identification and critical revision of the article. RS performed and performed molecular identification and data analysis and interpretation. JMPS performed collection and assembly of data. RPL performed collection and assembly of data. All the authors read and approved the manuscript.

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Endophytic fungi confirmed as entomopathogen of a new invasive pest, *Spodoptera frugiperda* infesting maize in South Sumatra, Indonesia

Abstract

Background: *Spodoptera frugiperda* (JE Smith) is a new invasive pest in Indonesia. It has caused financial losses. *S. frugiperda* larvae hide in the leaf midrib all day. To overcome the hidden larvae, it needs pathogenic endophytic fungi. The objective of this research was to molecularly identify the endophytic fungal species from South Sumatra and determine the most pathogenic species against *S. frugiperda* larvae. Endophytic fungal identification was based on morphological and molecular characteristics. The molecular identification was based on gene sequential analysis of Intergenic Transcribed Spacer (ITS) 1 and ITS 4. Bioassay of the endophytic fungal species was treated against the second larvae instar of *S. frugiperda*.

Result: The results of molecular identification were the endophytic fungal species consisted of *Chaetomium* sp. (GaTpeOi, JgTjPr, PiCrPga, and CaCjPga isolates), *Aspergillus niger* (L.) Van Tieghem (PsgTjPr, JgByU, and JaBuBys), *Beauveria bassiana* (Balsamo) Vuillemin (JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, and JaTpOi (1) isolates), *Curvularia lunata* (Wakker) Boed. (JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr isolates), *Aspergillus flavus* Link. (JgPWSR isolate), *Penicillium citrinum* Thom F 1539 (JaTpOi(2) isolate), and *Metarhizium anisopliae* (Metschn.) Sorokin (CaTpPGA isolate). All fungal isolates have been deposited in the GenBank. The endophytic fungal species of *B. bassiana* (JgSPK isolate) causing up to 22.67% mortality, *C. lunata* (JaSpkPga(3) isolate) causing 17.33% mortality, and *M. anisopliae* (CaTpPga isolate) causing 8% mortality were pathogenic against *S. frugiperda* larvae. First report of *B. bassiana* and *C. lunata* isolated from maize (*Zea mays* L.), and *M. anisopliae* isolated from red chili (*Capsicum annuum* L.) in Indonesia were found as entomopathogenic endophytic fungi against *S. frugiperda* larvae.

Conclusion:

The endophytic fungi species of *B. bassiana*, *C. lunata*, and *M. anisopliae* had potential as entomopathogens of *S. frugiperda*.

Keywords: *Aspergillus flavus*, *Aspergillus niger*, *Beauveria bassiana*, *Chaetomium* sp., *Curvularia lunata*, fall armyworm, *Metarhizium anisopliae*, *Penicillium citrinum*

Background

Fall armyworm, *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae) is a new invasive pest originating from South America (Otim et al. 2018). Currently, the fall armyworm has spread throughout the world and first entered Indonesia on March

2019 in West Sumatra (Sartiami et al. 2020; Nelly et al. 2021) and July 2019 in South Sumatra (Hutasoit et al. 2020) and now has been spreading throughout Indonesia, such as Bengkulu (Mukkun et al. 2021), Lampung (Trisyono et al. 2019; Lestari et al. 2020), West Java (Maharani et al. 2019), East Nusa Tenggara (Mukkun et al. 2021), Bali (Supartha et al. 2021). The pest destroys maize (*Zea mays* L.) and various other plant species (Montezano et al. 2018). The financial losses caused by this pest have been up to 250-630 million US dollars per year in Africa (Bateman et al. 2018). The fall armyworm attacks maize by means of its larvae stage eating leaves, stems, flowers, fruits, growing points, and the whole maize until it is bare (Montezano et al. 2018; Ginting et al. 2020).

Larvae of *S. frugiperda* can be found on the surface of leaves or maize stalks in the morning around 6.30 to 8.00 a.m. and after that the larvae hide in the leaf midrib (Gustianingtyas et al. 2021). It is difficult to control this pest because the larvae hides all day in the leaf midrib. However, the results of experiments in the laboratory showed that the entomopathogenic fungi, *Metarhizium* sp. can kill *S. frugiperda* larvae up to 78% (Herlinda et al. 2020a). *Beauveria bassiana* (Balsamo) Vuillemin, *Metarhizium anisopliae* (Metschn.) Sorokin, and *Metarhizium rileyi* (Farlow) Samson also kill *S. frugiperda* larvae more than 80% (Ramanujam et al. 2020), but these treatments were treated topically (contact) with the larvae exposed. Yet, in the field the presence of larvae hiding all day in the midrib of maize leaves causes the entomopathogenic fungi whose mode of action is contact to be less effective (Gustianingtyas et al. 2021).

To overcome the hidden larvae of *S. frugiperda*, it needs entomopathogenic fungi living in plant tissues or known as endophytic fungi (Ramos et al. 2020; Gustianingtyas et al. 2021). The endophytic fungi systemically colonize plant tissues and associate mutually with their host plants (Lira et al. 2020). The results of previous studies have proven that eight isolates of endophytic entomopathogenic fungi could topically kill *S. frugiperda* larvae (Gustianingtyas et al. 2021), however, the endophytic fungi in the study were only isolated from roots and the molecular identification had not yet been carried out. In this study, the fungi were isolated from leaves, shoots, and roots and identified molecularly. In addition, the application method in this study was not topical but by the larvae eating plants that had been colonized by the endophytic fungus. This study aimed to molecularly identify the endophytic fungal species from South Sumatra (Indonesia) and determine the most pathogenic fungal species against *S. frugiperda* larvae.

Methods

The fungal exploration was carried out in South Sumatra. The purification and identification, and bioassay of fungi were conducted from January to March 2021. The endophytic fungal species were identified based on the molecular analysis carried out at a laboratory accredited according to the ISO 17025 standard. Bioassay of the

fungi was carried out in an incubator at controlled and constant temperature and relative humidity, respectively 25 ºC and 97%.

Exploration, isolation, and purification of fungi

The exploration of endophytic fungi was carried out by taking the leaves, shoots, and roots of maize and other crop plants around it, such as bananas (*Musa* spp.), ridged gourd (*Luffa acutangula* (Roxb.) L.), and red chilies (*Capsicum annuum* L.). The selection of individual crop plant samples to obtain the endophytic fungi followed the method of Kasambala et al. (2018), which was the healthiest plant. The taken plant parts were put into an ice box, then taken to the laboratory. The exploration of endophytic fungi was carried out from the lowlands to the highlands of South Sumatra, Indonesia namely in Banyuasin District (2.8833°S 104.3831°E), Ogan Ilir District (3.43186°S 104.62727°E), Prabumulih City (3.4328°S 104.2356°E), Pagar Alam City (3°52′43.8″S 103°21′30″E) (Fig. 1 and Table 1).

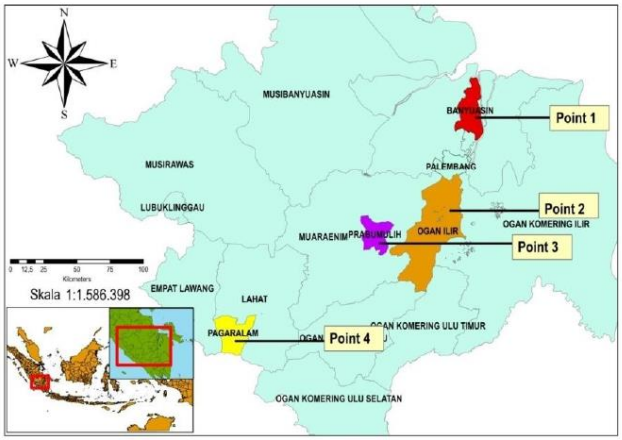

Fig. 1 Surveys locations for endophytic fungi in South Sumatra, Indonesia: Point 1 = Banyuasin District, Point 2 = Ogan Ilir District, Point 3 = Prabumulih City, and Point 4 = Pagar Alam City

The leaves, shoots, and roots of the sample plants were cleaned and washed aseptically with running tap water. Then, the plant parts were surface sterilized using method of Elfita et al. (2019) with 70% EtOH (Ethyl alcohol) and 1% NaOCl (Sodium hypochlorite), then rinsed three times. The last rinse water was used to check whether the microorganisms causing surface contamination were still present and carried out by growing them onto Sabouraud Dextrose Agar (SDA) (Russo et al. 2020).

To obtain endophytic fungi isolates, it started by growing onto the media the plant parts that have been sterilized earlier. The roots were grown on a specific medium, the malt extract agar (MEA) following the method of Silva et al. (2018). The shoots and leaves of the sample plants were grown on SDA media. The plant parts were grown as many as five pieces per petri dish with a length of 5 mm and a diameter of 1-5 mm (depending on the diameter of the leaves, shoots, and roots). Fungi growing from the leaves, shoots, and roots were purified to be one isolate per sample. The isolates were then observed for their macroscopic and microscopic characteristics and continued with the fungal colonization test into the maize seed tissue.

Table 1 The origin of isolates of endophytic fungi from South Sumatra, Indonesia

Location (village, district/city)	Isolate origin	Altitude (m)	Fungal species	Fungal isolate code	GenBank Acc. No.
Tanjung Pering. Ogan Ilir	Ridged gourd	36.0	*Chaetomium* sp.	GaTpeOi	MZ359734
Tanjung Pering. Ogan Ilir	Bananas	36.0	*Aspergillus niger*	PsgTjPr	MZ242060
Simpang Padang Karet. Pagar Alam	Maize	797.7	*Beauveria bassiana*	JgSPK	MZ356494
Tanjung Pering. Ogan Ilir	Maize	36.0	*Chaetomium* sp.	JgTjPr	MZ359736
Gunung Ibul. Prabumulih	Maize	53.0	*Beauveria bassiana*	JaGiP	MZ356495
Curup Jare. Pagar Alam	Bananas	806.0	*Chaetomium* sp.	PiCrPga	MZ359735
Gunung Ibul. Prabumulih	Maize	53.0	*Curvularia lunata*	JaGiPRB	MZ359815
Tanjung Payang. Pagar Alam	Red chilies	689.6	*Curvularia lunata*	CMTJP	MZ359816
Mulia Sari. Banyuasin	Maize	19.0	*Curvularia lunata*	JaMsBys	MZ359819
Simpang Padang Karet. Pagar Alam	Maize	789.5	*Beauveria bassiana*	JaSpkPGA(2)	MZ356496
Curup Jare. Pagar Alam	Maize	806.7	*Beauveria bassiana*	JgCrJr	MZ356497
Tanjung Pering. Ogan Ilir	Maize	36.0	*Beauveria bassiana*	JaTpOi (1)	MZ356498
Simpang Padang Karet. Pagar Alam	Maize	797.7	*Curvularia lunata*	JaSpkPga(3)	MZ359818
Curup Jare. Pagar Alam	Red chilies	806.0	*Chaetomium* sp.	CaCjPga	MZ359737
Banyuurip. Banyuasin	Maize	13.0	*Aspergillus niger*	JgByU	MZ242059
Telang Sari. Banyuasin	Maize	15.0	*Curvularia lunata*	JgTgSr	MZ359817
Banyuurip. Banyuasin	Maize	20.0	*Aspergillus niger*	JaBuBys	MZ242058
Purwosari. Banyuasin	Maize	15.0	*Aspergillus flavus*	JgPWSR	MZ359829
Tanjung Pering. Ogan Ilir	Maize	36.0	*Penicillium citrinum*	JaTpOi(2)	MZ359812
Tanjung Payang. Pagar Alam	Red chilies	689.6	*Metarhizium anisopliae*	CaTpPga	MZ242073

Assess endophytic fungal colonization

Fungal inoculation on maize seeds was carried out to observe the colonization of fungi into the maize seedling tissue and to further ensure that the isolates found were truly endophytic. The 15 seeds of maize were surface sterilized using 70% EtOH

(Ethyl alcohol) and 1% NaOCl (Sodium hypochlorite) (Elfita et al. 2019). The seeds were immersed in 10 mL of fungal suspension with a concentration of 1 x 10^6 conidia mL^{-1} for 6 hours, while for the control the sterilized seeds were only immersed in 10 mL of destilled water. Then, the seeds were grown in a sterile glass bottle (250 mL volume) with a sterile filter paper (whatman no. 42) at the bottom which was moistened with 1 mL destilled water and incubated for 10 days. All the treatments in this experiment were repeated three times. After the maize seedlings were 10 days old, the stems and leaves were sliced crosswise and longitudinally with a thickness of 0.02 mm each. The slices were observed with a light microscope at 40 x magnification with 0.05% lactofenol trypan blue dye. Mycelia endophytic fungi found in the maize seedling tissue were documented. The fungal isolates that were proven to be endophytic were used for bioassays. Prior to the bioassay, the isolates were observed for macroscopic and microscopic characteristics for morphological identification and followed by molecular identification.

Morphological and molecular identification of fungal species

Macroscopic and microscopic observation

Morphological observations were carried out based on the microscopic and macroscopic characteristics of the endophytic fungi. The macroscopic characteristics of the fungi were observed, such as the colony color and shape, while the microscopic observations were the shape and size of conidia and conidiophores following the method of Herlinda et al. (2020a).

DNA extraction and PCR amplification

The used endophytic fungal DNA extraction method refers to the Swibawa et al. (2020) and Lestari et al. (2020) method. DNA extraction was carried out on fungal conidia. The conidia were harvested from pure cultures of 7 days old fungus which was cultured in sterile petri dishes with a diameter of 9 cm. As much as 10 mL of sterile water was put into a petri dish containing fungal cultures and slowly harvested using a drigalski. The obtained conidia suspension was transferred to a centrifuge tube (volume 30 mL) and centrifuged using CF15RXII (Hitachi, Japan) for 10 minutes at a speed of 14,000 rpm. After being centrifuged, 1 mL of 70% ethanol was added to the centrifuge tube and then centrifuged again for 10 minutes. After that, the supernatant was removed and 1 mL of extraction buffer was added with a composition of 0.5 mL Tris HCl, 1 mL SDS 1% + 2.8 mL NaCl, 0.2 mL Mercaptho Ethanol, 2 mL EDTA, 3.5 mL sterile water and then homogenized. The suspension was transferred to a sterile mortal and incubated at -40 °C for 24 hours. After that, the frozen suspension was ground until it was smooth and melted. A total of 500 µL of suspension was then transferred into a 1.5 mL tube. A total of 400 µL of 2% cetyltrimethylammonium bromide (CTAB) was then added to the tube, homogenized and then incubated at 65 °C for 1 hour using a water bath (Brookfield TC 550 MX-230, USA). After the incubation, 500 µL of Phenol Chloroform Isoamyl alcohol (PCI) (25:24:1) was added, homogenized and centrifuged (Microspin12; Biosan,

Latvia) at 14,000 rpm for 10 minutes. A total of 600 µL of supernatant was taken and transferred to a new 1.5 mL tube. A total of 600 µL Chloroform Isoamyl Alcohol (CI) (24:1) was added to the tube, homogenized and centrifuged (Microspin12; Biosan, Latvia) at 14,000 rpm for 10 minutes. A total of 400 µL of supernatant was then transferred to a new 1.5 mL tube and 400 µL of cold isopropanol was added, homogenized, and incubated at -40 ºC for 20 minutes. After the incubation, it was centrifuged (Microspin12; Biosan, Latvia) at 14,000 rpm for 15 minutes. The supernatant was then discarded. After the supernatant was removed, 500 µL of 70% cold ethanol was added and centrifuged (Microspin12; Biosan, Latvia) at 14,000 rpm for 5 minutes. The supernatant was then discarded and the pellets obtained were dried at room temperature for 24 hours. After drying, the tube was then added as much as 50 µL 1x Tris-HCL EDTA (TE) pH 8.0 (1st Base Malaysia).

PCR amplification was carried out using the Sensoquest Thermal Cycler (Germany) PCR machine on the Internal Transcribed Spacer (ITS) using ITS1 and ITS4 primers (White et al. 1990). The PCR was carried out with a total volume of 25 µL consisting of a mixture of Master Mix (Red Mix) (bioline) as much as 12.5 µL, 10 µM of primer ITS 1 (5'TCC GTA GGT GAA CCT TGC GG 3 ') and ITS 4 (5'TCC TCC GCT TAT TGA TAT GC 3') 1 µL each, 1 L of template DNA and 9.5 µL of sterile water. The DNA amplification stage consisted of 1 initiation cycle at 95 ºC for 5 minutes, followed by 30 cycles consisting of denaturation at 95 ºC for 1 minute, primer attachment at 52 ºC for 1 minute, primer extension at 72 ºC for 1 minute, and 1 elongation cycle at 72 ºC for 5 minutes. The PCR results were then electrophoresed using 0.5% agarose in 20 mL of 1x Tris-Boric Acid-EDTA (TBE) buffer (1st Base Malaysia) and added 1 µL of Ethidium Bromide (EtBr 10 mg/mL). The electrophoresis was carried out in 1x TBE buffer solution at 50 volts for 70 minutes. Electrophoresis results were visualized using a DigiDoc UV transilluminator (UVP, USA).

Sequencing and analysis of the results

The PCR results were then sent to 1st Base Malaysia for the sequencing process. The results of the sequencing were analyzed using Bio Edit ver. 7.2.6 for windows. Then, the results were submitted to the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to find out the genus or species that had the greatest homology or similarity and molecularly. The phylogeny tree was created using the Mega 7 for Windows program (Kumar *et al.* 2016) using the UPGMA (jukes and cantor model) method. The ITS region sequences for several strains used as a reference in this study were obtained from NCBI (https://www.ncbi.nlm.nih.gov/).

Calculation of conidial density and viability

All the isolates of endophytic fungi were counted for their conidial density and viability. The conidial density of the seven old days fungi was calculated according to the method of Sumikarsih et al. (2019) using a haemocytometer and observed with

a light microscope at 40 x magnification. The viability was observed by growing 1 mL of conidia fungal suspension (1 x 10^6 conidia mL^{-1}) onto 2% agar-water medium, then the culture was incubated for 1 x 24 hours and 2 x 24 hours under a light microscope at 40 x magnification.

The mass rearing of *Spodoptera frugiperda*

The mass-rearing of *S. frugiperda* was carried out following the method of Herlinda et al. (2020a). The larvae were collected from the maize plants grown around the campus (3˚13′25″S 104˚39′51″E), then they were reared in the laboratory for more than five generations to obtain the larvae with relatively homogeneous genetics and to eliminate the effects of exposure to synthetic insecticides from the fields. In the laboratory, the larvae of *S. frugiperda* were kept individually in a porous plastic cup (Ø 6.5 cm, height 4.6 cm) because the larvae were cannibals. The larvae were fed daily with the maize leaves measuring 2 cm x 5 cm. During the prepupae stage, all the prepupae were transferred to a plastic container (Ø 15 cm, height 25 cm) on which its bottom had been given sterile soil (5 cm thick). The plastic container containing the prepupae was placed in a wire mesh cage (30 x 30 x 30 cm^3) and inside this cage were also put fresh maize leaves which were changed daily for the adults to lay eggs. The one day old second instar larvae were used for the bioassay.

The bioassay of endophytic fungi against larvae of *Spodoptera frugiperda*

The bioassay of endophytic fungi followed the method of Zea et al. (2019). The young maize (seedling) already inoculated with the endophytic fungi and not-inoculated (control) aged 10 days old were given to the second instar larvae of *S. frugiperda*. Each individual larvae was given one maize seedling whose cotyledons were already removed. The larvae were allowed to eat the leaves and stems of the young maize for 6 hours in a sterile room (a laminar air flow cabinet) and each replication was tested for 25 individuals of *S. frugiperda* larvae. Then, the larvae were transferred to a porous plastic cup (Ø 6.5 cm, height 4.6 cm) and were fed healthy non-inoculated leaves measuring 2 cm x 5 cm per day per larvae. This experiment was designed using completely randomized designs with treatments of 20 isolates, three replications per treatment. The dead larvae were recorded daily for 12 days following the method of Herlinda et al. (2020b). The dead larvae were grown in the agar-water medium to confirm whether the infection was caused by the endophytic fungi or not. The number of dead larvae were used to calculate the mortality.

Data Analysis

The differences in the mortality data of *S. frugiperda* larvae were analyzed using analysis of variance (ANOVA). Tukey's Honestly Significant Difference (HSD) test (Tukey's test) was employed to test for the significant differences among the

treatments (isolates) at P = 0.05. All data were calculated using software of SAS University Edition 2.7 9.4 M5.

Results

Colonization of endophytic fungi on maize tissues

All fungal isolates isolated from the leaves, shoots, and roots of the maize, bananas, ridged gourd, and red chilies were found to be 124 isolates. However, after assessing for the fungal colonization in young maize (seedlings) tissue, there were only 20 isolates of the fungi confirmed as endophytic fungi (Fig. 2). Evidence of fungal colonization that entered the maize seedlings tissue was that mycelia fungi were seen entering and growing spread throughout the stem and leaf tissue. Mycelia endophytic fungi were able to invade the stem and leaf of the maize tissue, the mycelia appeared to be branched. The size of mycelia varied day by day from 13.51 µm to 34.25 µm. In the maize tissue which their seeds were not inoculated with the endophytic fungi (control) there was no mycelia of endophytic fungus and the cells in the maize tissue were clean and clear without fungal colonization.

Identification results of the endophytic fungal isolates

The colony morphology of 20 isolates of the endophytic fungi showed different colors (Fig. 3) and likewise the shapes of hyphae and conidia of each isolate showed their own characteristics (Fig. 4). The colony isolates of GaTpeOi, JgTjPr, PiCrPga, and CaCjPga grown on SDA media showed similarities, namely the colony was pale gray, hyphae and mycelia were septate, the conidia were in the shape of letter D (asymmetric/elliptical) and non-septation. Based on the phylogenetic tree, these 4 isolates were placed within the group of *Chaetomium* sp. (Fig. 5). The isolates have been deposited in the GenBank with the accession number MZ359734 (GaTpeOi), MZ359736 (JgTjPr), MZ359735 (PiCrPga), MZ359737 (CaCjPga) (Table 1).

The colony isolates of PsgTjPr, JgByU, and JaBuBys showed similarities, their colonies were black, the hyphae and mycelia were also black, the conidia were non-septate, globose in shape. The three isolates were placed within the group of *Aspergillus niger* (L.) Van Tieghem (Fig. 5). The isolates have been deposited in the GenBank with the accession number MZ242060 (PsgTjPr), MZ242059 (JgByu), and MZ242058 (JaBUBys) (Table 1).

The isolates of JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, and JaTpOi (1) had white colony, white hyphae and mycelia, and the globose conidia which were non-septate conidia. The isolates were placed within the group of *B. bassiana* (Fig. 5). The isolates have been deposited in the GenBank with the accession number MZ356494 (JgSPK), MZ356495 (JaGiP), MZ356496 (JaSpkPGA(2)), MZ356497 (JgCrJr), and MZ356498 (JaTpOi1) (Table 1).

The isolates of JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr had black colony, black hyphae and mycelia, two-septate boomerang-shaped conidia. All the isolates were placed within the group of *Curvularia lunata* (Wakker) Boed. (Fig. 5).

The isolates heve been deposited in the GenBank with the accession number MZ359815 (JaGiPRB), MZ359816 (CMTJP), MZ359819 (JaMsBys), MZ359818 (JaSpkPga(3)), and MZ359817 (JgTgSr) (Table 1).

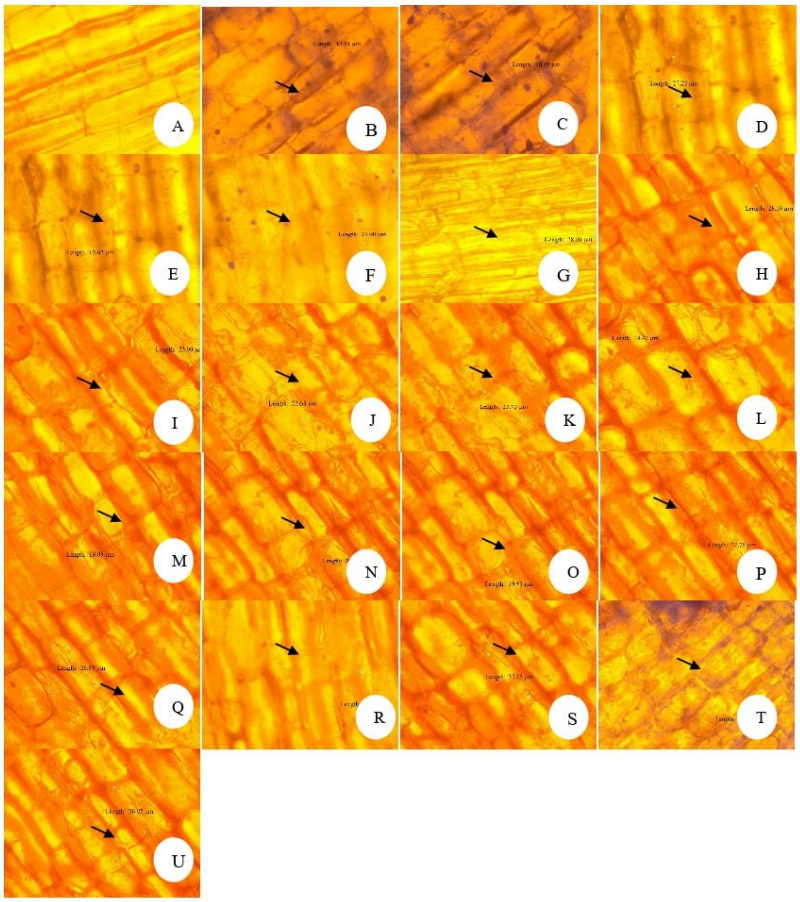

Fig 2 Young maize tissues colonized by endophytic fungal species: Control (A); *Chaetomium* sp. isolates of GaTpeOi (B), JgTjPr (E), PiCrPga (G), and CaCjPga (O); *Aspergillus niger* isolates of PsgTjPr (C), JgByU (P), and JaBuBys (R); *Beauveria bassiana* isolates of JgSPK (D), JaGiP (F JaSpkPGA(2) (K), JgCrJr (L), and JaTpOi(1) (M); *Curvularia lunata* isolates of JaGiPRB (H), CMTjP (I), JaMsBys (J), JaSpkPga(3) (N), and JgTgSr (Q); *Aspergillus flavus* isolate of JgPWSR (S); *Penicillium citrinum* isolate of JaTpOi(2) (T); *Metarhizium anisopliae* isolate of CaTpPga (U)

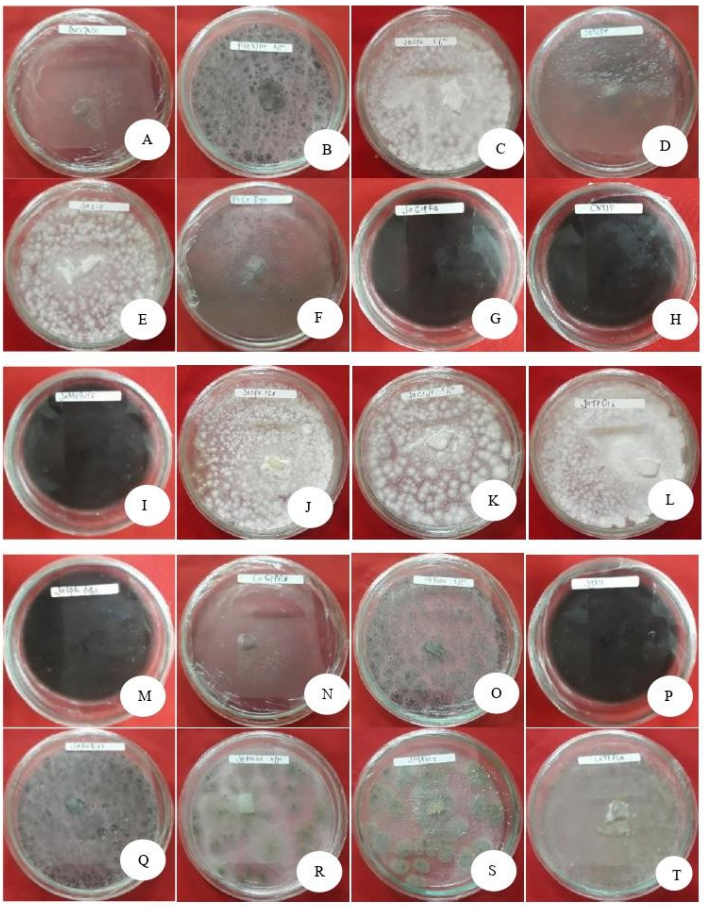

Fig. 3 Colony morphology of endophytic fungal species: *Chaetomium* sp. isolates of GaTpeOi (A), JgTjPr (D), PiCrPga (F), and CaCjPga (M); *Aspergillus niger* isolates of PsgTjPr (B), JgByU (O), and JaBuBys (Q); *Beauveria bassiana* isolates of JgSPK (C), JaGiP (D), JaSpkPGA(2) (J), JgCrJr (K), and JaTpOi(1) (L); *Curvularia lunata* isolates of JaGiPRB (G), CMTjP (H), JaMsBys (I), JaSpkPga(3) (M), and JgTgSr (P); *Aspergillus flavus* isolate of JgPWSR (R); *Penicillium citrinum* isolate of JaTpOi(2) (S); *Metarhizium anisopliae* isolate of CaTpPga (T)

The isolate of JgPWSR had green colony, green hyphae and mycelia, non-septate globose conidia. The JgPwSr isolate was placed within the group of *Aspergillus flavus* Link (Fig. 5). The isolate has been deposited in the GenBank with the accession number MZ359829 (JgPWSR) (Table 1).

The color of the colony JaTpOi(2) isolate was initially white, as the fungus got older it turned bluish green with white margin, rounded conidia. The isolate was placed within the group of *Penicillium citrinum* Thom F 1539 (Fig. 5). The isolate has been deposited in the GenBank with the accession number MZ359812 (JaTpOi(2)) (Table 1).

The isolate of CaTpPGA had a white colony and as the fungus got older it turned greenish white to dark green, green hyphae and mycelia, the conidia are clear, cylindrical and non-septation. The isolate was placed within the group of *M. anisopliae* (Fig. 5). The isolate has been deposited in the GenBank with the accession number MZ242073 (CaTpPGA) (Table 1).

The result of BLAST search revealed that the isolates of GaTpeOi, JgTjPr, PiCrPga, and CaCjPga had a similarity (100%) with the *Chaetomium* sp. isolate A13 (Acc. No. MH34803.1), isolate MJ51 (Acc No. KM203618.1), and isolate CP-2009 (Acc. No. AB50680.1). The isolates of PsgTjPr, JgByU, and JaBuBys showed 100% of similarity with *A. niger* NBRC:105649 (Acc. No LC573609.1), strain WM04.470 (Acc No. AJ853742.1) and isolate IFM61597 (Acc. No. LC602036.1). The isolates of JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, and JaTpOi (1) showed (100%) similarity to the *B. bassiana* isolate GZMS-28 (Acc. No. KT715480.1), strain TF6-1B (Acc No. JX122736.1), and isolate BSwTd4 (Acc. No. MT4487322.1). The isolates of JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr showed 99.81% of similarity to *C. lunata* strain D25A (Acc. No MH010917.1), strain AN 2 (Acc No. KY859790.1), and isolate 1z -7 (Ac. No. MN213745.1). The isolate of JgPWSR showed 100% of similarity to *A. flavus* strain GFRS9 (Acc. No MT447477.1), strain GFRS06 (Acc. No MT447474.1), and strain aT3 (Acc. No KU561920.1). The isolate of JaTpOi(2) showed 100% of similarity to *P. citrinum* strain yx-001 (Acc. No. MN826202.1), strain AKF2-KU (Acc No. MN879404.1), and strain J (Acc. No. MK791668.1). The isolate of CaTpPGA had a similarity (99.29%) to *M. anisopliae* isolate MSwTp3 (Acc. No MT448733.1), strain STBMa-001 (Acc No. KF766520.1), and isolate C1 (Acc. No. KX809520.1).

There were 7 species from the 20 isolates of the endophytic fungi found in this study. The four isolates (GaTpeOi, JgTjPr, PiCrPga, and CaCjPga) were in the group of *Chaetomium* sp., the three isolates (PsgTjPr, JgByU, and JaBuBys) were in the group of *Aspergillus niger*, the five isolates (JgSPK, JaGiP, JaSpkPGA(2) , JgCrJr, and JaTpOi (1)) were placed within the group of *B. bassiana*, the five isolates (JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr) were in the group of *C. lunata*, an isolate (JgPWSR) was in the group of *A. flavus*, an isolate (JaTpOi(2)) was in the group of *P. citrinum*, and an isolate (CaTpPGA) was in the group of *M. anisopliae*.

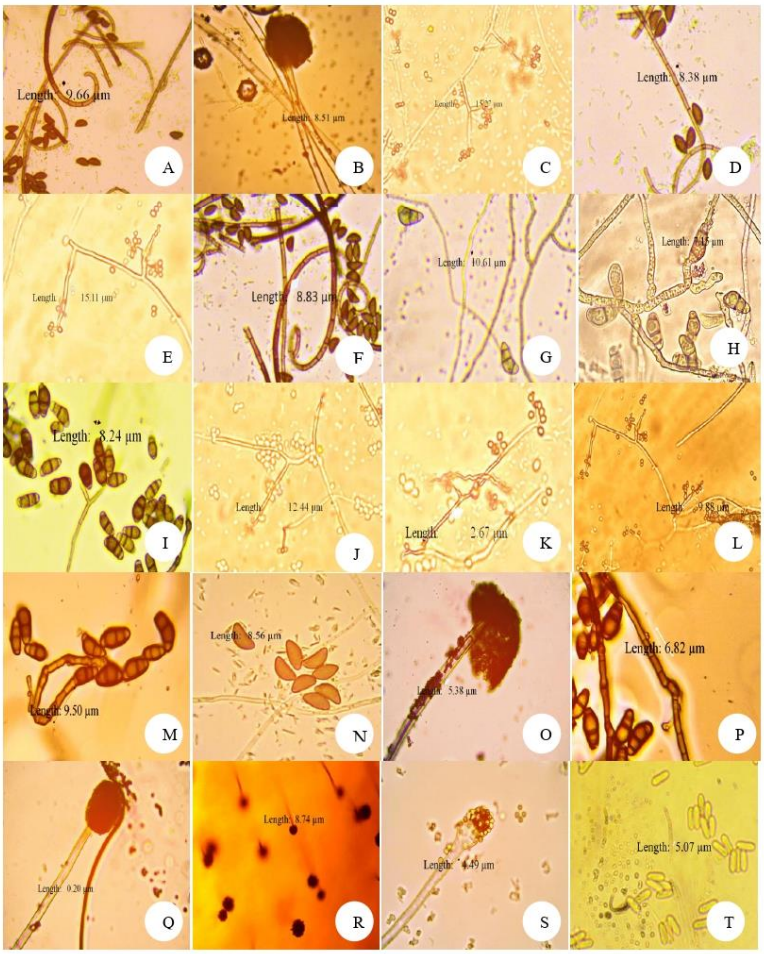

Fig. 4 Conidial and hyphal morphology of endophytic fungal species: *Chaetomium* sp. isolates of GaTpeOi (A), JgTjPr (D), PiCrPga (F), and CaCjPga (M); *Aspergillus niger* isolates of PsgTjPr (B), JgByU (O), and JaBuBys (Q); *Beauveria bassiana* isolates of JgSPK (C), JaGiP (D), JaSpkPGA(2) (J), JgCrJr (K), and JaTpOi(1) (L); *Curvularia lunata* isolates of JaGiPRB (G), CMTjP (H), JaMsBys (I), JaSpkPga(3) (M), and JgTgSr (P); *Aspergillus flavus* isolate of JgPWSR (R); *Penicillium citrinum* isolate of JaTpOi(2) (S); *Metarhizium anisopliae* isolate of CaTpPga (T)

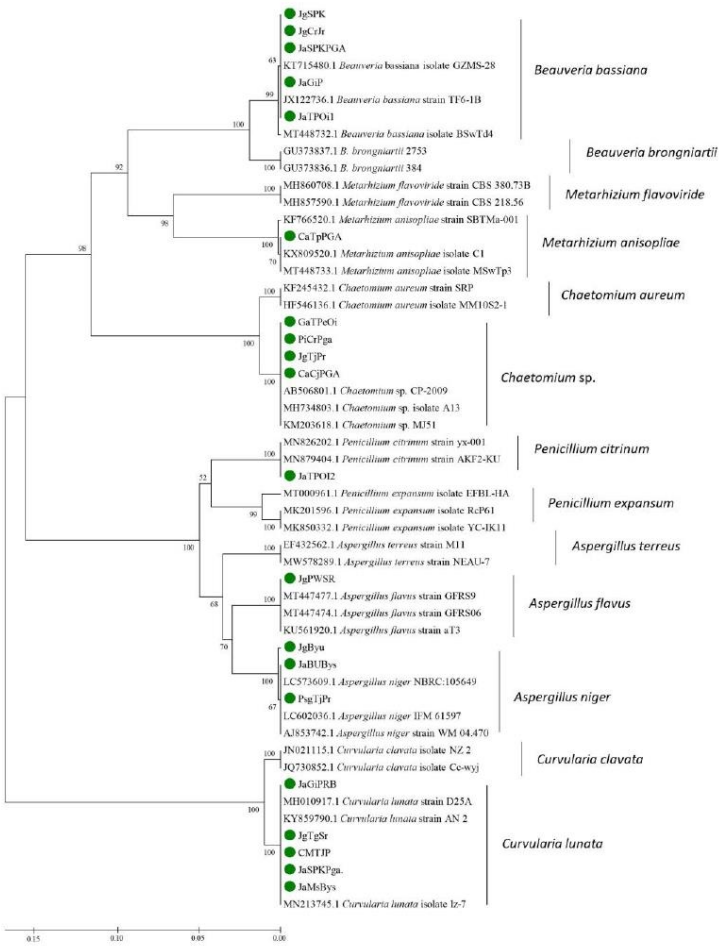

Fig. 5 Phylogenetic tree based on the sequencing of Internal Transcribed Spacer (ITS) region using UPGMA method (jukes and cantor model) performed with Mega 7 program for windows (Kumar et al 2016). This research found 20 isolates of entomopathogenic fungi: 4 isolates placed within the group of *Chaetomium* sp., 3 isolates placed within the group of *Aspergillus niger*, 5 isolates placed within the group of *Beauveria bassiana*, 5 isolates placed within the group of *Curvularia lunata*, 1 isolate placed within the group of *Aspergillus flavus*, 1 isolate placed within the group of *Penicillium citrinum*, and 1 isolate placed within the group of *Metarhizium anisopliae*. ● = fungal isolates obtained from this research

Table 2 Mean mortality of *Spodoptera frugiperda* larvae treated with endophytic fungi (1 x 10^6 conidia mL^{-1}), and conidial density and viability of endophytic fungi

Fungal isolate code	Fungal species	Mortality ± SE (%)	Conidial viability ± SE (%) for 1 x 24 hours	Conidial viability ± SE (%) for 2 x 24 hours
Control	-	0.00 ± 0.00a	-	-
GaTpeOi	*Chaetomium* sp.	4.00 ± 1.89bcd	45.17±1.51bcd	47.00±0.34a
PsgTjPr	*Aspergillus niger*	2.67 ± 2.18abc	38.20±0.79ab	51.63±0.31abcd
JgSPK	*Beauveria bassiana*	22.67 ± 2.88f	47.59±1.33efgh	55.23±2.12cde
JgTjPr	*Chaetomium* sp.	2.67 ± 1.08abcd	35.74±2.36a	55.76±1.90de
JaGiP	*Beauveria bassiana*	21.33 ± 2.18f	42.86±3.39abc	52.91±1.03abcd
PiCrPga	*Chaetomium* sp.	5.33 ± 1.09cd	44.59±1.37cde	47.99±2.87abc
JaGiPRB	*Curvularia lunata*	4.00 ± 1.89bcd	45.18±1.76bcd	52.75±2.51abcd
CMTJP	*Curvularia lunata*	2.67 ± 1.09abcd	45.25±2.65efg	54.94±2.62bcde
JaMsBys	*Curvularia lunata*	6.67 ± 1.09d	49.98±1.64gh	58.60±3.00de
JaSpkPGA(2)	*Beauveria bassiana*	2.67 ± 1.09abcd	47.20±1.77cde	48.15±1.95abc
JgCrJr	*Beauveria bassiana*	21.33 ± 3.93f	46.50±1.50cd	52.63±2.31abcd
JaTpOi (1)	*Beauveria bassiana*	18.67 ± 1.09f	54.02±1.28efg	56.55±2.03de
JaSpkPga(3)	*Curvularia lunata*	17.33 ± 2.18ef	50.85±3.75def	56.93±1.59de
CaCjPga	*Chaetomium* sp.	2.67 ± 1.09abcd	37.24±3.40a	47.77±1.68ab
JgByU	*Aspergillus niger*	1.33 ± 1.09ab	58.84±1.72gh	69.57±3.81g
JgTgSr	*Curvularia lunata*	4.00 ± 0.00bcd	55.33±1.75efgh	61.24±1.12ef
JaBuBys	*Aspergillus niger*	2.67 ± 1.09abcd	58.45±0.20gh	76.50±2.08h
JgPWSR	*Aspergillus flavus*	4.00 ± 0.00bcd	62.54±2.08h	68.39±0.34g
JaTpOi(2)	*Penicillium citrinum*	6.67 ± 2.17cd	57.44±0.27fgh	65.98±1.35fg
CaTpPga	*Metarhizium anisophiae*	8.00 ± 1.88de	50.76±0.81def	68.75±3.29f
F-value		7.27*	9.05*	15.67*
P-value		0.00	0.00	0.00
HSD value		8.8	4.27	4.07

Note: * = significantly different; values within a column followed by the same letters were not significantly different at P < 0.05 according to Tukey's HSD test

Pathogenicity of the endophytic fungi against *Spodoptera frugiperda* larvae

From seven species of 20 isolates of the endophytic fungi found in this study, there were 6 isolates that were the most pathogenic against *S. frugiperda* larvae (Table 2). The four isolates (JgSPK, JaGiP, JgCrJr, and JaTpOi1) with the resulting mortality of 22.67%, 21.33%, 21.33%, and 17.33% consecutively was *B. bassiana*. In addition, the isolate JaSpkPga(3) isolate of *C. lunata* could cause a fairly high mortality (18.67%) and was not significantly different from the mortality caused by the isolates of JgSPK, JaGiP, JgCrJr, JaTpOi1. The CaTpPga isolate of *M.*

anisopliae caused 8% larvae mortality and was not significantly different from the mortality caused JaSpkPga(3) isolate. The *B. bassiana* and *C. lunata* isolates were isolated from maize, and the *M. anisopliae* isolate was isolated from red chili. The JaSpkPGA(2) isolate of *B. bassiana* resulting mortality was only 2.67%. Likewise, *P. citrinum*, *Chaetomium* sp., *A. niger,* and *A. flavus* caused the lowest larvae mortality of less than 8%.

The larvae of *S. frugiperda* that died by eating the leaves colonized by the endophytic fungi had characteristics, i.e. dull and dark integument, shriveled body, dry, and odorless. The feces excreted by sick larvae was wetter than the those of untreated larvae (control). The larvae that died as a result of treatment by the endophytic fungi when grown in SDA media, their cadavers were not overgrown with mycelia fungi.

Discussion

The isolates of the endophytic fungi found in this study had an rDNA sequence similarity value of more than 99% to the reference species (BLAST), meaning that the isolates had a high phylogenetic relationship and were in the same species. Bich et al. (2021) state that ribosomal DNA sequences are used to determine the phylogenetic relationships of organisms to taxa species. Based on the similarity value, it is stated that if the similarity value is 100%, it means that the isolates are the same strain, whereas if a similarity value of 99% indicates the isolates are the same species, and if the similarity value of the isolates is 89-99%, it means they are the same genus (Henry et al. 2000). An organism is declared the same species when the difference in DNA sequences is between 0.2–1% (Shenoy et al. 2007).

The results of identification based on the morphological characters of several species of fungi showed the similarities, for example the morphology of the colony and the shape of the conidia isolates of *B. bassiana* were similar to the morphology of *P. citrinum*, but after the molecular identification, they showed different species. According to Minarni et al. (2021) proper species identification is very important in determining a species as an entomopathogen.

The fungi in this study proved to be endophytic based on the characteristics of maize stalks and leaves colonized by the fungal mycelia. According to Barra-Bucarei et al. (2020), endophytic fungus (*B. bassiana*) has a systemic mode of action ability to colonize their host plant (tomato). The endophytic fungi can still be found on roots, stems, and leaves of tomato up to 30 days after inoculation (Carolina et al. 2020). Endophytic *B. bassiana* colonized 100% of leaves, 80% of stems, and 60% of roots of maize seven days after foliar spray and caused significant reductions in *S. frugiperda* growth and reproduction (Russo et al. 2020).The research results of Shikano (2018) showed the endophytic fungi were able to live in the tissue for several months depending on the age of the host plant.

Of seven species of 20 isolates of the endophytic fungi found in this study, there were only 6 isolates the most pathogenic against the larvae of *S. frugiperda*. The

isolates JgSPK, JaGiP, JgCrJr, JaTpOi1 belong to *B. bassiana*, the JaSpkPga(3) and CaTpPga isolates belong to *C. lunata* and *M. anisopliae*, respectively. First report of *B. bassiana* and *C. lunata* isolated from maize, and *M. anisopliae* isolated from red chili in Indonesia were found as entomopathogenic endophytic fungi against *S. frugiperda*. The endophytic *B. bassiana* has been reported to be effective in killing several species of the insect pests, such as *S. frugiperda* (Ramos et al. 2020), *Diaphorina citri* (Bamisile et al. 2019), and *Trialeurodes vaporariorum* (Barra-Bucarei et al. 2020). *Curvularia* spp. also proves to suppress insect populations (Assaf et al. 2011), but Gao et al. (2014) stated that *C. lunata* is an important maize foliar fungal pathogen. The endophytic *M. anisopliae* effectively killed the larvae of *S. frugiperda* (Ramos et al. 2020) and *Agrotis ipsilon* (Ahmad et al. 2020).

The endophytic fungi species of *B. bassiana* (JgSPK, JaGiP, JgCrJr, JaTpOi1 isolates), *C. lunata* (JaSpkPga(3) isolate), and *M. anisopliae* (CaTpPga isolate) had potential as entomopathogens of *S. frugiperda* larvae. *S. frugiperda* larvae that died from consuming plants colonized by the endophytic fungi showed that the fungi could be applied through seed treatment, especially for maize seeds. The seed treatment causes the endophytic fungi to colonize plants (Gustianingtyas et al. 2021) and if *S. frugiperda* larvae eat them it can cause them die (Ramos et al. 2020).

The *B. bassiana* of JaSpkPGA(2) isolate and all isolates of *P. citrinum, Chaetomium* sp., *A. niger*, and *A. flavus* showed no potential as entomopathogens of *S. frugiperda* with their resulting mortality of less than 8%. In contrast to the research results of Gustianingtyas et al. (2021) which showed the endophytic fungi species (*Aspergillus* sp. and *Chaetomium* sp.) applied topically (contact) caused mortality of *S. frugiperda* larvae as many as 18.67% and 14.67%, respectively and the mortality caused by *Beauveria* sp. reached 29%. This difference in mortality was due to different ways of application of fungi, in this study the larvae of *S. frugiperda* consumed the plants that had been colonized by the endophytic fungi which were not applied topically.

The larvae of *S. frugiperda* that were sick and died by eating leaves colonized by the endophytic fungus had different characteristics from the larvae that were sick and died due to the topical suspension of the endophytic fungus. The sick larvae of *S. frugiperda* in this study had wetter feces and the cadavers were not overgrown and covered by the fungal mycelia, while the sick larvae caused by contact with suspension of the endophytic fungi according to Gustianingtyas et al. (2021) had dry feces and the cadavers were covered with the fungal mycelia. The dead larvae treated by the endophytic fungi were not overgrown with mycelia fungi because the cadavers were too dry. According to El-Ghany (2015), the success of a fungus in germinating was affected by several external factors, such as humidity (more than 98%). So, if the cadavers were too dry, the fungal conidia could not germinate on the cadavers.

Conclusions

Finally, the results of molecular identification were found 7 species of the endophytic fungi, namely *Chaetomium* sp. (GaTpeOi, JgTjPr, PiCrPga, and CaCjPga isolates), *Aspergillus niger* (PsgTjPr, JgByU, and JaBuBys), *Beauveria bassiana* (JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, and JaTpOi (1) isolates), *Curvularia lunata* (JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr isolates), *Aspergillus flavus* (JgPWSR isolate), *Penicillium citrinum* (JaTpOi(2) isolate), and *Metarhizium anisopliae* (CaTpPGA isolate). The endophytic fungal species of *B. bassiana* (JgSPK isolate) causing up to 22.67% mortality, *C. lunata* (JaSpkPga(3) isolate) causing 17.33% mortality, and *M. anisopliae* (CaTpPga isolate) causing 8% mortality were pathogenic against *S. frugiperda* larvae. The *B. bassiana*, *C. lunata*, and *M. anisopliae* were pathogenic against *S. frugiperda* larvae. First report of *B. bassiana* and *C. lunata* isolated from maize, and *M. anisopliae* isolated from red chili in Indonesia were found as entomopathogenic endophytic fungi against *S. frugiperda*. The endophytic fungi species of *B. bassiana*, *C. lunata*, and *M. anisopliae* had potential as entomopathogens of *S. frugiperda* larvae.

List of abbreviations

ANOVA: analysis of variance; BLAST: Basic Local Alignment Search Tool; CTAB: cetyltrimethylammonium bromide; DNA: Deoxyribonucleic acid; EtOH: Ethyl alcohol; HSD: Tukey's Honestly Significant Difference; ITS: Internal Transcribed Spacer; MEA: the malt extract agar; NaOCl: Sodium hypochlorite; SDA: Sabouraud Dextrose Agar; TBE: Tris-Boric Acid-EDTA.

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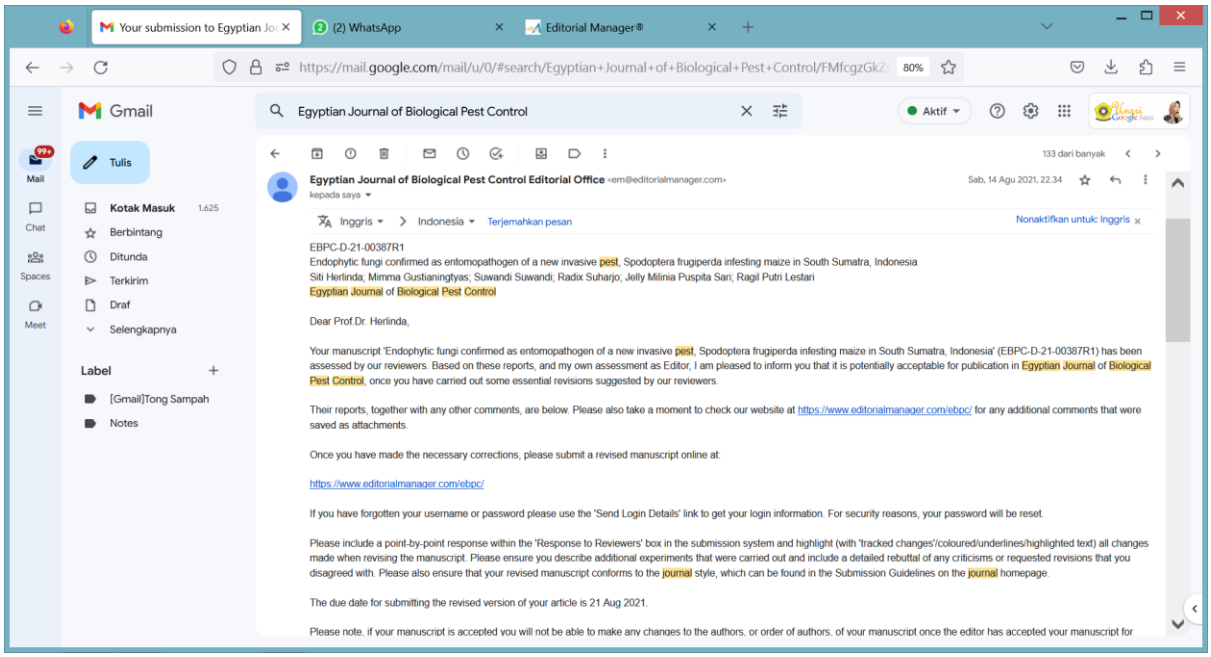

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Endophytic fungi confirmed as entomopathogen of a new invasive pest, Spodoptera frugiperda infesting maize in South Sumatra, Indonesia

Siti Herlinda; Mimma Gustianingtyas; Suwandi Suwandi; Radix Suharjo; Jelly Milinia Puspita Sari; Ragil Putri Lestari

Egyptian Journal of Biological Pest Control

Dear Prof.Dr. Herlinda,

Your manuscript 'Endophytic fungi confirmed as entomopathogen of a new invasive pest, Spodoptera frugiperda infesting maize in South Sumatra, Indonesia' (EBPC-D-21-00387R1) has been assessed by our reviewers. Based on these reports, and my own assessment as Editor, I am pleased to inform you that it is potentially acceptable for publication in Egyptian Journal of Biological Pest Control, once you have carried out some essential revisions suggested by our reviewers.

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Egyptian Journal of Biological Pest Control

Endophytic fungi confirmed as entomopathogens of the new invasive pest, the fall armyworm, Spodoptera frugiperda (JE Smith) (Lepidoptera: Noctuidae), infesting maize in South Sumatra, Indonesia

--Manuscript Draft--

Manuscript Number:	EBPC-D-21-00387R2
Full Title:	Endophytic fungi confirmed as entomopathogens of the new invasive pest, the fall armyworm, Spodoptera frugiperda (JE Smith) (Lepidoptera: Noctuidae), infesting maize in South Sumatra, Indonesia
Article Type:	Research
Funding Information:	ministry of education, culture, research, and technology, republic of indonesia (150/E4.1/AK.04.PT/2021) — Prof. Dr. Siti Herlinda
Abstract:	Background: The fall armyworm, Spodoptera frugiperda (JE Smith) (Lepidoptera: Noctuidae) is a new invasive pest in Indonesia causing financial losses. S. frugiperda larvae hide in the leaf midrib all day. To overcome the hidden larvae, pathogenic endophytic fungi are needed. The objective of this research was to molecularly identify the endophytic fungal species from South Sumatra and determine the most pathogenic species against S. frugiperda larvae. Endophytic fungal identification was based on morphological and molecular characteristics. The molecular identification was based on gene sequential analysis of Intergenic Transcribed Spacer (ITS) 1 and ITS 4. Bioassay of the endophytic fungal species was treated against the 2nd larvae instar of S. frugiperda. Result: The results of molecular identification revealed endophytic fungal species consisted of Chaetomium sp. (GaTpeOi, JgTjPr, PiCrPga, and CaCjPga isolates), Aspergillus niger (L.) Van Tieghem (PsgTjPr, JgByU, and JaBuBys), Beauveria bassiana (Balsamo) Vuillemin (JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, and JaTpOi (1) isolates), Curvularia lunata (Wakker) Boed. (JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr isolates), Aspergillus flavus Link. (JgPWSR isolate), Penicillium citrinum Thom F 1539 (JaTpOi(2) isolate), and Metarhizium anisopliae (Metschn.) Sorokin (CaTpPGA isolate). All fungal isolates were deposited in the GenBank. The endophytic fungal species of B. bassiana (JgSPK isolate) caused up to (22.67%) mortality, C. lunata (JaSpkPga(3) isolate) caused (17.33%) mortality, and M. anisopliae (CaTpPga isolate) caused (8%) mortality of the pest's larvae. First report of B. bassiana and C. lunata isolated from maize (Zea mays L.), while M. anisopliae was isolated from red chili (Capsicum annuum L.) as entomopathogenic endophytic fungi against S. frugiperda larvae in Indonesia. Conclusion: The endophytic fungi species of B. bassiana, C. lunata, and M. anisopliae had potentials as entomopathogens of S. frugiperda.
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Response to Reviewers:	Dear Editors, we have revised all suggestions from editors/reviewers. The revisions in the manuscript were highlighted with yellow colour. We have also responded to editor/reviewer comments: 1.Comment [A1]: Authors should not use an excessive number of citations to support one point. Response to Comment [A1]: We have revised them and used a citation to support one point. The revision in the manuscript was highlighted with yellow colour. 2.Comment [A2]: Delete the figure?? As all its information was mentioned in the table?? Response to Comment [A2]: We have deleted the figure 1. The revision in the manuscript was highlighted with yellow colour. 3.Comment [A 3]: Prepupa IS not a stage???? Response to Comment [A3]: We have revised it. The revision in the manuscript was highlighted with yellow colour. 4.Comment [A 4]: Fig. 1 was deleted? Response to Comment [A4]: We have deleted the figure 1. The revision in the manuscript was highlighted with yellow colour. 5.Comment [A5]: Authors should not use an excessive number of citations to support one point. Response to Comment [A5]: We have revised them and used a citation to support one point. The revision in the manuscript was highlighted with yellow colour. 6.Comment [A6]: Discussion should include the target pest and /or same pathogens species? Different hosts have different susceptibility?? Response to Comment [A6]: We have revised the discussion as follows: “C. lunata infected and killed grain insect pests, such as Tribolium castaneum (Herbst.) (Coleoptera: Tenebrionidae), Trogoderma granarium (Everts) (Coleoptera: Dermestidae), Rhyzopertha dominica (F.) (Coleoptera: Bostrichidae), and Cryptolestes ferrugineus (Stephens) (Coleoptera: Cucujidae) (Wakil et al. 2014).” This revision in the manuscript was highlighted with yellow colour. 7.Comment [A7]: Conclusions should touch the significant results and suggest recommendations?? Response to Comment [A7]: We have added the recommendations as follows: “The endophytic fungi species of B. bassiana, C. lunata, and M. anisopliae had potentials as entomopathogens of S. frugiperda.” This revision in the manuscript was highlighted with yellow colour. 8.Comment [A8 and A9]: References list should be written by a simple way that allows doing corrections??? Response to Comment [A8 and A9]: We have revised the references.
Additional Information:	
Question	**Response**
Is this study a clinical trial? <p>A clinical trial is defined by the World Health Organisation as ‘any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes’.	No

Endophytic fungi confirmed as entomopathogens of the new invasive pest, the fall armyworm, *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae), infesting maize in South Sumatra, Indonesia

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Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data are available in the article and the materials used in this work are of high quality and grade.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

SH performed research concept and design, writing the article, and final approval of article. MG performed collection and assembly of data. SS prepared and performed morphological identification and critical revision of the article. RS performed and performed molecular identification and data analysis and interpretation. JMPS performed collection and assembly of data. RPL performed collection and assembly of data. All the authors read and approved the manuscript.

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Endophytic fungi confirmed as entomopathogens of the new invasive pest, the fall armyworm, *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae), infesting maize in South Sumatra, Indonesia

Abstract

Background: The fall armyworm, *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae) is a new invasive pest in Indonesia causing financial losses. *S. frugiperda* larvae hide in the leaf midrib all day. To overcome the hidden larvae, pathogenic endophytic fungi are needed. The objective of this research was to molecularly identify the endophytic fungal species from South Sumatra and determine the most pathogenic species against *S. frugiperda* larvae. Endophytic fungal identification was based on morphological and molecular characteristics. The molecular identification was based on gene sequential analysis of Intergenic Transcribed Spacer (ITS) 1 and ITS 4. Bioassay of the endophytic fungal species was treated against the 2nd larvae instar of *S. frugiperda*.

Result: The results of molecular identification revealed endophytic fungal species consisted of *Chaetomium* sp. (GaTpeOi, JgTjPr, PiCrPga, and CaCjPga isolates), *Aspergillus niger* (L.) Van Tieghem (PsgTjPr, JgByU, and JaBuBys), *Beauveria bassiana* (Balsamo) Vuillemin (JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, and JaTpOi (1) isolates), *Curvularia lunata* (Wakker) Boed. (JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr isolates), *Aspergillus flavus* Link. (JgPWSR isolate), *Penicillium citrinum* Thom F 1539 (JaTpOi(2) isolate), and *Metarhizium anisopliae* (Metschn.) Sorokin (CaTpPGA isolate). All fungal isolates were deposited in the GenBank. The endophytic fungal species of *B. bassiana* (JgSPK isolate) caused up to (22.67%) mortality, *C. lunata* (JaSpkPga(3) isolate) caused (17.33%) mortality, and *M. anisopliae* (CaTpPga isolate) caused (8%) mortality of the pest's larvae. First report of *B. bassiana* and *C. lunata* isolated from maize (*Zea mays* L.), while *M. anisopliae* was isolated from red chili (*Capsicum annuum* L.) as entomopathogenic endophytic fungi against *S. frugiperda* larvae in Indonesia.

Conclusion: The endophytic fungi species of *B. bassiana*, *C. lunata*, and *M. anisopliae* had potentials as entomopathogens of *S. frugiperda*.

Keywords: Endophytic fungi, Invasive pest, *Spodoptera frugiperda*, Morphological and molecular identifications, Indonesia.

Background

The fall armyworm, *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae) is a new invasive pest, originating from South America (Otim *et al.* 2018). Currently, it has spread throughout the world and its first record in Indonesia was in March 2019 in West Sumatra (Sartiami *et al.* 2020) and in July 2019 in South Sumatra (Hutasoit *et al.* 2020). Now it has been spread all over Indonesia. The pest attacks maize (*Zea mays* L.) and various other plant species by means of its larval stage, eating leaves, stems, flowers, fruits, growing points, and the whole maize

plant until it becomes bare (Montezano *et al.* 2018). Larvae are found on the surface of leaves or maize stalks in the morning and then hide in the leaves' midribs (Gustianingtyas *et al.* 2021). Such behavior makes it difficult to be controlled.

Laboratory experiments of Herlinda *et al.* (2020a) showed that the entomopathogenic fungus (EPF), *Metarhizium* sp. could kill (78%) of *S. frugiperda* larvae. *Beauveria bassiana* (Balsamo) Vuillemin, *Metarhizium anisopliae* (Metschn.) Sorokin, and *M. rileyi* (Farlow) Samson also killed more than (80%) *S. frugiperda* larvae (Ramanujam *et al.* 2020). Their treatments were applied topically (direct contact) with the larvae exposed. In the field, the presence of hiding larvae in the midribs all the day makes the contacted EPF less effective (Gustianingtyas *et al.* 2021). To overcome such behavior, EPF that found in plant tissues (endophytic fungi) are needed (Ramos *et al.* 2020). The endophytic fungi systemically colonize plant tissues and associate mutually their host plants (Lira *et al.* 2020). The results of previous studies have proven that 8 isolates of endophytic EPF could topically kill *S. frugiperda* larvae (Gustianingtyas *et al.* 2021), however, the endophytic fungi found in their studies were only isolated from the roots and their molecular identification had not yet been carried out.

In this study, the fungi isolated from leaves, shoots, and roots of maize plants from South Sumatra (Indonesia) were identified morphologically and molecularly and their pathogenicity to *S. frugiperda* larvae was evaluated.

Methods

Purification, identification, and bioassay of fungi were conducted from January to March 2021. The endophytic fungal species were identified based on the molecular analysis carried out at a laboratory accredited according to the ISO 17025 standard. Bioassay of the fungi was carried out in an incubator at controlled and constant temperature and relative humidity, respectively 25ºC and 97%.

Exploration, isolation, and purification of fungi

Exploration of endophytic fungi was carried out by collecting leaves, shoots, and roots of maize and other crop plants around it such as: bananas (*Musa* spp.), ridged gourd (*Luffa acutangula* (Roxb.) L.) and red chilies (*Capsicum annuum* L.). Selection of individual crop plant samples to obtain the endophytic fungi, followed the method of Kasambala *et al.* (2018). Samples of the plant parts were placed into an ice box, then taken to the laboratory. The exploration of endophytic fungi was carried out from the lowlands to the highlands of South Sumatra, Indonesia namely in Banyuasin District (2.8833°S 104.3831°E), Ogan Ilir District (3.43186°S 104.62727°E), Prabumulih City (3.4328°S 104.2356°E), Pagar Alam City (3°52′43.8″S 103°21′30″E) (Table 1).

Table 1 Origin of isolates of endophytic fungi from South Sumatra, Indonesia

Location (village, district/city)	Isolate origin	Altitude (m)	Fungal species	Fungal isolate code	GenBank Acc. No.
Tanjung Pering. Ogan Ilir	Ridged gourd	36.0	*Chaetomium* sp.	GaTpeOi	MZ359734
Tanjung Pering. Ogan Ilir	Bananas	36.0	*Aspergillus niger*	PsgTjPr	MZ242060
Simpang Padang Karet. Pagar Alam	Maize	797.7	*Beauveria bassiana*	JgSPK	MZ356494
Tanjung Pering. Ogan Ilir	Maize	36.0	*Chaetomium* sp.	JgTjPr	MZ359736
Gunung Ibul. Prabumulih	Maize	53.0	*Beauveria bassiana*	JaGiP	MZ356495
Curup Jare. Pagar Alam	Bananas	806.0	*Chaetomium* sp.	PiCrPga	MZ359735
Gunung Ibul. Prabumulih	Maize	53.0	*Curvularia lunata*	JaGiPRB	MZ359815
Tanjung Payang. Pagar Alam	Red chilies	689.6	*Curvularia lunata*	CMTJP	MZ359816
Mulia Sari. Banyuasin	Maize	19.0	*Curvularia lunata*	JaMsBys	MZ359819
Simpang Padang Karet. Pagar Alam	Maize	789.5	*Beauveria bassiana*	JaSpkPGA(2)	MZ356496
Curup Jare. Pagar Alam	Maize	806.7	*Beauveria bassiana*	JgCrJr	MZ356497
Tanjung Pering. Ogan Ilir	Maize	36.0	*Beauveria bassiana*	JaTpOi (1)	MZ356498
Simpang Padang Karet. Pagar Alam	Maize	797.7	*Curvularia lunata*	JaSpkPga(3)	MZ359818
Curup Jare. Pagar Alam	Red chilies	806.0	*Chaetomium* sp.	CaCjPga	MZ359737
Banyuurip. Banyuasin	Maize	13.0	*Aspergillus niger*	JgByU	MZ242059
Telang Sari. Banyuasin	Maize	15.0	*Curvularia lunata*	JgTgSr	MZ359817
Banyuurip. Banyuasin	Maize	20.0	*Aspergillus niger*	JaBuBys	MZ242058
Purwosari. Banyuasin	Maize	15.0	*Aspergillus flavus*	JgPWSR	MZ359829
Tanjung Pering. Ogan Ilir	Maize	36.0	*Penicillium citrinum*	JaTpOi(2)	MZ359812
Tanjung Payang. Pagar Alam	Red chilies	689.6	*Metarhizium anisopliae*	CaTpPga	MZ242073

The leaves, shoots, and roots of the sample plants were cleaned and washed aseptically with running tap water. Then, the plant parts were surface sterilized using the method of Elfita *et al.* (2019) with 70% EtOH (Ethyl alcohol) and 1% NaOCl (Sodium hypochlorite), then rinsed 3 times.

The last rinse water was used to check whether the microorganisms causing surface contamination were still present and carried out by growing them onto Sabouraud Dextrose Agar (SDA) (Russo *et al.* 2020). Obtaining the endophytic fungi isolates started with growing the plant parts that sterilized earlier onto a media. The roots were grown on a specific medium, the malt extract agar (MEA), following the method of Silva *et al.* (2018). The shoots and leaves of the sample plants were grown on SDA media. The plant parts were grown as many as 5 pieces per Petri dish with a length of 5 mm and a diameter of 1-5 mm (depending on the diameter of the leaves, shoots, and roots). Fungi growing from the leaves, shoots, and roots were purified to be one isolate per sample. The isolates were then observed for their microscopic characteristics and continued with the fungal colonization test into the maize seed tissue.

Assess endophytic fungal colonization

Fungal inoculation on maize seeds was carried out to observe the colonization of fungi into the maize seedling tissue and to further ensure that the isolates found were truly endophytic. The 15 seeds of maize were surface sterilized, using 70% EtOH (Ethyl alcohol) and 1% NaOCl (Sodium hypochlorite) (Elfita *et al.* 2019). The seeds were immersed in 10 ml of fungal suspension, with a concentration of 1 x 10^6 conidia ml^{-1} for 6 hrs, while for the control, the sterilized seeds were only immersed in 10 ml of destilled water. Then, the seeds were grown in a sterile glass bottle (250 ml volume) with a sterile filter paper (whatman no. 42) at the bottom, which was moistened with 1 ml destilled water and incubated for 10 days. All the treatments in this experiment were repeated 3 times. After the maize seedlings were 10 days old, the stems and leaves were sliced crosswise and longitudinally with a thickness of 0.02 mm each. The slices were observed with a light microscope at 40 x magnification with 0.05% lactofenol trypan blue dye. Mycelia endophytic fungi found in the maize seedling tissue were documented. The fungal isolates that were proven to be endophytic were used for bioassays. Prior to the bioassay, the isolates were observed for microscopic characteristics for morphological identification, followed by molecular identification.

Morphological and molecular identification of fungal species

Macroscopic and microscopic observation

Morphological observations were carried out based on the macroscopic characteristics of the endophytic fungi that included colony color and shape, shape and size of conidia and conidiophores, following the method of Herlinda *et al.* (2020a).

DNA extraction and PCR amplification

The used endophytic fungal DNA extraction method refers to the Swibawa *et al.* (2020) method. DNA extraction was carried out on fungal conidia. The conidia were harvested from pure cultures of 7 days old fungus, which was cultured in sterile Petri dishes with a diameter of 9 cm. As much as 10 ml of sterile water was placed into a Petri dish containing fungal cultures and slowly harvested, using a drigalski. The obtained conidia suspension was transferred to a

centrifuge tube (volume 30 ml) and centrifuged using CF15RXII (Hitachi, Japan) for 10 min at a speed of 14,000 rpm. After being centrifuged, 1 ml of 70% ethanol was added to the centrifuge tube and then centrifuged again for 10 min. After that, the supernatant was removed and 1 ml of extraction buffer was added with a composition of 0.5 ml Tris HCl, 1 mL SDS 1% + 2.8 mL NaCl, 0.2 ml Mercaptho Ethanol, 2 ml EDTA, 3.5 ml sterile water and then homogenized. The suspension was transferred to a sterile mortal and incubated at -40 °C for 24 hrs. After that, the frozen suspension was ground until it was smooth and melted. A total of 500 µl of suspension was then transferred into a 1.5 ml tube. A total of 400 µl of 2% cetyltrimethylammonium bromide (CTAB) was then added to the tube, homogenized and then incubated at 65 °C for 1 hr using a water bath (Brookfield TC 550 MX-230, USA). After the incubation, 500 µl of Phenol Chloroform Isoamyl alcohol (PCI) (25:24:1) was added, homogenized and centrifuged (Microspin12; Biosan, Latvia) at 14,000 rpm for 10 min. A total of 600 µl of supernatant was taken and transferred to a new 1.5 ml tube. A total of 600 µl Chloroform Isoamyl Alcohol (CI) (24:1) was added to the tube, homogenized and centrifuged (Microspin12; Biosan, Latvia) at 14,000 rpm for 10 min. A total of 400 µl of supernatant was then transferred to a new 1.5 ml tube and 400 µl of cold isopropanol was added, homogenized, and incubated at -40 ºC for 20 min. After the incubation, it was centrifuged (Microspin12; Biosan, Latvia) at 14,000 rpm for 15 min. The supernatant was then discarded. After the supernatant was removed, 500 µl of 70% cold ethanol was added and centrifuged (Microspin12; Biosan, Latvia) at 14,000 rpm for 5 min. The supernatant was then discarded and the pellets obtained were dried at room temperature for 24 hrs. After drying, the tube was then added as much as 50 µl 1x Tris-HCL EDTA (TE) pH 8.0 (1st Base Malaysia).

PCR amplification was carried out using the Sensoquest Thermal Cycler (Germany) PCR machine on the Internal Transcribed Spacer (ITS) using ITS1 and ITS4 primers (White *et al.* 1990). The PCR was carried out with a total volume of 25 µl consisting of a mixture of Master Mix (Red Mix) (bioline) as much as 12.5 µl, 10 µM of primer ITS 1 (5'TCC GTA GGT GAA CCT TGC GG 3 ') and ITS 4 (5'TCC TCC GCT TAT TGA TAT GC 3') 1 µL each, 1 L of template DNA and 9.5 µl of sterile water. The DNA amplification stage consisted of 1 initiation cycle at 95 ºC for 5 min, followed by 30 cycles consisting of denaturation at 95 ºC for 1 min, primer attachment at 52 ºC for 1 min, primer extension at 72 ºC for 1 min, and 1 elongation cycle at 72 ºC for 5 min. The PCR results were then electrophoresed, using 0.5% agarose in 20 ml of 1x Tris-Boric Acid-EDTA (TBE) buffer (1st Base Malaysia) and added 1 µl of Ethidium Bromide (EtBr 10 mg/ml). The electrophoresis was carried out in 1x TBE buffer solution at 50 volts for 70 min. Electrophoresis results were visualized using a DigiDoc UV transilluminator (UVP, USA).

Sequencing and analysis of the results

The PCR results were then sent to 1st Base Malaysia for the sequencing process. The results of the sequencing were analyzed, using Bio Edit ver. 7.2.6 for windows. Then, the results were submitted to the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to find out the genus or species that had the greatest

homology or similarity and molecularly. The phylogeny tree was created using the Mega 7 for Windows program (Kumar *et al*. 2016), using the UPGMA (jukes and cantor model) method. The ITS region sequences for several strains used as a reference in this study were obtained from NCBI (https://www.ncbi.nlm.nih.gov/).

Calculation of conidial density and viability

All the isolates of endophytic fungi were counted for their conidial density and viability. The conidial density of the 7 old days fungi was calculated according to the method of Sumikarsih *et al.* (2019), using a haemocytometer and observed with a light microscope at 40 x magnification. The viability was observed by growing 1 ml of conidia fungal suspension (1 x 10^6 conidia ml^{-1}) onto 2% agar-water medium, then the culture was incubated for 1 x 24 and 2 x 24 hrs under a light microscope at 40 x magnification.

Mass-rearing of *Spodoptera frugiperda*

The mass-rearing of *S. frugiperda* was carried out, following the method of Herlinda *et al.* (2020a). The larvae were collected from the maize plants grown around the campus (3°13′25″S 104°39′51″E), were reared in the laboratory for more than 5 generations to obtain the larvae with relatively homogeneous genetics and to eliminate the effects of exposure to synthetic insecticides from the fields. In the laboratory, the larvae of *S. frugiperda* were kept individually in a porous plastic cup (Ø 6.5 cm, height 4.6 cm) because the larvae were cannibals. The larvae were fed daily by maize leaves (measuring 2 cm x 5 cm). Prepupae were transferred to a plastic container (Ø 15 cm, height 25 cm) on which its bottom had a sterile soil (5 cm thick). The plastic container containing the prepupae was placed in a wire mesh cage (30 x 30 x 30 cm^3) and inside this cage placed also fresh maize leaves for the adults to lay eggs, which were changed daily. The one day old 2nd instar larvae were used for the bioassay.

Bioassay of endophytic fungi against larvae of *Spodoptera frugiperda*

The bioassay of endophytic fungi, followed the method of Zea *et al.* (2019). The young maize (seedling) already inoculated with the endophytic fungi and not-inoculated (control) aged 10 days old were given to the 2nd instar larvae of *S. frugiperda*. Each individual larvae was given one maize seedling whose cotyledons were already removed. The larvae were allowed to feed on the leaves and stems of the young maize for 6 hrs. in a sterile room (a laminar air flow cabinet) and each replication was tested for 25 individuals of *S. frugiperda* larvae. Then, the larvae were transferred to a porous plastic cup (Ø 6.5 cm, height 4.6 cm) and fed on healthy non-inoculated leaves measuring 2 cm x 5 cm per day per larvae. The experiment was designed, using completely randomized designs with treatments of 20 isolates, 3 replications per treatment. The dead larvae were recorded daily for 12 days, following the method of Herlinda *et al.* (2020b). The dead larvae were grown in the agar-water medium to confirm whether the infection was caused by the endophytic fungi or not. The number of dead larvae were used to calculate the mortality.

Data analysis

The differences in the mortality data of *S. frugiperda* larvae were analyzed using analysis of variance (ANOVA). Tukey's Honestly Significant Difference (HSD) test (Tukey's test) was employed to test for the significant differences among the treatments (isolates) at P = 0.05. All data were calculated using software of SAS University Edition 2.7 9.4 M5.

Results

Colonization of endophytic fungi on maize tissues

All fungal isolates, isolated from the leaves, shoots, and roots of the maize, bananas, ridged gourd, and red chilies were found to be 124 isolates. However, after assessing for the fungal colonization in young maize (seedlings) tissue, only 20 isolates of the fungi were confirmed as endophytic fungi (Fig. 1). Evidence of fungal colonization that entered the maize seedlings tissue was that mycelia fungi were seen entering and growing spread throughout the stem and leaf tissue. Mycelia endophytic fungi were able to invade the stem and leaf of the maize tissue, the mycelia appeared to be branched. The size of mycelia varied daily from 13.51 to 34.25 µm. In the maize tissue, which their seeds were not inoculated by the endophytic fungi (control), the cells in the maize tissue were clean and clear without fungal colonization.

Identification results of the endophytic fungal isolates

The colony morphology of 20 isolates of the endophytic fungi showed different colors (Fig. 2) and likewise the shapes of hyphae and conidia of each isolate showed their own characteristics (Fig. 3). The colony isolates of GaTpeOi, JgTjPr, PiCrPga, and CaCjPga grown on SDA media showed similarities, namely the colony was pale gray, hyphae and mycelia were septate, the conidia were in the shape of letter D (asymmetric/elliptical) and non-septation. Based on the phylogenetic tree, these 4 isolates were placed within the group of *Chaetomium* sp. (Fig. 4). The isolates were deposited in the GenBank with the accession number MZ359734 (GaTpeOi), MZ359736 (JgTjPr), MZ359735 (PiCrPga), MZ359737 (CaCjPga) (Table 1).

The colony isolates of PsgTjPr, JgByU, and JaBuBys showed similarities, their colonies were black, the hyphae and mycelia were also black, the conidia were non-septate, globose in shape. The 3 isolates were placed within the group of *Aspergillus niger* (L.) Van Tieghem (Fig. 4). The isolates were deposited in the GenBank with the accession number MZ242060 (PsgTjPr), MZ242059 (JgByu), and MZ242058 (JaBUBys) (Table 1).

The isolates of JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, and JaTpOi (1) had a white colony, white hyphae and mycelia, and the globose conidia, which were non-septate conidia. The isolates were placed within the group of *B. bassiana* (Fig. 4). The isolates were deposited in the GenBank with the accession number MZ356494 (JgSPK), MZ356495 (JaGiP), MZ356496 (JaSpkPGA(2)), MZ356497 (JgCrJr), and MZ356498 (JaTpOi1) (Table 1).

The isolates of JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr had a black colony, black hyphae and mycelia, two-septate boomerang-shaped conidia. All the isolates were placed within the group of *Curvularia lunata* (Wakker) Boed. (Fig. 4). The isolates were deposited in the GenBank with the accession number MZ359815 (JaGiPRB), MZ359816 (CMTJP), MZ359819 (JaMsBys), MZ359818 (JaSpkPga(3)), and MZ359817 (JgTgSr) (Table 1).

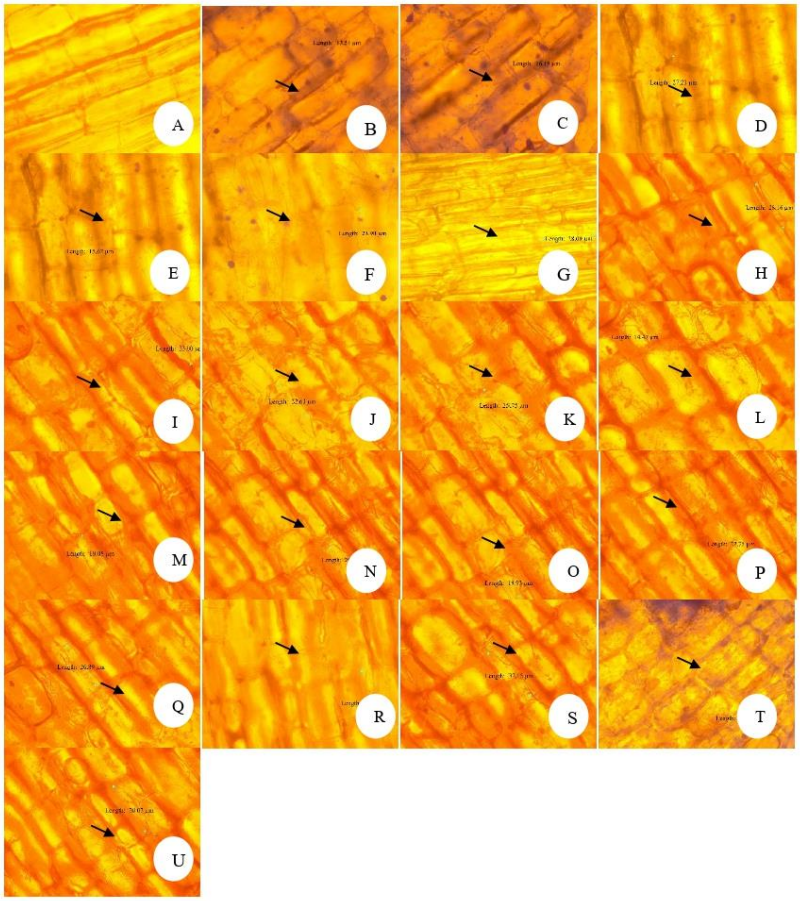

Fig. 1 Young maize tissues colonized by endophytic fungal species: Control (A); *Chaetomium* sp. isolates of GaTpeOi (B), JgTjPr (E), PiCrPga (G), and CaCjPga (O); *Aspergillus niger* isolates of PsgTjPr (C), JgByU (P), and JaBuBys (R); *Beauveria bassiana* isolates of JgSPK (D), JaGiP (F

JaSpkPGA(2) (K), JgCrJr (L), and JaTpOi(1) (M); *Curvularia lunata* isolates of JaGiPRB (H), CMTjP (I), JaMsBys (J), JaSpkPga(3) (N), and JgTgSr (Q); *Aspergillus flavus* isolate of JgPWSR (S); *Penicillium citrinum* isolate of JaTpOi(2) (T); *Metarhizium anisopliae* isolate of CaTpPga (U)

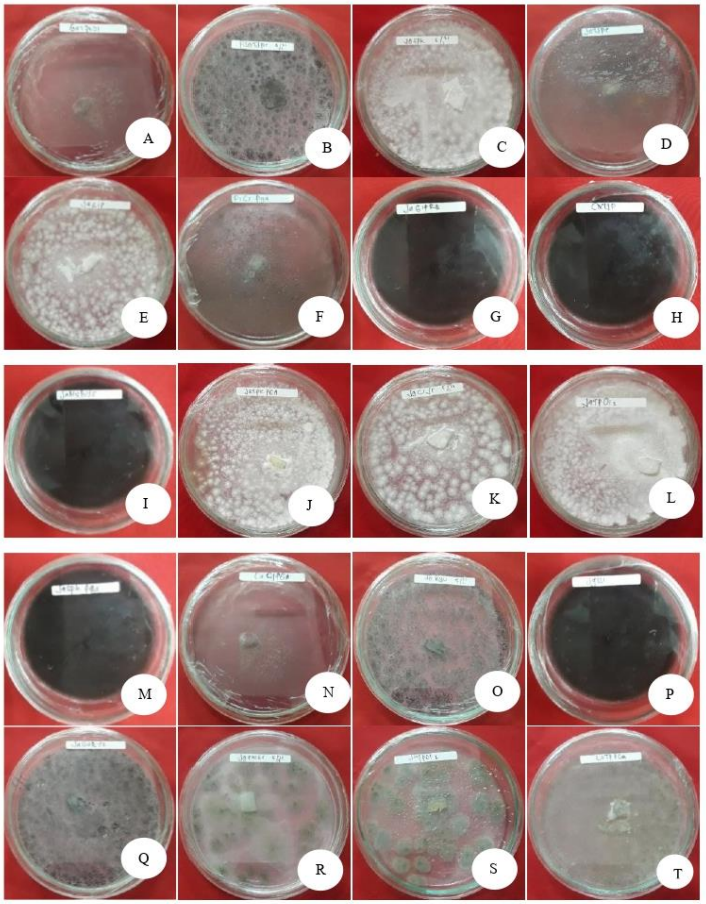

Fig. 2 Colony morphology of endophytic fungal species: *Chaetomium* sp. isolates of GaTpeOi (A), JgTjPr (D), PiCrPga (F), and CaCjPga (M); *Aspergillus niger* isolates of PsgTjPr (B), JgByU (O), and JaBuBys (Q); *Beauveria bassiana* isolates of JgSPK (C), JaGiP (D), JaSpkPGA(2) (J), JgCrJr (K), and JaTpOi(1) (L); *Curvularia lunata* isolates of JaGiPRB (G), CMTjP (H), JaMsBys

(I), JaSpkPga(3) (M), and JgTgSr (P); *Aspergillus flavus* isolate of JgPWSR (R); *Penicillium citrinum* isolate of JaTpOi(2) (S); *Metarhizium anisopliae* isolate of CaTpPga (T)

The isolate of JgPWSR had a green colony, green hyphae and mycelia, non-septate globose conidia. The JgPwSr isolate was placed within the group of *Aspergillus flavus* Link. (Fig. 4). The isolate was deposited in the GenBank with the accession number MZ359829 (JgPWSR) (Table 1).

The color of the colony JaTpOi(2) isolate was initially white, as the fungus got older, it turned bluish green with white margin, rounded conidia. The isolate was placed within the group of *Penicillium citrinum* Thom F 1539 (Fig. 4). The isolate was deposited in the GenBank with the accession number MZ359812 (JaTpOi (2)) (Table 1).

The isolate of CaTpPGA had a white colony and as the fungus got older it turned greenish white to dark green, green hyphae and mycelia, the conidia are clear, cylindrical and non-septation. The isolate was placed within the group of *M. anisopliae* (Fig. 4). The isolate was deposited in the GenBank with the accession number MZ242073 (CaTpPGA) (Table 1).

The result of BLAST search revealed that the isolates of GaTpeOi, JgTjPr, PiCrPga, and CaCjPga had a similarity (100%) with the *Chaetomium* sp. isolate A13 (Acc. No. MH34803.1), isolate MJ51 (Acc No. KM203618.1), and isolate CP-2009 (Acc. No. AB50680.1). The isolates of PsgTjPr, JgByU, and JaBuBys showed 100% of similarity with *A. niger* NBRC: 105649 (Acc. No LC573609.1), strain WM04.470 (Acc No. AJ853742.1) and isolate IFM61597 (Acc. No. LC602036.1). The isolates of JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, and JaTpOi (1) showed (100%) similarity to the *B. bassiana* isolate GZMS-28 (Acc. No. KT715480.1), strain TF6-1B (Acc No. JX122736.1), and isolate BSwTd4 (Acc. No. MT4487322.1). The isolates of JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr showed 99.81% of similarity to *C. lunata* strain D25A (Acc. No MH010917.1), strain AN 2 (Acc No. KY859790.1), and isolate 1z -7 (Ac. No. MN213745.1). The isolate of JgPWSR showed 100% of similarity to *A. flavus* strain GFRS9 (Acc. No MT447477.1), strain GFRS06 (Acc. No MT447474.1), and strain aT3 (Acc. No KU561920.1). The isolate of JaTpOi(2) showed 100% of similarity to *P. citrinum* strain yx-001 (Acc. No. MN826202.1), strain AKF2-KU (Acc No. MN879404.1), and strain J (Acc. No. MK791668.1). The isolate of CaTpPGA had a similarity (99.29%) to *M. anisopliae* isolate MSwTp3 (Acc. No MT448733.1), strain STBMa-001 (Acc No. KF766520.1), and isolate C1 (Acc. No. KX809520.1).

There were 7 species from the 20 isolates of the endophytic fungi found in this study. The 4 isolates (GaTpeOi, JgTjPr, PiCrPga, and CaCjPga) were in the group of *Chaetomium* sp., the three isolates (PsgTjPr, JgByU, and JaBuBys) were in the group of *Aspergillus niger*, the 5 isolates (JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, and JaTpOi (1)) were placed within the group of *B. bassiana*, the 5 isolates (JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr) were in the group of *C. lunata*, an isolate (JgPWSR) was in the group of *A. flavus*, an isolate (JaTpOi(2)) was in the group of *P. citrinum*, and an isolate (CaTpPGA) was in the group of *M. anisopliae*.

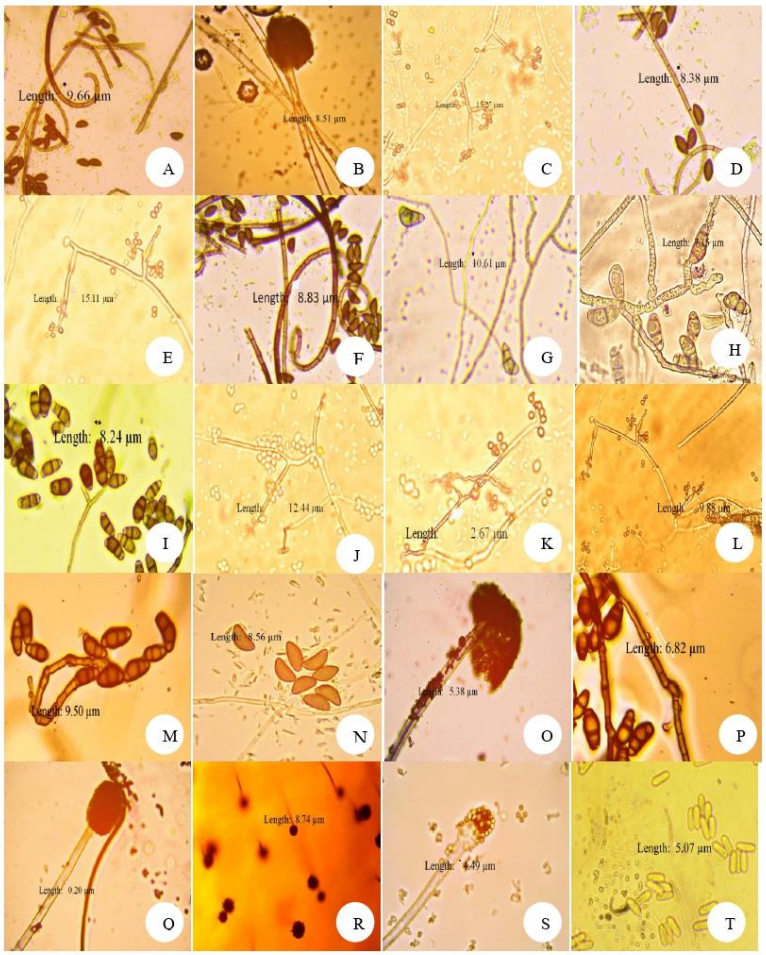

Fig. 3 Conidial and hyphal morphology of endophytic fungal species: *Chaetomium* sp. isolates of GaTpeOi (A), JgTjPr (D), PiCrPga (F), and CaCjPga (M); *Aspergillus niger* isolates of PsgTjPr (B), JgByU (O), and JaBuBys (Q); *Beauveria bassiana* isolates of JgSPK (C), JaGiP (D), JaSpkPGA(2) (J), JgCrJr (K), and JaTpOi(1) (L); *Curvularia lunata* isolates of JaGiPRB (G), CMTjP (H), JaMsBys (I), JaSpkPga(3) (M), and JgTgSr (P); *Aspergillus flavus* isolate of JgPWSR (R); *Penicillium citrinum* isolate of JaTpOi(2) (S); *Metarhizium anisopliae* isolate of CaTpPga (T)

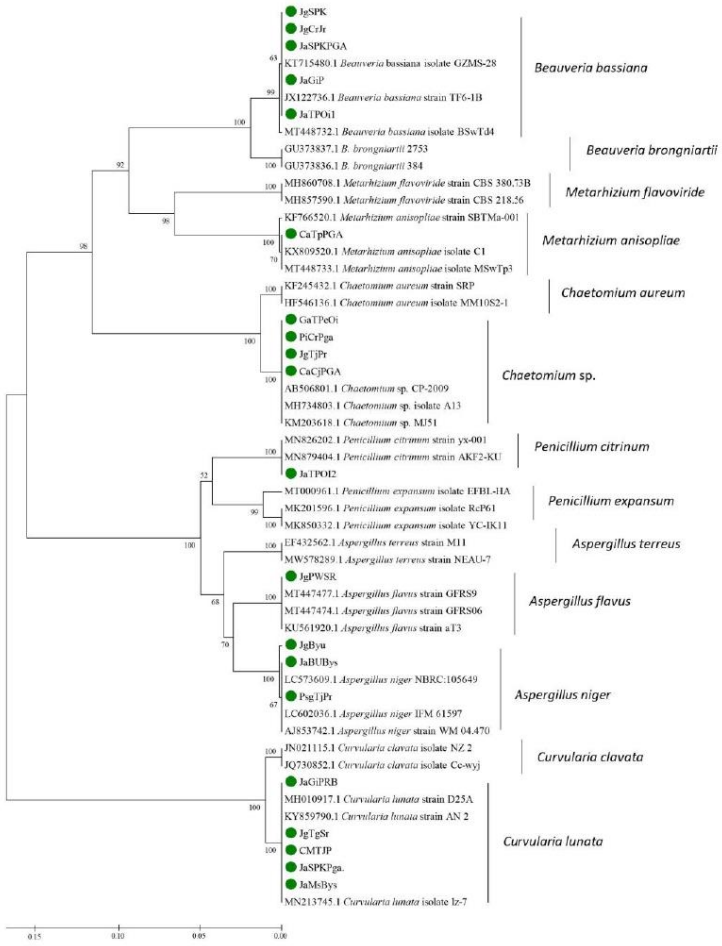

Fig. 4 Phylogenetic tree based on the sequencing of Internal Transcribed Spacer (ITS) region using UPGMA method (jukes and cantor model) performed with Mega 7 program for windows (Kumar *et al.,* 2016). This research found 20 isolates of entomopathogenic fungi: 4 isolates placed within the group of *Chaetomium* sp., 3 isolates placed within the group of *Aspergillus niger*, 5 isolates placed within the group of *Beauveria bassiana*, 5 isolates placed within the group of *Curvularia lunata*, 1 isolate placed within the group of *Aspergillus flavus*, 1 isolate placed within the group of

Penicillium citrinum, and 1 isolate placed within the group of *Metarhizium anisopliae*. ● = fungal isolates obtained from this research

Table 2 Mean mortality of *Spodoptera frugiperda* larvae treated with endophytic fungi (1 x 10^6 conidia ml^{-1}), and conidial density and viability of endophytic fungi

Fungal isolate code	Fungal species	Mortality ± SE (%)	Conidial viability ± SE (%) for 1 x 24 hrs	Conidial viability ± SE (%) for 2 x 24 hrs
Control	-	0.00 ± 0.00a	-	-
GaTpeOi	*Chaetomium* sp.	4.00 ± 1.89bcd	45.17±1.51bcd	47.00±0.34a
PsgTjPr	*Aspergillus niger*	2.67 ± 2.18abc	38.20±0.79ab	51.63±0.31abcd
JgSPK	*Beauveria bassiana*	22.67 ± 2.88f	47.59±1.33efgh	55.23±2.12cde
JgTjPr	*Chaetomium* sp.	2.67 ± 1.08abcd	35.74±2.36a	55.76±1.90de
JaGiP	*Beauveria bassiana*	21.33 ± 2.18f	42.86±3.39abc	52.91±1.03abcd
PiCrPga	*Chaetomium* sp.	5.33 ± 1.09cd	44.59±1.37cde	47.99±2.87abc
JaGiPRB	*Curvularia lunata*	4.00 ± 1.89bcd	45.18±1.76bcd	52.75±2.51abcd
CMTJP	*Curvularia lunata*	2.67 ± 1.09abcd	45.25±2.65efg	54.94±2.62bcde
JaMsBys	*Curvularia lunata*	6.67 ± 1.09d	49.98±1.64gh	58.60±3.00de
JaSpkPGA(2)	*Beauveria bassiana*	2.67 ± 1.09abcd	47.20±1.77cde	48.15±1.95abc
JgCrJr	*Beauveria bassiana*	21.33 ± 3.93f	46.50±1.50cd	52.63±2.31abcd
JaTpOi (1)	*Beauveria bassiana*	18.67 ± 1.09f	54.02±1.28efg	56.55±2.03de
JaSpkPga(3)	*Curvularia lunata*	17.33 ± 2.18ef	50.85±3.75def	56.93±1.59de
CaCjPga	*Chaetomium* sp.	2.67 ± 1.09abcd	37.24±3.40a	47.77±1.68ab
JgByU	*Aspergillus niger*	1.33 ± 1.09ab	58.84±1.72gh	69.57±3.81g
JgTgSr	*Curvularia lunata*	4.00 ± 0.00bcd	55.33±1.75efgh	61.24±1.12ef
JaBuBys	*Aspergillus niger*	2.67 ± 1.09abcd	58.45±0.20gh	76.50±2.08h
JgPWSR	*Aspergillus flavus*	4.00 ± 0.00bcd	62.54±2.08h	68.39±0.34g
JaTpOi(2)	*Penicillium citrinum*	6.67 ± 2.17cd	57.44±0.27fgh	65.98±1.35fg
CaTpPga	*Metarhizium anisopliae*	8.00 ± 1.88de	50.76±0.81def	68.75±3.29f
F-value		7.27*	9.05*	15.67*
P-value		0.00	0.00	0.00
HSD value		8.8	4.27	4.07

Note: * = significantly different; values within a column followed by the same letters were not significantly different at $P < 0.05$ according to Tukey's HSD test

Pathogenicity of the endophytic fungi against *Spodoptera frugiperda* larvae

Out of the 7 species of the 20 isolates of the endophytic fungi found in this study, there were 6 isolates that were the most pathogenic against *S. frugiperda* larvae (Table 2). The 4 isolates (JgSPK, JaGiP, JgCrJr, and JaTpOi1) resulted to mortality rates of 22.67, 21.3%, 21.33, and 17.33% consecutively was *B. bassiana*. In addition, the isolate JaSpkPga(3) isolate of *C. lunata* caused a fairly high mortality (18.67%) and was non-significantly different than the mortality caused by the isolates of JgSPK, JaGiP, JgCrJr, JaTpOi1. The CaTpPga isolate of *M. anisopliae* caused (8%) larval mortality and was non-significantly different than the mortality caused by JaSpkPga(3) isolate. The *B. bassiana* and *C. lunata* isolates were isolated from maize, while the *M. anisopliae* isolate was isolated from red chili. The JaSpkPGA(2) isolate of *B. bassiana* resulting a mortality rate of only (2.67%). Likewise, *P. citrinum*, *Chaetomium* sp., *A. niger,* and *A. flavus* caused the lowest larvae mortality of less than (8%).

The larvae of *S. frugiperda* that died because of feeding on leaves colonized by the endophytic fungi had the characteristics, *i.e.* dull and dark integument, shriveled body, dry, and odorless. The feces excreted by sick larvae was wetter than those of untreated larvae (control). The larvae that died as a result of treatment with the the endophytic fungi when grown in SDA media, their cadavers were not overgrown with mycelia fungi.

Discussion

The isolates of the endophytic fungi found in this study had an rDNA sequence similarity value of more than 99% to the reference species (BLAST), meaning that the isolates had a high phylogenetic relationship and were in the same species. Bich *et al.* (2021) stated that ribosomal DNA sequences were used to determine the phylogenetic relationships of organisms to taxa species. Based on the similarity value, it is stated that if the similarity value is 100%, it means that the isolates are the same strain, whereas if the similarity value of 99% indicates that the isolates are the same species, and if the similarity value of the isolates is 89-99%, that means they are the same genus (Henry *et al.* 2000). An organism is declared the same species when the difference in DNA sequences is between 0.2–1% (Shenoy *et al.* 2007).

The results of identification based on the morphological characters of several species of fungi showed the similarities, for example the morphology of the colony and the shape of the conidia isolates of *B. bassiana* were similar to that of *P. citrinum*, but after the molecular identification, they showed them different species. According to Minarni *et al.* (2021) proper species identification is very important in determining a species as an entomopathogen.

The fungi in this study proved to be endophytic based on the characteristics of maize stalks and leaves colonized by the fungal mycelia. According to Barra-Bucarei *et al.* (2020), endophytic fungus (*B. bassiana*) had a systemic mode of action ability to colonize their host plant (tomato). The endophytic fungi can still be found on roots, stems, and leaves of tomato up to 30 days after inoculation (Carolina *et al.* 2020). Endophytic *B. bassiana* colonized 100% of leaves, 80% of stems, and 60% of roots of maize 7 days after foliar spray and caused significant reductions in *S. frugiperda* growth and reproduction (Russo *et al.* 2020). The research results of Shikano (2018)

showed the endophytic fungi were able to live in the tissue for several months depending on the age of the host plant.

Out of the 7 species of 20 isolates of the endophytic fungi found in this study, there were only 6 pathogenic isolates against the larvae of *S. frugiperda*. The isolates JgSPK, JaGiP, JgCrJr, JaTpOi1 belong to *B. bassiana*, the JaSpkPga(3) and CaTpPga isolates belong to *C. lunata* and *M. anisopliae*, respectively. First report of *B. bassiana* and *C. lunata* isolated from maize, and *M. anisopliae* isolated from red chili in Indonesia were found as entomopathogenic endophytic fungi against *S. frugiperda*. *C. lunata* infected and killed grain insect pests, such as *Tribolium castaneum* (Herbst.) (Coleoptera: Tenebrionidae), *Trogoderma granarium* (Everts) (Coleoptera: Dermestidae), *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae), and *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Cucujidae) (Wakil *et al.* 2014). The endophytic *M. anisopliae* effectively killed the larvae of *S. frugiperda* (Ramos *et al.* 2020).

The endophytic fungi species of *B. bassiana* (JgSPK, JaGiP, JgCrJr, JaTpOi1 isolates), *C. lunata* (JaSpkPga(3) isolate), and *M. anisopliae* (CaTpPga isolate) had potential as entomopathogens of *S. frugiperda* larvae. *S. frugiperda* larvae that died from consuming plants colonized by the endophytic fungi showed that the fungi could be applied through seed treatment, especially for maize seeds. The seed treatment causes the endophytic fungi to colonize plants (Gustianingtyas et al. 2021) and if *S. frugiperda* larvae eat them it can cause them die (Ramos *et al.* 2020).

The *B. bassiana* of JaSpkPGA(2) isolate and all isolates of *P. citrinum, Chaetomium* sp., *A. niger*, and *A. flavus* showed no potential as entomopathogens of *S. frugiperda* with their resulting mortality rates of less than 8%. In contrast to the research results of Gustianingtyas *et al.* (2021) reported that the endophytic fungi species (*Aspergillus* sp. and *Chaetomium* sp.) applied topically (contact) caused mortality rates of *S. frugiperda* larvae as many as 18.67 and 14.67%, respectively and the mortality rate caused by *Beauveria* sp. reached 29%. This difference in the rates was due to different ways of application of the fungi, in this study the larvae of *S. frugiperda* consumed the plants that had been colonized by the endophytic fungi which were not applied topically.

The larvae of *S. frugiperda* that were sick and died by feeding on the leaves colonized by the endophytic fungus had different characteristics from the larvae that were sick and died due to the topical suspension of the endophytic fungus. The sick larvae of *S. frugiperda* in this study had wetter feces and the cadavers were not overgrown and covered by the fungal mycelia, while the sick larvae caused by contact with suspension of the endophytic fungi according to Gustianingtyas *et al.* (2021) had dry feces and the cadavers were covered with the fungal mycelia. The dead larvae treated by the endophytic fungi were not overgrown by mycelia fungi because the cadavers were too dry. According to El-Ghany (2015), the success of a fungus in germinating was affected by several external factors, such as humidity (more than 98%). So, if the cadavers were too dry, the fungal conidia could not germinate on the cadavers.

Conclusions

Molecular identifications recorded 7 species of the endophytic fungi, namely *Chaetomium* sp. (GaTpeOi, JgTjPr, PiCrPga, and CaCjPga isolates), *Aspergillus niger* (PsgTjPr, JgByU, and JaBuBys), *Beauveria bassiana* (JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, and JaTpOi (1) isolates), *Curvularia lunata* (JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr isolates), *Aspergillus flavus* (JgPWSR isolate), *Penicillium citrinum* (JaTpOi(2) isolate), and *Metarhizium anisopliae* (CaTpPGA isolate). The endophytic fungal species of *B. bassiana* (JgSPK isolate) caused up to (22.67%) mortality, *C. lunata* (JaSpkPga(3) isolate) caused (17.33%) mortality, and *M. anisopliae* (CaTpPga isolate) caused (8%) mortality against *S. frugiperda* larvae. First report of *B. bassiana* and *C. lunata* isolated from maize, and *M. anisopliae* isolated from red chili in Indonesia were found as entomopathogenic endophytic fungi against *S. frugiperda* larvae. The endophytic fungi species of *B. bassiana*, *C. lunata*, and *M. anisopliae* had potentials as entomopathogens of *S. frugiperda*.

List of abbreviations

ANOVA: analysis of variance; BLAST: Basic Local Alignment Search Tool; CTAB: cetyltrimethylammonium bromide; DNA: Deoxyribonucleic acid; EtOH: Ethyl alcohol; HSD: Tukey's Honestly Significant Difference; ITS: Internal Transcribed Spacer; MEA: the malt extract agar; NaOCl: Sodium hypochlorite; SDA: Sabouraud Dextrose Agar; TBE: Tris-Boric Acid-EDTA.

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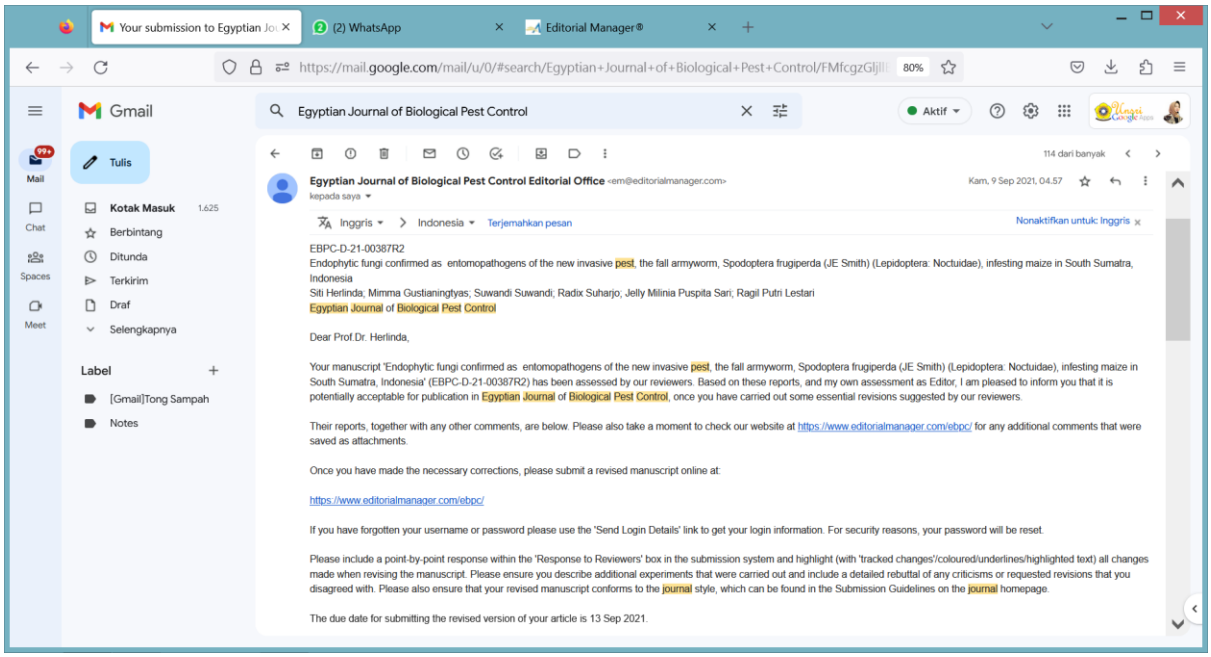
6. Bukti konfirmasi review ketiga 9 September 2021



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Endophytic fungi confirmed as entomopathogens of the new invasive pest, the fall armyworm, Spodoptera frugiperda (JE Smith) (Lepidoptera: Noctuidae), infesting maize in South Sumatra, Indonesia

Siti Herlinda; Mimma Gustianingtyas; Suwandi Suwandi; Radix Suharjo; Jelly Milinia Puspita Sari; Ragil Putri Lestari

Egyptian Journal of Biological Pest Control

Dear Prof.Dr. Herlinda,

Your manuscript 'Endophytic fungi confirmed as entomopathogens of the new invasive pest, the fall armyworm, Spodoptera frugiperda (JE Smith) (Lepidoptera: Noctuidae), infesting maize in South Sumatra, Indonesia' (EBPC-D-21-00387R2) has been assessed by our reviewers. Based on these reports, and my own assessment as Editor, I am pleased to inform you that it is potentially acceptable for publication in Egyptian Journal of Biological Pest Control, once you have carried out some essential revisions suggested by our reviewers.

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Endophytic fungi confirmed as entomopathogens of the new invasive pest, the fall armyworm, Spodoptera frugiperda (JE Smith) (Lepidoptera: Noctuidae), infesting maize in South Sumatra, Indonesia

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Abstract:	Background: The fall armyworm, Spodoptera frugiperda (JE Smith) (Lepidoptera: Noctuidae) is a new invasive pest in Indonesia causing financial losses. S. frugiperda larvae hide in the leaf midrib all day. To overcome the hidden larvae, pathogenic endophytic fungi are needed. The objective of this research was to molecularly identify the endophytic fungal species from South Sumatra and determine the most pathogenic species against S. frugiperda larvae. Endophytic fungal identification was based on morphological and molecular characteristics. The molecular identification was based on gene sequential analysis of Intergenic Transcribed Spacer (ITS) 1 and ITS 4. Bioassay of the endophytic fungal species was treated against the 2nd larval instar of S. frugiperda. Result: The results of molecular identification revealed endophytic fungal species consisted of Chaetomium sp. (GaTpeOi, JgTjPr, PiCrPga, and CaCjPga isolates), Aspergillus niger (L.) Van Tieghem (PsgTjPr, JgByU, and JaBuBys), Beauveria bassiana (Balsamo) Vuillemin (JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, and JaTpOi (1) isolates), Curvularia lunata (Wakker) Boed. (JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr isolates), Aspergillus flavus Link. (JgPWSR isolate), Penicillium citrinum Thom F 1539 (JaTpOi(2) isolate), and Metarhizium anisopliae (Metschn.) Sorokin (CaTpPGA isolate). All fungal isolates were deposited in the GenBank. The endophytic fungal species of B. bassiana (JgSPK isolate) caused up to (22.67%) mortality, C. lunata (JaSpkPga(3) isolate) caused (17.33%) mortality, and M. anisopliae (CaTpPga isolate) caused (8%) mortality of the pest's larvae. First report of B. bassiana and C. lunata isolated from maize (Zea mays L.), while M. anisopliae was isolated from red chili (Capsicum annuum L.) as entomopathogenic endophytic fungi against S. frugiperda larvae in Indonesia. Conclusion: The endophytic fungi species of B. bassiana, C. lunata, and M. anisopliae had potentials as entomopathogens of S. frugiperda.
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Endophytic fungi confirmed as entomopathogens of the new invasive pest, the fall armyworm, *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae), infesting maize in South Sumatra, Indonesia

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Declarations

Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests
The authors declare that they have no competing interests

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Authors' contributions
SH performed research concept and design, writing the article, and final approval of article. MG performed collection and assembly of data. SS prepared and performed morphological identification and critical revision of the article. RS performed and performed molecular identification and data analysis and interpretation. JMPS performed collection and assembly of data. RPL performed collection and assembly of data. All the authors read and approved the manuscript.

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Endophytic fungi confirmed as entomopathogens of the new invasive pest, the fall armyworm, *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae), infesting maize in South Sumatra, Indonesia

Abstract

Background: The fall armyworm, *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae) is a new invasive pest in Indonesia causing financial losses. *S. frugiperda* larvae hide in the leaf midrib all day. To overcome the hidden larvae, pathogenic endophytic fungi are needed. The objective of this research was to molecularly identify the endophytic fungal species from South Sumatra and determine the most pathogenic species against *S. frugiperda* larvae. Endophytic fungal identification was based on morphological and molecular characteristics. The molecular identification was based on gene sequential analysis of Intergenic Transcribed Spacer (ITS) 1 and ITS 4. Bioassay of the endophytic fungal species was treated against the 2nd larval instar of *S. frugiperda.*

Result: The results of molecular identification revealed endophytic fungal species consisted of *Chaetomium* sp. (GaTpeOi, JgTjPr, PiCrPga, and CaCjPga isolates), *Aspergillus niger* (L.) Van Tieghem (PsgTjPr, JgByU, and JaBuBys), *Beauveria bassiana* (Balsamo) Vuillemin (JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, and JaTpOi (1) isolates), *Curvularia lunata* (Wakker) Boed. (JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr isolates), *Aspergillus flavus* Link. (JgPWSR isolate), *Penicillium citrinum* Thom F 1539 (JaTpOi(2) isolate), and *Metarhizium anisopliae* (Metschn.) Sorokin (CaTpPGA isolate). All fungal isolates were deposited in the GenBank. The endophytic fungal species of *B. bassiana* (JgSPK isolate) caused up to (22.67%) mortality, *C. lunata* (JaSpkPga(3) isolate) caused (17.33%) mortality, and *M. anisopliae* (CaTpPga isolate) caused (8%) mortality of the pest's larvae. First report of *B. bassiana* and *C. lunata* isolated from maize (*Zea mays* L.), while *M. anisopliae* was isolated from red chili (*Capsicum annuum* L.) as entomopathogenic endophytic fungi against *S. frugiperda* larvae in Indonesia.

Conclusion: The endophytic fungi species of *B. bassiana*, *C. lunata*, and *M. anisopliae* had potentials as entomopathogens of *S. frugiperda*.

Keywords: Endophytic fungi, Invasive pest, *Spodoptera frugiperda*, Morphological and molecular identifications, Indonesia.

Background

The fall armyworm, *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae) is a new invasive pest, originating from South America (Otim *et al.* 2018). Currently, it has spread throughout the world and its first record in Indonesia was in March 2019 in West Sumatra (Sartiami *et al.* 2020) and in July 2019 in South Sumatra (Hutasoit *et al.* 2020). Now it has been spread all over Indonesia. The pest attacks maize (*Zea mays* L.) and various other plant species by means of its larval stage, eating leaves, stems, flowers, fruits, growing points, and the whole maize

plant until it becomes bare (Montezano *et al.* 2018). Larvae are found on the surface of leaves or maize stalks in the morning and then hide in the leaves' midribs (Gustianingtyas *et al.* 2021). Such behavior makes it difficult to be controlled.

Laboratory experiments of Herlinda *et al. (*2020a) showed that the entomopathogenic fungus (EPF), *Metarhizium* sp. could kill (78%) of *S. frugiperda* larvae. *Beauveria bassiana* (Balsamo) Vuillemin, *Metarhizium anisopliae* (Metschn.) Sorokin, and *M. rileyi* (Farlow) Samson also killed more than (80%) *S. frugiperda* larvae (Ramanujam *et al.* 2020). Their treatments were applied topically (direct contact) with the larvae exposed. In the field, the presence of hiding larvae in the midribs all the day makes the contacted EPF less effective (Gustianingtyas *et al*. 2021). To overcome such behavior, EPF that found in plant tissues (endophytic fungi) are needed (Ramos *et al.* 2020). The endophytic fungi systemically colonize plant tissues and associate mutually their host plants (Lira *et al.* 2020). The results of previous studies have proven that 8 isolates of endophytic EPF could topically kill *S. frugiperda* larvae (Gustianingtyas *et al.* 2021), however, the endophytic fungi found in their studies were only isolated from the roots and their molecular identification had not yet been carried out.

In this study, the fungi isolated from leaves, shoots, and roots of maize plants from South Sumatra (Indonesia) were identified morphologically and molecularly and their pathogenicity to *S. frugiperda* larvae was evaluated.

Methods

Purification, identification, and bioassay of fungi were conducted from January to March 2021. The endophytic fungal species were identified based on the molecular analysis carried out at a laboratory accredited according to the ISO 17025 standard. Bioassay of the fungi was carried out in an incubator at controlled and constant temperature and relative humidity, respectively 25ºC and 97%.

Exploration, isolation, and purification of fungi

Exploration of endophytic fungi was carried out by collecting leaves, shoots, and roots of maize and other crop plants around it such as: bananas (*Musa* spp.), ridged gourd (*Luffa acutangula* (Roxb.) L.) and red chilies (*Capsicum annuum* L.). Selection of individual crop plant samples to obtain the endophytic fungi, followed the method of Kasambala *et al.* (2018). Samples of the plant parts were placed into an ice box, then taken to the laboratory. The exploration of endophytic fungi was carried out from the lowlands to the highlands of South Sumatra, Indonesia namely in Banyuasin District (2.8833°S 104.3831°E), Ogan Ilir District (3.43186°S 104.62727°E), Prabumulih City (3.4328°S 104.2356°E), Pagar Alam City (3°52′43.8″S 103°21′30″E) (Table 1).

Table 1 Origin of isolates of endophytic fungi from South Sumatra, Indonesia

Location (village, district/city)	Isolate origin	Altitude (m)	Fungal species	Fungal isolate code	GenBank Acc. No.
Tanjung Pering. Ogan Ilir	Ridged gourd	36.0	*Chaetomium* sp.	GaTpeOi	MZ359734
Tanjung Pering. Ogan Ilir	Bananas	36.0	*Aspergillus niger*	PsgTjPr	MZ242060
Simpang Padang Karet. Pagar Alam	Maize	797.7	*Beauveria bassiana*	JgSPK	MZ356494
Tanjung Pering. Ogan Ilir	Maize	36.0	*Chaetomium* sp.	JgTjPr	MZ359736
Gunung Ibul. Prabumulih	Maize	53.0	*Beauveria bassiana*	JaGiP	MZ356495
Curup Jare. Pagar Alam	Bananas	806.0	*Chaetomium* sp.	PiCrPga	MZ359735
Gunung Ibul. Prabumulih	Maize	53.0	*Curvularia lunata*	JaGiPRB	MZ359815
Tanjung Payang. Pagar Alam	Red chilies	689.6	*Curvularia lunata*	CMTJP	MZ359816
Mulia Sari. Banyuasin	Maize	19.0	*Curvularia lunata*	JaMsBys	MZ359819
Simpang Padang Karet. Pagar Alam	Maize	789.5	*Beauveria bassiana*	JaSpkPGA(2)	MZ356496
Curup Jare. Pagar Alam	Maize	806.7	*Beauveria bassiana*	JgCrJr	MZ356497
Tanjung Pering. Ogan Ilir	Maize	36.0	*Beauveria bassiana*	JaTpOi (1)	MZ356498
Simpang Padang Karet. Pagar Alam	Maize	797.7	*Curvularia lunata*	JaSpkPga(3)	MZ359818
Curup Jare. Pagar Alam	Red chilies	806.0	*Chaetomium* sp.	CaCjPga	MZ359737
Banyuurip. Banyuasin	Maize	13.0	*Aspergillus niger*	JgByU	MZ242059
Telang Sari. Banyuasin	Maize	15.0	*Curvularia lunata*	JgTgSr	MZ359817
Banyuurip. Banyuasin	Maize	20.0	*Aspergillus niger*	JaBuBys	MZ242058
Purwosari. Banyuasin	Maize	15.0	*Aspergillus flavus*	JgPWSR	MZ359829
Tanjung Pering. Ogan Ilir	Maize	36.0	*Penicillium citrinum*	JaTpOi(2)	MZ359812
Tanjung Payang. Pagar Alam	Red chilies	689.6	*Metarhizium anisopliae*	CaTpPga	MZ242073

The leaves, shoots, and roots of the sample plants were cleaned and washed aseptically with running tap water. Then, the plant parts were surface sterilized using the method of Elfita *et al.* (2019) with 70% EtOH (Ethyl alcohol) and 1% NaOCl (Sodium hypochlorite), then rinsed 3 times.

The last rinse water was used to check whether the microorganisms causing surface contamination were still present and carried out by growing them onto Sabouraud Dextrose Agar (SDA) (Russo *et al.* 2020). Obtaining the endophytic fungi isolates started with growing the plant parts that sterilized earlier onto a media. The roots were grown on a specific medium, the malt extract agar (MEA), following the method of Silva *et al.* (2018). The shoots and leaves of the sample plants were grown on SDA media. The plant parts were grown as many as 5 pieces per Petri dish with a length of 5 mm and a diameter of 1-5 mm (depending on the diameter of the leaves, shoots, and roots). Fungi growing from the leaves, shoots, and roots were purified to be one isolate per sample. The isolates were then observed for their microscopic characteristics and continued with the fungal colonization test into the maize seed tissue.

Assess endophytic fungal colonization

Fungal inoculation on maize seeds was carried out to observe the colonization of fungi into the maize seedling tissue and to further ensure that the isolates found were truly endophytic. The 15 seeds of maize were surface sterilized, using 70% EtOH (Ethyl alcohol) and 1% NaOCl (Sodium hypochlorite) (Elfita *et al.* 2019). The seeds were immersed in 10 ml of fungal suspension, with a concentration of 1 x 10^6 conidia ml^{-1} for 6 hrs, while for the control, the sterilized seeds were only immersed in 10 ml of destilled water. Then, the seeds were grown in a sterile glass bottle (250 ml volume) with a sterile filter paper (whatman no. 42) at the bottom, which was moistened with 1 ml destilled water and incubated for 10 days. All the treatments in this experiment were repeated 3 times. After the maize seedlings were 10 days old, the stems and leaves were sliced crosswise and longitudinally with a thickness of 0.02 mm each. The slices were observed with a light microscope at 40 x magnification with 0.05% lactofenol trypan blue dye. Mycelia endophytic fungi found in the maize seedling tissue were documented. The fungal isolates that were proven to be endophytic were used for bioassays. Prior to the bioassay, the isolates were observed for microscopic characteristics for morphological identification, followed by molecular identification.

Morphological and molecular identification of fungal species

Macroscopic and microscopic observation

Morphological observations were carried out based on the macroscopic characteristics of the endophytic fungi that included colony color and shape, shape and size of conidia and conidiophores, following the method of Herlinda *et al*. (2020a).

DNA extraction and PCR amplification

The used endophytic fungal DNA extraction method refers to the Swibawa *et al.* (2020) method. DNA extraction was carried out on fungal conidia. The conidia were harvested from pure cultures of 7 days old fungus, which was cultured in sterile Petri dishes with a diameter of 9 cm. As much as 10 ml of sterile water was placed into a Petri dish containing fungal cultures and slowly harvested, using a drigalski. The obtained conidia suspension was transferred to a

centrifuge tube (volume 30 ml) and centrifuged using CF15RXII (Hitachi, Japan) for 10 min at a speed of 14,000 rpm. After being centrifuged, 1 ml of 70% ethanol was added to the centrifuge tube and then centrifuged again for 10 min. After that, the supernatant was removed and 1 ml of extraction buffer was added with a composition of 0.5 ml Tris HCl, 1 mL SDS 1% + 2.8 mL NaCl, 0.2 ml Mercaptho Ethanol, 2 ml EDTA, 3.5 ml sterile water and then homogenized. The suspension was transferred to a sterile mortal and incubated at -40 °C for 24 hrs. After that, the frozen suspension was ground until it was smooth and melted. A total of 500 µl of suspension was then transferred into a 1.5 ml tube. A total of 400 µl of 2% cetyltrimethylammonium bromide (CTAB) was then added to the tube, homogenized and then incubated at 65 °C for 1 hr using a water bath (Brookfield TC 550 MX-230, USA). After the incubation, 500 µl of Phenol Chloroform Isoamyl alcohol (PCI) (25:24:1) was added, homogenized and centrifuged (Microspin12; Biosan, Latvia) at 14,000 rpm for 10 min. A total of 600 µl of supernatant was taken and transferred to a new 1.5 ml tube. A total of 600 µl Chloroform Isoamyl Alcohol (CI) (24:1) was added to the tube, homogenized and centrifuged (Microspin12; Biosan, Latvia) at 14,000 rpm for 10 min. A total of 400 µl of supernatant was then transferred to a new 1.5 ml tube and 400 µl of cold isopropanol was added, homogenized, and incubated at -40 ºC for 20 min. After the incubation, it was centrifuged (Microspin12; Biosan, Latvia) at 14,000 rpm for 15 min. The supernatant was then discarded. After the supernatant was removed, 500 µl of 70% cold ethanol was added and centrifuged (Microspin12; Biosan, Latvia) at 14,000 rpm for 5 min. The supernatant was then discarded and the pellets obtained were dried at room temperature for 24 hrs. After drying, the tube was then added as much as 50 µl 1x Tris-HCL EDTA (TE) pH 8.0 (1st Base Malaysia).

PCR amplification was carried out using the Sensoquest Thermal Cycler (Germany) PCR machine on the Internal Transcribed Spacer (ITS) using ITS1 and ITS4 primers (White *et al.* 1990). The PCR was carried out with a total volume of 25 µl consisting of a mixture of Master Mix (Red Mix) (bioline) as much as 12.5 µl, 10 µM of primer ITS 1 (5'TCC GTA GGT GAA CCT TGC GG 3 ') and ITS 4 (5'TCC TCC GCT TAT TGA TAT GC 3') 1 µL each, 1 L of template DNA and 9.5 µl of sterile water. The DNA amplification stage consisted of 1 initiation cycle at 95 ºC for 5 min, followed by 30 cycles consisting of denaturation at 95 ºC for 1 min, primer attachment at 52 ºC for 1 min, primer extension at 72 ºC for 1 min, and 1 elongation cycle at 72 ºC for 5 min. The PCR results were then electrophoresed, using 0.5% agarose in 20 ml of 1x Tris-Boric Acid-EDTA (TBE) buffer (1st Base Malaysia) and added 1 µl of Ethidium Bromide (EtBr 10 mg/ml). The electrophoresis was carried out in 1x TBE buffer solution at 50 volts for 70 min. Electrophoresis results were visualized using a DigiDoc UV transilluminator (UVP, USA).

Sequencing and analysis of the results

The PCR results were then sent to 1st Base Malaysia for the sequencing process. The results of the sequencing were analyzed, using Bio Edit ver. 7.2.6 for windows. Then, the results were submitted to the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to find out the genus or species that had the greatest

homology or similarity and molecularly. The phylogeny tree was created using the Mega 7 for Windows program (Kumar *et al*. 2016), using the UPGMA (jukes and cantor model) method. The ITS region sequences for several strains used as a reference in this study were obtained from NCBI (https://www.ncbi.nlm.nih.gov/).

Calculation of conidial density and viability

All the isolates of endophytic fungi were counted for their conidial density and viability. The conidial density of the 7 old days fungi was calculated according to the method of Sumikarsih *et al.* (2019), using a haemocytometer and observed with a light microscope at 40 x magnification. The viability was observed by growing 1 ml of conidia fungal suspension (1 x 10^6 conidia ml^{-1}) onto 2% agar-water medium, then the culture was incubated for 1 x 24 and 2 x 24 hrs under a light microscope at 40 x magnification.

Mass-rearing of *S. frugiperda*

The mass-rearing of *S. frugiperda* was carried out, following the method of Herlinda *et al.* (2020a). The larvae were collected from the maize plants grown around the campus (3°13′25″S 104°39′51″E), were reared in the laboratory for more than 5 generations to obtain the larvae with relatively homogeneous genetics and to eliminate the effects of exposure to synthetic insecticides from the fields. In the laboratory, the larvae of *S. frugiperda* were kept individually in a porous plastic cup (Ø 6.5 cm, height 4.6 cm) because the larvae were cannibals. The larvae were fed daily by maize leaves (measuring 2 cm x 5 cm). Prepupae were transferred to a plastic container (Ø 15 cm, height 25 cm) on which its bottom had a sterile soil (5 cm thick). The plastic container containing the prepupae was placed in a wire mesh cage (30 x 30 x 30 cm^3) and inside this cage placed also fresh maize leaves for the adults to lay eggs, which were changed daily. The one day old 2nd instar larvae were used for the bioassay.

Bioassay of endophytic fungi against larvae of *S. frugiperda*

The bioassay of endophytic fungi, followed the method of Zea *et al.* (2019). The young maize (seedling) already inoculated with the endophytic fungi and not-inoculated (control) aged 10 days old were given to the 2nd instar larvae of *S. frugiperda*. Each larva was given one maize seedling whose cotyledons were already removed. The larvae were allowed to feed on the leaves and stems of the young maize for 6 hrs. in a sterile room (a laminar air flow cabinet) and each replication was tested for 25 individuals of *S. frugiperda* larvae. Then, the larvae were transferred to a porous plastic cup (Ø 6.5 cm, height 4.6 cm) and fed on healthy non-inoculated leaves measuring 2 cm x 5 cm per day per larvae. The experiment was designed, using completely randomized designs with treatments of 20 isolates, 3 replications per treatment. The dead larvae were recorded daily for 12 days, following the method of Herlinda *et al*. (2020b). The dead larvae were grown in the agar-water medium to confirm whether the infection was caused by the endophytic fungi or not. The number of dead larvae was used to calculate the mortality.

Data analysis

The differences in the mortality data of *S. frugiperda* larvae were analyzed using analysis of variance (ANOVA). Tukey's Honestly Significant Difference (HSD) test (Tukey's test) was employed to test for the significant differences among the treatments (isolates) at P = 0.05. All data were calculated using software of SAS University Edition 2.7 9.4 M5.

Results

Colonization of endophytic fungi on maize tissues

All fungal isolates, isolated from the leaves, shoots, and roots of the maize, bananas, ridged gourd, and red chilies were found to be 124 isolates. However, after assessing for the fungal colonization in young maize (seedlings) tissue, only 20 isolates of the fungi were confirmed as endophytic fungi (Fig. 1). Evidence of fungal colonization that entered the maize seedlings tissue was that mycelia fungi were seen entering and growing spread throughout the stem and leaf tissue. Mycelia endophytic fungi were able to invade the stem and leaf of the maize tissue, the mycelia appeared to be branched. The size of mycelia varied daily from 13.51 to 34.25 µm. In the maize tissue, which their seeds were not inoculated by the endophytic fungi (control), the cells in the maize tissue were clean and clear without fungal colonization.

Identification results of the endophytic fungal isolates

The colony morphology of 20 isolates of the endophytic fungi showed different colors (Fig. 2) and likewise the shapes of hyphae and conidia of each isolate showed their own characteristics (Fig. 3). The colony isolates of GaTpeOi, JgTjPr, PiCrPga, and CaCjPga grown on SDA media showed similarities, namely the colony was pale gray, hyphae and mycelia were septate, the conidia were in the shape of letter D (asymmetric/elliptical) and non-septation. Based on the phylogenetic tree, these 4 isolates were placed within the group of *Chaetomium* sp. (Fig. 4). The isolates were deposited in the GenBank with the accession number MZ359734 (GaTpeOi), MZ359736 (JgTjPr), MZ359735 (PiCrPga), MZ359737 (CaCjPga) (Table 1).

The colony isolates of PsgTjPr, JgByU, and JaBuBys showed similarities, their colonies were black, the hyphae and mycelia were also black, the conidia were non-septate, globose in shape. The 3 isolates were placed within the group of *Aspergillus niger* (L.) Van Tieghem (Fig. 4). The isolates were deposited in the GenBank with the accession number MZ242060 (PsgTjPr), MZ242059 (JgByu), and MZ242058 (JaBUBys) (Table 1).

The isolates of JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, and JaTpOi (1) had a white colony, white hyphae and mycelia, and the globose conidia, which were non-septate conidia. The isolates were placed within the group of *B. bassiana* (Fig. 4). The isolates were deposited in the GenBank with the accession number MZ356494 (JgSPK), MZ356495 (JaGiP), MZ356496 (JaSpkPGA(2)), MZ356497 (JgCrJr), and MZ356498 (JaTpOi1) (Table 1).

The isolates of JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr had a black colony, black hyphae and mycelia, two-septate boomerang-shaped conidia. All the isolates were placed within the group of *Curvularia lunata* (Wakker) Boed. (Fig. 4). The isolates were deposited in the GenBank with the accession number MZ359815 (JaGiPRB), MZ359816 (CMTJP), MZ359819 (JaMsBys), MZ359818 (JaSpkPga(3)), and MZ359817 (JgTgSr) (Table 1).

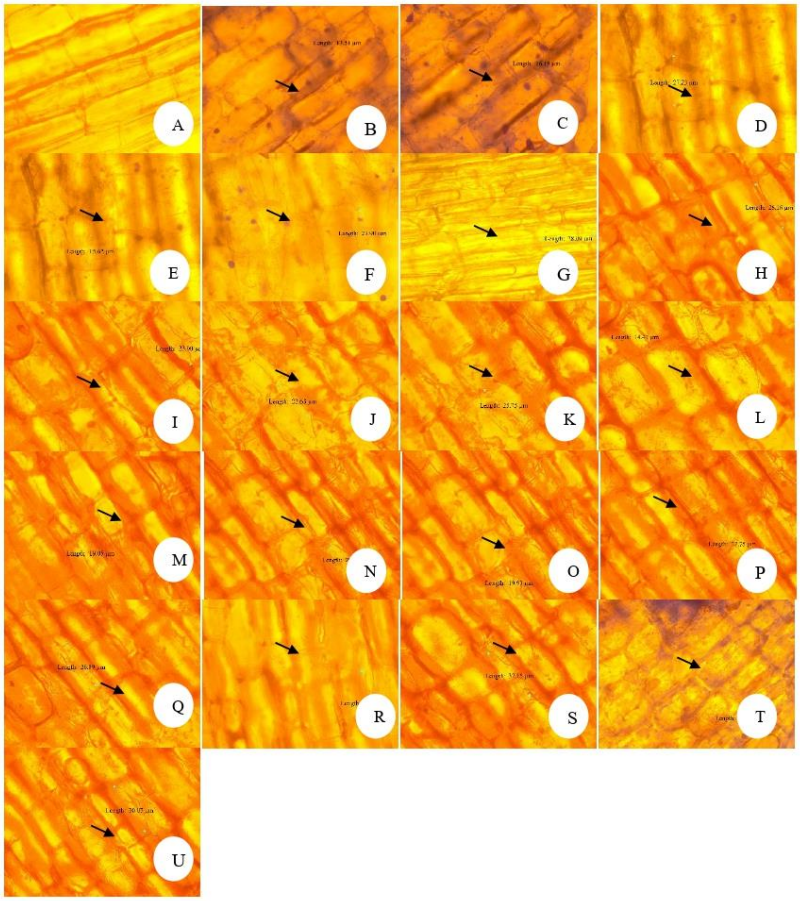

Fig. 1 Young maize tissues colonized by endophytic fungal species: Control (A); *Chaetomium* sp. isolates of GaTpeOi (B), JgTjPr (E), PiCrPga (G), and CaCjPga (O); *Aspergillus niger* isolates of PsgTjPr (C), JgByU (P), and JaBuBys (R); *Beauveria bassiana* isolates of JgSPK (D), JaGiP (F

JaSpkPGA(2) (K), JgCrJr (L), and JaTpOi(1) (M); *Curvularia lunata* isolates of JaGiPRB (H), CMTjP (I), JaMsBys (J), JaSpkPga(3) (N), and JgTgSr (Q); *Aspergillus flavus* isolate of JgPWSR (S); *Penicillium citrinum* isolate of JaTpOi(2) (T); *Metarhizium anisopliae* isolate of CaTpPga (U)

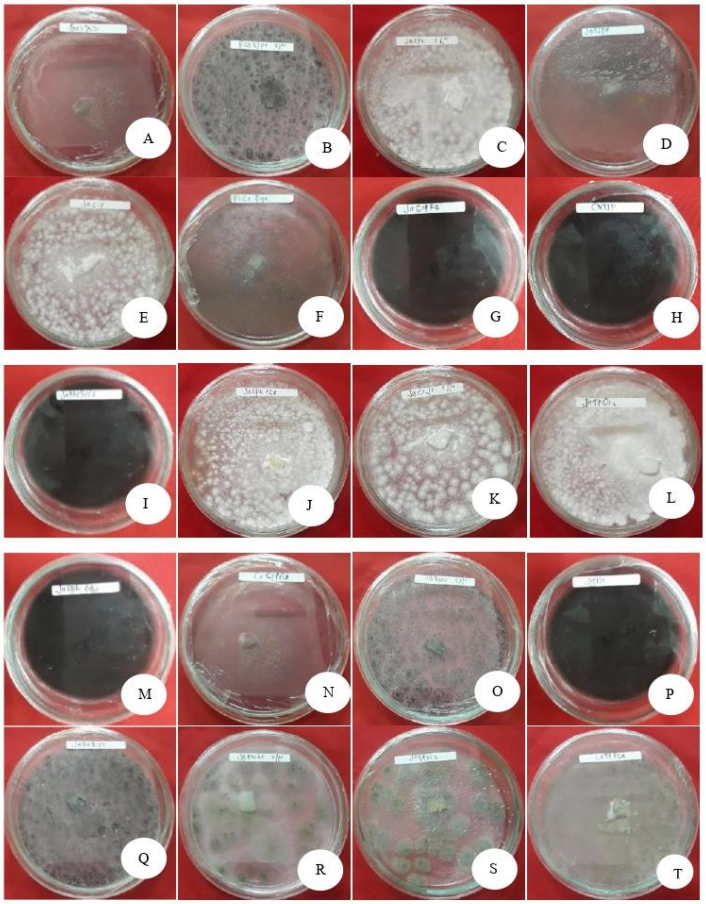

Fig. 2 Colony morphology of endophytic fungal species: *Chaetomium* sp. isolates of GaTpeOi (A), JgTjPr (D), PiCrPga (F), and CaCjPga (M); *Aspergillus niger* isolates of PsgTjPr (B), JgByU (O), and JaBuBys (Q); *Beauveria bassiana* isolates of JgSPK (C), JaGiP (D), JaSpkPGA(2) (J), JgCrJr (K), and JaTpOi(1) (L); *Curvularia lunata* isolates of JaGiPRB (G), CMTjP (H), JaMsBys

(I), JaSpkPga(3) (M), and JgTgSr (P); *Aspergillus flavus* isolate of JgPWSR (R); *Penicillium citrinum* isolate of JaTpOi(2) (S); *Metarhizium anisopliae* isolate of CaTpPga (T)

The isolate of JgPWSR had a green colony, green hyphae and mycelia, non-septate globose conidia. The JgPwSr isolate was placed within the group of *Aspergillus flavus* Link. (Fig. 4). The isolate was deposited in the GenBank with the accession number MZ359829 (JgPWSR) (Table 1).

The color of the colony JaTpOi(2) isolate was initially white, as the fungus got older, it turned bluish green with white margin, rounded conidia. The isolate was placed within the group of *Penicillium citrinum* Thom F 1539 (Fig. 4). The isolate was deposited in the GenBank with the accession number MZ359812 (JaTpOi (2)) (Table 1).

The isolate of CaTpPGA had a white colony and as the fungus got older it turned greenish white to dark green, green hyphae and mycelia, the conidia are clear, cylindrical and non-septation. The isolate was placed within the group of *M. anisopliae* (Fig. 4). The isolate was deposited in the GenBank with the accession number MZ242073 (CaTpPGA) (Table 1).

The result of BLAST search revealed that the isolates of GaTpeOi, JgTjPr, PiCrPga, and CaCjPga had a similarity (100%) with the *Chaetomium* sp. isolate A13 (Acc. No. MH34803.1), isolate MJ51 (Acc No. KM203618.1), and isolate CP-2009 (Acc. No. AB50680.1). The isolates of PsgTjPr, JgByU, and JaBuBys showed 100% of similarity with *A. niger* NBRC: 105649 (Acc. No LC573609.1), strain WM04.470 (Acc No. AJ853742.1) and isolate IFM61597 (Acc. No. LC602036.1). The isolates of JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, and JaTpOi (1) showed (100%) similarity to the *B. bassiana* isolate GZMS-28 (Acc. No. KT715480.1), strain TF6-1B (Acc No. JX122736.1), and isolate BSwTd4 (Acc. No. MT4487322.1). The isolates of JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr showed 99.81% of similarity to *C. lunata* strain D25A (Acc. No MH010917.1), strain AN 2 (Acc No. KY859790.1), and isolate 1z -7 (Ac. No. MN213745.1). The isolate of JgPWSR showed 100% of similarity to *A. flavus* strain GFRS9 (Acc. No MT447477.1), strain GFRS06 (Acc. No MT447474.1), and strain aT3 (Acc. No KU561920.1). The isolate of JaTpOi(2) showed 100% of similarity to *P. citrinum* strain yx-001 (Acc. No. MN826202.1), strain AKF2-KU (Acc No. MN879404.1), and strain J (Acc. No. MK791668.1). The isolate of CaTpPGA had a similarity (99.29%) to *M. anisopliae* isolate MSwTp3 (Acc. No MT448733.1), strain STBMa-001 (Acc No. KF766520.1), and isolate C1 (Acc. No. KX809520.1).

There were 7 species from the 20 isolates of the endophytic fungi found in this study. The 4 isolates (GaTpeOi, JgTjPr, PiCrPga, and CaCjPga) were in the group of *Chaetomium* sp., the three isolates (PsgTjPr, JgByU, and JaBuBys) were in the group of *Aspergillus niger*, the 5 isolates (JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, and JaTpOi (1)) were placed within the group of *B. bassiana*, the 5 isolates (JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr) were in the group of *C. lunata*, an isolate (JgPWSR) was in the group of *A. flavus*, an isolate (JaTpOi(2)) was in the group of *P. citrinum*, and an isolate (CaTpPGA) was in the group of *M. anisopliae*.

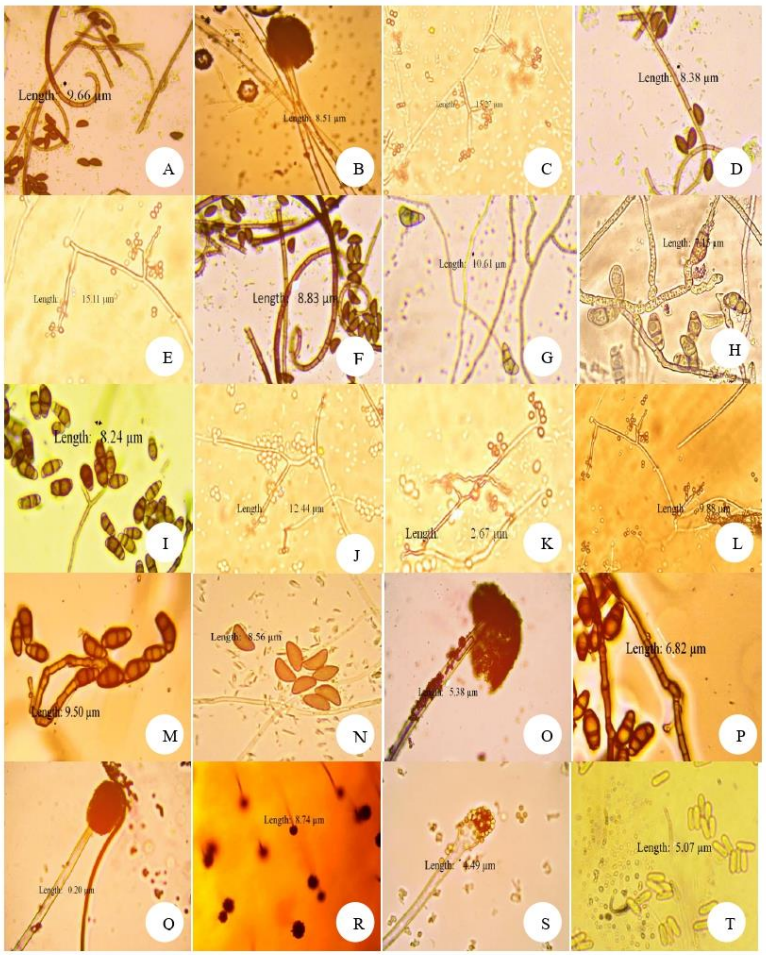

Fig. 3 Conidial and hyphal morphology of endophytic fungal species: *Chaetomium* sp. isolates of GaTpeOi (A), JgTjPr (D), PiCrPga (F), and CaCjPga (M); *Aspergillus niger* isolates of PsgTjPr (B), JgByU (O), and JaBuBys (Q); *Beauveria bassiana* isolates of JgSPK (C), JaGiP (D), JaSpkPGA(2) (J), JgCrJr (K), and JaTpOi(1) (L); *Curvularia lunata* isolates of JaGiPRB (G), CMTjP (H), JaMsBys (I), JaSpkPga(3) (M), and JgTgSr (P); *Aspergillus flavus* isolate of JgPWSR (R); *Penicillium citrinum* isolate of JaTpOi(2) (S); *Metarhizium anisopliae* isolate of CaTpPga (T)

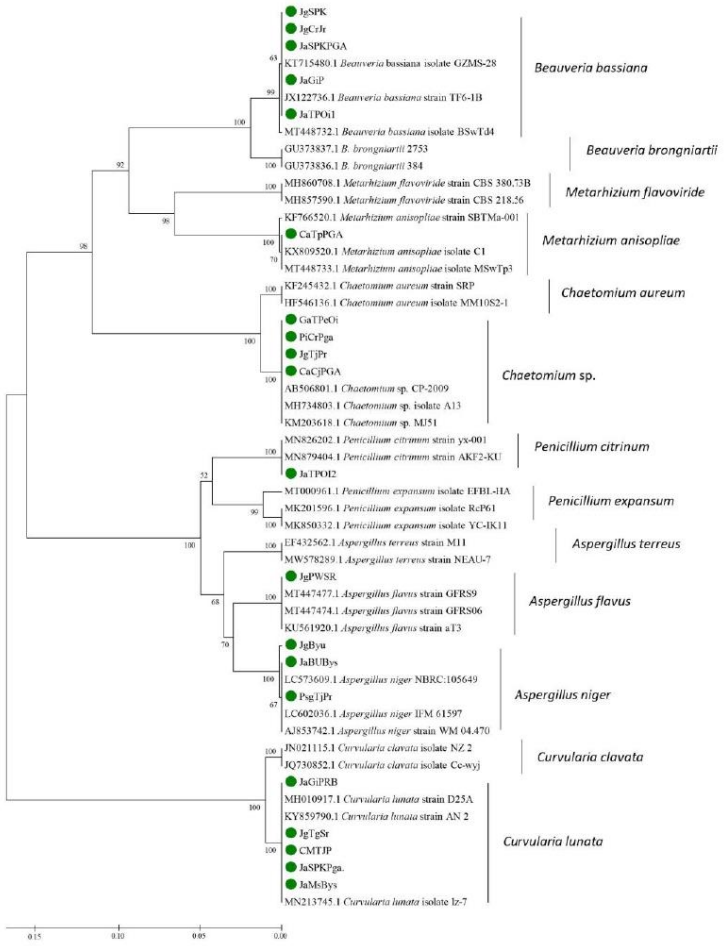

Fig. 4 Phylogenetic tree based on the sequencing of Internal Transcribed Spacer (ITS) region using UPGMA method (jukes and cantor model) performed with Mega 7 program for windows (Kumar *et al.,* 2016). This research found 20 isolates of entomopathogenic fungi: 4 isolates placed within the group of *Chaetomium* sp., 3 isolates placed within the group of *Aspergillus niger*, 5 isolates placed within the group of *Beauveria bassiana*, 5 isolates placed within the group of *Curvularia lunata*, 1 isolate placed within the group of *Aspergillus flavus*, 1 isolate placed within the group of

Penicillium citrinum, and 1 isolate placed within the group of *Metarhizium anisopliae.* ● = fungal isolates obtained from this research

Table 2 Mean mortality of *Spodoptera frugiperda* larvae treated with endophytic fungi (1 x 10^6 conidia ml^{-1}), and conidial density and viability of endophytic fungi

Fungal isolate code	Fungal species	Mortality (%)	Conidial viability (%) for 1 x 24 hrs	Conidial viability (%) for 2 x 24 hrs
Control	-	0.00a	-	-
GaTpeOi	*Chaetomium* sp.	4.00bcd	45.17bcd	47.00a
PsgTjPr	*Aspergillus niger*	2.67abc	38.20ab	51.63abcd
JgSPK	*Beauveria bassiana*	22.67f	47.59efgh	55.23cde
JgTjPr	*Chaetomium* sp.	2.67abcd	35.74a	55.76de
JaGiP	*Beauveria bassiana*	21.33f	42.86abc	52.91abcd
PiCrPga	*Chaetomium* sp.	5.33cd	44.59cde	47.99abc
JaGiPRB	*Curvularia lunata*	4.00bcd	45.18bcd	52.75abcd
CMTJP	*Curvularia lunata*	2.67abcd	45.25efg	54.94bcde
JaMsBys	*Curvularia lunata*	6.67d	49.98gh	58.60de
JaSpkPGA(2)	*Beauveria bassiana*	2.67abcd	47.20cde	48.15abc
JgCrJr	*Beauveria bassiana*	21.33f	46.50cd	52.63abcd
JaTpOi (1)	*Beauveria bassiana*	18.67f	54.02efg	56.55de
JaSpkPga(3)	*Curvularia lunata*	17.33ef	50.85def	56.93de
CaCjPga	*Chaetomium* sp.	2.67abcd	37.24a	47.77ab
JgByU	*Aspergillus niger*	1.33ab	58.84gh	69.57g
JgTgSr	*Curvularia lunata*	4.00bcd	55.33efgh	61.24ef
JaBuBys	*Aspergillus niger*	2.67abcd	58.45gh	76.50h
JgPWSR	*Aspergillus flavus*	4.00bcd	62.54h	68.39g
JaTpOi(2)	*Penicillium citrinum*	6.67cd	57.44fgh	65.98fg
CaTpPga	*Metarhizium anisopliae*	8.00de	50.76def	68.75f
F-value		7.27*	9.05*	15.67*
P-value		0.00	0.00	0.00
HSD value		8.8	4.27	4.07

Note: * = significantly different; values within a column followed by the same letters were not significantly different at $P < 0.05$ according to Tukey's HSD test

Pathogenicity of the endophytic fungi against *S. frugiperda* larvae

Out of the 7 species of the 20 isolates of the endophytic fungi found in this study, there were 6 isolates that were the most pathogenic against *S. frugiperda* larvae (Table 2). The 4 isolates (JgSPK, JaGiP, JgCrJr, and JaTpOi1) resulted to mortality rates of 22.67, 21.3%, 21.33, and

17.33% consecutively was *B. bassiana*. In addition, the isolate JaSpkPga(3) isolate of *C. lunata* caused a fairly high mortality (18.67%) and was non-significantly different than the mortality caused by the isolates of JgSPK, JaGiP, JgCrJr, JaTpOi1. The CaTpPga isolate of *M. anisopliae* caused (8%) larval mortality and was non-significantly different than the mortality caused by JaSpkPga(3) isolate. The *B. bassiana* and *C. lunata* isolates were isolated from maize, while the *M. anisopliae* isolate was isolated from red chili. The JaSpkPGA(2) isolate of *B. bassiana* resulting a mortality rate of only (2.67%). Likewise, *P. citrinum*, *Chaetomium* sp., *A. niger,* and *A. flavus* caused the lowest larval mortality of less than (8%).

The larvae of *S. frugiperda* that died because of feeding on leaves colonized by the endophytic fungi had the characteristics, *i.e.* dull and dark integument, shriveled body, dry, and odorless. The feces excreted by sick larvae were wetter than those of untreated larvae (control). The larvae that died as a result of treatment with the endophytic fungi when grown in SDA media, their cadavers were not overgrown with mycelia fungi.

Discussion

The isolates of the endophytic fungi found in this study had an rDNA sequence similarity value of more than 99% to the reference species (BLAST), meaning that the isolates had a high phylogenetic relationship and were in the same species. Bich *et al.* (2021) stated that ribosomal DNA sequences were used to determine the phylogenetic relationships of organisms to taxa species. Based on the similarity value, it is stated that if the similarity value is 100%, it means that the isolates are the same strain, whereas if the similarity value of 99% indicates that the isolates are the same species, and if the similarity value of the isolates is 89-99%, that means they are the same genus (Henry *et al.* 2000). An organism is declared the same species when the difference in DNA sequences is between 0.2–1% (Shenoy *et al.* 2007).

The results of identification based on the morphological characters of several species of fungi showed the similarities, for example the morphology of the colony and the shape of the conidia isolates of *B. bassiana* were similar to that of *P. citrinum*, but after the molecular identification, they showed them different species. According to Minarni *et al.* (2021) proper species identification is very important in determining a species as an entomopathogen.

The fungi in this study proved to be endophytic based on the characteristics of maize stalks and leaves colonized by the fungal mycelia. According to Barra-Bucarei *et al.* (2020), endophytic fungus (*B. bassiana*) had a systemic mode of action ability to colonize their host plant (tomato). The endophytic fungi can still be found on roots, stems, and leaves of tomato up to 30 days after inoculation (Carolina *et al.* 2020). Endophytic *B. bassiana* colonized 100% of leaves, 80% of stems, and 60% of roots of maize 7 days after foliar spray and caused significant reductions in *S. frugiperda* growth and reproduction (Russo *et al.* 2020). The research results of Shikano (2018) showed the endophytic fungi were able to live in the tissue for several months depending on the age of the host plant.

Out of the 7 species of 20 isolates of the endophytic fungi found in this study, there were only 6 pathogenic isolates against the larvae of *S. frugiperda*. The isolates JgSPK, JaGiP, JgCrJr, JaTpOi1 belong to *B. bassiana*, the JaSpkPga(3) and CaTpPga isolates belong to *C. lunata* and *M. anisopliae*, respectively. First report of *B. bassiana* and *C. lunata* isolated from maize, and *M. anisopliae* isolated from red chili in Indonesia were found as entomopathogenic endophytic fungi against *S. frugiperda*. *C. lunata* infected and killed grain insect pests, such as *Tribolium castaneum* (Herbst.) (Coleoptera: Tenebrionidae), *Trogoderma granarium* (Everts) (Coleoptera: Dermestidae), *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae), and *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Cucujidae) (Wakil *et al.* 2014). The endophytic *M. anisopliae* effectively killed the larvae of *S. frugiperda* (Ramos *et al.* 2020).

The endophytic fungi species of *B. bassiana* (JgSPK, JaGiP, JgCrJr, JaTpOi1 isolates), *C. lunata* (JaSpkPga(3) isolate), and *M. anisopliae* (CaTpPga isolate) had potential as entomopathogens of *S. frugiperda* larvae. *S. frugiperda* larvae that died from consuming plants colonized by the endophytic fungi showed that the fungi could be applied through seed treatment, especially for maize seeds. The seed treatment causes the endophytic fungi to colonize plants (Gustianingtyas et al. 2021) and if *S. frugiperda* larvae eat them it can cause them die (Ramos *et al.* 2020).

The *B. bassiana* of JaSpkPGA(2) isolate and all isolates of *P. citrinum, Chaetomium* sp., *A. niger*, and *A. flavus* showed no potential as entomopathogens of *S. frugiperda* with their resulting mortality rates of less than 8%. In contrast to the research results of Gustianingtyas *et al.* (2021) reported that the endophytic fungi species (*Aspergillus* sp. and *Chaetomium* sp.) applied topically (contact) caused mortality rates of *S. frugiperda* larvae as many as 18.67 and 14.67%, respectively and the mortality rate caused by *Beauveria* sp. reached 29%. This difference in the rates was due to different ways of application of the fungi, in this study the larvae of *S. frugiperda* consumed the plants that had been colonized by the endophytic fungi which were not applied topically.

The larvae of *S. frugiperda* that were sick and died by feeding on the leaves colonized by the endophytic fungus had different characteristics from the larvae that were sick and died due to the topical suspension of the endophytic fungus. The sick larvae of *S. frugiperda* in this study had wetter feces and the cadavers were not overgrown and covered by the fungal mycelia, while the sick larvae caused by contact with suspension of the endophytic fungi according to Gustianingtyas *et al.* (2021) had dry feces and the cadavers were covered with the fungal mycelia. The dead larvae treated by the endophytic fungi were not overgrown by mycelia fungi because the cadavers were too dry. According to El-Ghany (2015), the success of a fungus in germinating was affected by several external factors, such as humidity (more than 98%). So, if the cadavers were too dry, the fungal conidia could not germinate on the cadavers.

Conclusions

Molecular identifications recorded 7 species of the endophytic fungi, namely *Chaetomium* sp. (GaTpeOi, JgTjPr, PiCrPga, and CaCjPga isolates), *Aspergillus niger* (PsgTjPr, JgByU, and

JaBuBys), *B. bassiana* (JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, and JaTpOi (1) isolates), *Curvularia lunata* (JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr isolates), *Aspergillus flavus* (JgPWSR isolate), *Penicillium citrinum* (JaTpOi(2) isolate), and *Metarhizium anisopliae* (CaTpPGA isolate). The endophytic fungal species of *B. bassiana* (JgSPK isolate) caused up to (22.67%) mortality, *C. lunata* (JaSpkPga(3) isolate) caused (17.33%) mortality, and *M. anisopliae* (CaTpPga isolate) caused (8%) mortality against *S. frugiperda* larvae. First report of *B. bassiana* and *C. lunata* isolated from maize, and *M. anisopliae* isolated from red chili in Indonesia were found as entomopathogenic endophytic fungi against *S. frugiperda* larvae. The endophytic fungi species of *B. bassiana*, *C. lunata*, and *M. anisopliae* had potentials as entomopathogens of *S. frugiperda*.

List of abbreviations

ANOVA: analysis of variance; BLAST: Basic Local Alignment Search Tool; CTAB: cetyltrimethylammonium bromide; DNA: Deoxyribonucleic acid; EtOH: Ethyl alcohol; HSD: Tukey's Honestly Significant Difference; ITS: Internal Transcribed Spacer; MEA: the malt extract agar; NaOCl: Sodium hypochlorite; SDA: Sabouraud Dextrose Agar; TBE: Tris-Boric Acid-EDTA.

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Abstract

The fall armyworm, *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae), is a new invasive pest in Indonesia causing financial losses. *S. frugiperda* larvae hide in the leaf midrib all day. To overcome the hidden larvae, pathogenic endophytic fungi are needed. The objective of this research was to molecularly identify the endophytic fungal species from South Sumatra and determine the most pathogenic species against *S. frugiperda* larvae. Endophytic fungal identification was based on morphological and molecular characteristics. The molecular identification was based on gene sequential analysis of Intergenic Transcribed Spacer (ITS) 1 and ITS 4. Bioassay of the endophytic fungal species

was treated against the 2nd larval instar of *S. frugiperda*.The results of molecular identification revealed endophytic fungal species consisted of *Chaetomium* sp. (GaTpeOi, JgTjPr, PiCrPga, and CaCjPga isolates), *Aspergillus niger* (L.) Van Tieghem (PsgTjPr, JgByU, and JaBuBys), *Beauveria bassiana* (Balsamo) Vuillemin (JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, and JaTpOi (1) isolates), *Curvularia lunata* (Wakker) Boed. (JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr isolates), *Aspergillus flavus* Link. (JgPWSR isolate), *Penicillium citrinum* Thom F 1539 (JaTpOi(2) isolate), and *Metarhizium anisopliae* (Metschn.) Sorokin (CaTpPGA isolate). All fungal isolates were deposited in the GenBank. The endophytic fungal species of *B. bassiana* (JgSPK isolate) caused up to (22.67%) mortality, *C. lunata* (JaSpkPga(3) isolate) caused (17.33%) mortality, and *M. anisopliae* (CaTpPga isolate) caused (8%) mortality of the pest's larvae. First report of *B. bassiana* and *C. lunata* isolated from maize (*Zea mays* L.), while *M. anisopliae* was isolated from red chili (*Capsicum annuum* L.) as entomopathogenic endophytic fungi against *S. frugiperda* larvae in Indonesia.The endophytic fungi species of *B. bassiana*, *C. lunata*, and *M. anisopliae* had potentials as entomopathogens of *S. frugiperda*.

Keywords (separated by '-')	Endophytic fungi - Invasive pest - *Spodoptera frugiperda* - Morphological and molecular identifications - Indonesia
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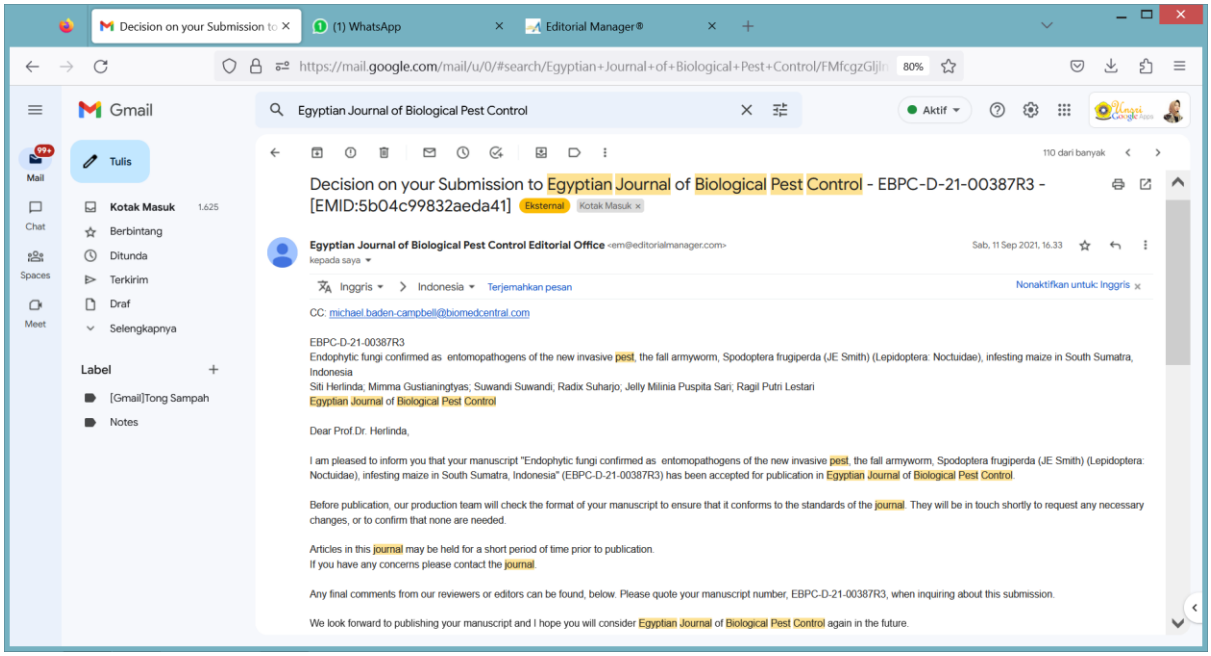

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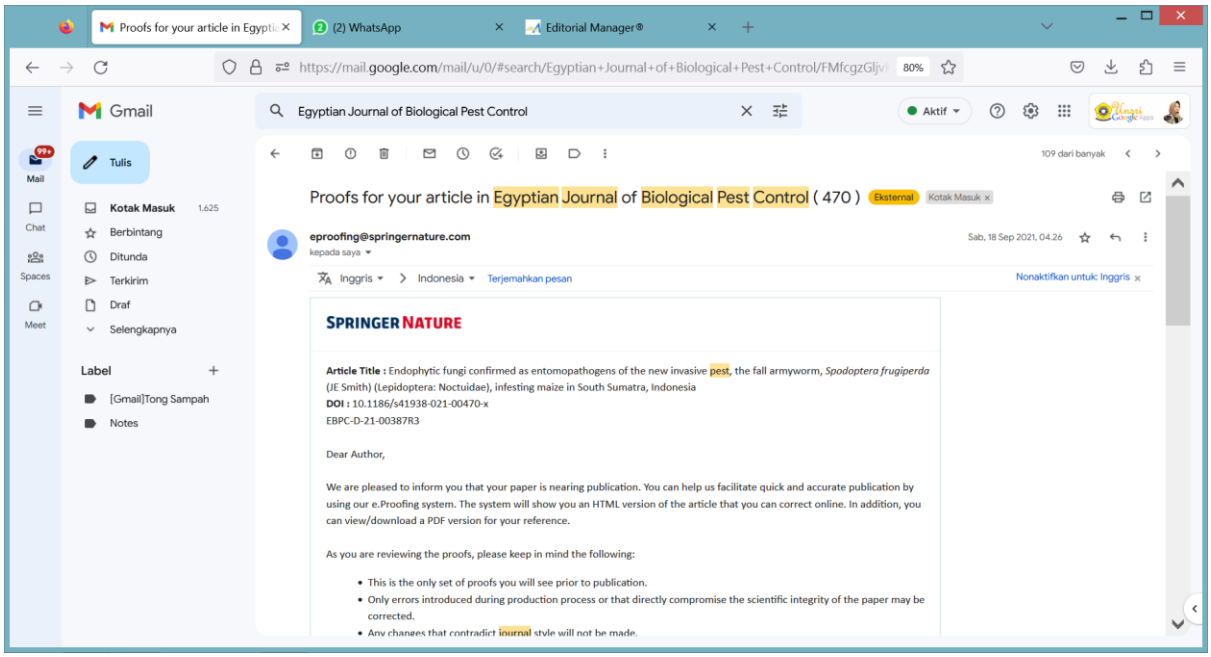

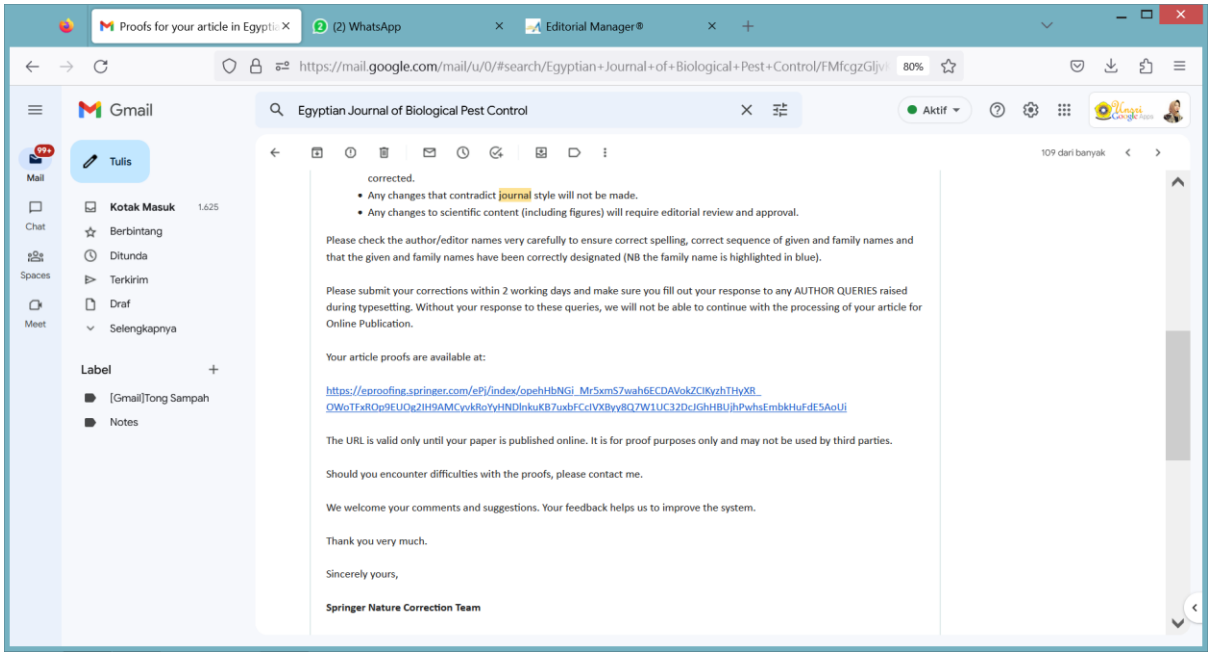

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Endophytic fungi confirmed as entomopathogens of the new invasive pest, the fall armyworm, *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae), infesting maize in South Sumatra, Indonesia

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Abstract

Background: The fall armyworm, *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae), is a new invasive pest in Indonesia causing financial losses. *S. frugiperda* larvae hide in the leaf midrib all day. To overcome the hidden larvae, pathogenic endophytic fungi are needed. The objective of this research was to molecularly identify the endophytic fungal species from South Sumatra and determine the most pathogenic species against *S. frugiperda* larvae. Endophytic fungal identification was based on morphological and molecular characteristics. The molecular identification was based on gene sequential analysis of Intergenic Transcribed Spacer (ITS) 1 and ITS 4. Bioassay of the endophytic fungal species was treated against the 2nd larval instar of *S. frugiperda*.

Result: The results of molecular identification revealed endophytic fungal species consisted of *Chaetomium* sp. (GaTpeOi, JgTjPr, PiCrPga, and CaCjPga isolates), *Aspergillus niger* (L.) Van Tieghem (PsgTjPr, JgByU, and JaBuBys), *Beauveria bassiana* (Balsamo) Vuillemin (JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, and JaTpOi (1) isolates), *Curvularia lunata* (Wakker) Boed. (JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr isolates), *Aspergillus flavus* Link. (JgPWSR isolate), *Penicillium citrinum* Thom F 1539 (JaTpOi(2) isolate), and *Metarhizium anisopliae* (Metschn.) Sorokin (CaTpPGA isolate). All fungal isolates were deposited in the GenBank. The endophytic fungal species of *B. bassiana* (JgSPK isolate) caused up to (22.67%) mortality, *C. lunata* (JaSpkPga(3) isolate) caused (17.33%) mortality, and *M. anisopliae* (CaTpPga isolate) caused (8%) mortality of the pest's larvae. First report of *B. bassiana* and *C. lunata* isolated from maize (*Zea mays* L.), while *M. anisopliae* was isolated from red chili (*Capsicum annuum* L.) as entomopathogenic endophytic fungi against *S. frugiperda* larvae in Indonesia.

Conclusion: The endophytic fungi species of *B. bassiana*, *C. lunata*, and *M. anisopliae* had potentials as entomopathogens of *S. frugiperda*.

Keywords: Endophytic fungi, Invasive pest, *Spodoptera frugiperda*, Morphological and molecular identifications, Indonesia

Background

The fall armyworm, *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae), is a new invasive pest, originating from South America (Otim et al. 2018). Currently,

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it has spread throughout the world and its first record in Indonesia was in March 2019 in West Sumatra (Sartiami et al. 2020) and in July 2019 in South Sumatra (Hutasoit et al. 2020). Now, it has been spread all over Indonesia. The pest attacks maize (*Zea mays* L.) and various other plant species by means of its larval stage, eating leaves, stems, flowers, fruits, growing points, and the whole maize plant until it becomes bare (Montezano et al. 2018). Larvae are found on the surface of leaves or maize stalks in the morning and then hide in the leaves' midribs (Gustianingtyas et al. 2021). Such behavior makes it difficult to be controlled.

Laboratory experiments of Herlinda et al. (2020a) showed that the entomopathogenic fungus (EPF), *Metarhizium* sp., could kill (78%) of *S. frugiperda* larvae. *Beauveria bassiana* (Balsamo) Vuillemin, *Metarhizium anisopliae* (Metschn.) Sorokin, and *M. rileyi* (Farlow) Samson also killed more than (80%) *S. frugiperda* larvae (Ramanujam et al. 2020). Their treatments were applied topically (direct contact) with the larvae exposed. In the field, the presence of hiding larvae in the midribs all the day makes the contacted EPF less effective (Gustianingtyas et al. 2021). To overcome such behavior, EPF found in plant tissues (endophytic fungi) is needed (Ramos et al. 2020). The endophytic fungi systemically colonize plant tissues and associate mutually their host plants (Lira et al. 2020). The results of previous studies have proven that 8 isolates of endophytic EPF could topically kill *S. frugiperda* larvae (Gustianingtyas et al. 2021); however, the endophytic fungi found in their studies were only isolated from the roots and their molecular identification had not yet been carried out.

In this study, the fungi isolated from leaves, shoots, and roots of maize plants from South Sumatra (Indonesia) were identified morphologically and molecularly and their pathogenicity to *S. frugiperda* larvae was evaluated.

Methods

Purification, identification, and bioassay of fungi were conducted from January to March 2021. The endophytic fungal species were identified based on the molecular analysis carried out at a laboratory accredited according to the ISO 17025 standard. Bioassay of the fungi was carried out in an incubator at controlled and constant temperature and relative humidity, respectively, 25 °C and 97%.

Exploration, isolation, and purification of fungi

Exploration of endophytic fungi was carried out by collecting leaves, shoots, and roots of maize and other crop plants around it such as bananas (*Musa* spp.), ridged gourd (*Luffa acutangula* (Roxb.) L.), and red chilies (*Capsicum annuum* L.). Selection of individual crop plant samples to obtain the endophytic fungi followed the method of Kasambala et al. (2018). Samples of the plant parts were placed into an ice box and then taken to the laboratory. The exploration of endophytic fungi was carried out from the lowlands to the highlands of South Sumatra, Indonesia, namely in Banyuasin District (2.8833°S 104.3831°E), Ogan Ilir District (3.43186°S 104.62727°E), Prabumulih City (3.4328°S 104.2356°E), Pagar Alam City (3°52′43.8″S 103°21′30″E) (Table 1).

The leaves, shoots, and roots of the sample plants were cleaned and washed aseptically with running tap water. Then, the plant parts were surface sterilized using the method of Elfita et al. (2019) with 70% EtOH (ethyl alcohol) and 1% NaOCl (sodium hypochlorite) and then rinsed 3 times. The last rinse water was used to check whether the microorganisms causing surface contamination were still present and carried out by growing them onto sabouraud dextrose agar (SDA) (Russo et al. 2020). Obtaining the endophytic fungi isolates started with growing the plant parts that sterilized earlier onto a media. The roots were grown on a specific medium, the malt extract agar (MEA), following the method of Silva et al. (2018). The shoots and leaves of the sample plants were grown on SDA media. The plant parts were grown as many as 5 pieces per Petri dish with a length of 5 mm and a diameter of 1–5 mm (depending on the diameter of the leaves, shoots, and roots). Fungi growing from the leaves, shoots, and roots were purified to be one isolate per sample. The isolates were then observed for their microscopic characteristics and continued with the fungal colonization test into the maize seed tissue.

Assess endophytic fungal colonization

Fungal inoculation on maize seeds was carried out to observe the colonization of fungi into the maize seedling tissue and to further ensure that the isolates found were truly endophytic. The 15 seeds of maize were surface sterilized, using 70% EtOH (ethyl alcohol) and 1% NaOCl (sodium hypochlorite) (Elfita et al. 2019). The seeds were immersed in 10 ml of fungal suspension, with a concentration of 1×10^6 conidia ml^{-1} for 6 h, while for the control, the sterilized seeds were only immersed in 10 ml of distilled water. Then, the seeds were grown in a sterile glass bottle (250 ml volume) with a sterile filter paper (Whatman no. 42) at the bottom, which was moistened with 1 ml distilled water and incubated for 10 days. All the treatments in this experiment were repeated 3 times. After the maize seedlings were 10 days old, the stems and leaves were sliced crosswise and longitudinally with a thickness of 0.02 mm each. The slices were observed with a light microscope at 40 × magnification with 0.05% lactofenol trypan blue dye. Mycelia endophytic fungi found in the maize seedling tissue were documented. The

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Table 1 Origin of isolates of endophytic fungi from South Sumatra, Indonesia

Location (village, district/city)	Isolate origin	Altitude (m)	Fungal species	Fungal isolate code	GenBank acc. no
Tanjung Pering. Ogan Ilir	Ridged gourd	36.0	*Chaetomium* sp.	GaTpeOI	MZ359734
Tanjung Pering. Ogan Ilir	Bananas	36.0	*Aspergillus niger*	PsgTjPr	MZ242060
Simpang Padang Karet. Pagar Alam	Maize	797.7	*Beauveria bassiana*	JgSPK	MZ356494
Tanjung Pering. Ogan Ilir	Maize	36.0	*Chaetomium* sp.	JgTjPr	MZ359736
Gunung Ibul. Prabumulih	Maize	53.0	*Beauveria bassiana*	JaGIP	MZ356495
Curup Jare. Pagar Alam	Bananas	806.0	*Chaetomium* sp.	PICrPga	MZ359735
Gunung Ibul. Prabumulih	Maize	53.0	*Curvularia lunata*	JaGIPRB	MZ359815
Tanjung Payang. Pagar Alam	Red chilies	689.6	*Curvularia lunata*	CMTJP	MZ359816
Mulia Sari. Banyuasin	Maize	19.0	*Curvularia lunata*	JaMsBys	MZ359819
Simpang Padang Karet. Pagar Alam	Maize	789.5	*Beauveria bassiana*	JaSpkPGA(2)	MZ356496
Curup Jare. Pagar Alam	Maize	806.7	*Beauveria bassiana*	JgCrJr	MZ356497
Tanjung Pering. Ogan Ilir	Maize	36.0	*Beauveria bassiana*	JaTpOI (1)	MZ356498
Simpang Padang Karet. Pagar Alam	Maize	797.7	*Curvularia lunata*	JaSpkPga(3)	MZ359818
Curup Jare. Pagar Alam	Red chilies	806.0	*Chaetomium* sp.	CaCJPga	MZ359737
Banyuurip. Banyuasin	Maize	13.0	*Aspergillus niger*	JgByU	MZ242059
Telang Sari. Banyuasin	Maize	15.0	*Curvularia lunata*	JgTgSr	MZ359817
Banyuurip. Banyuasin	Maize	20.0	*Aspergillus niger*	JaBuBys	MZ242058
Purwosari. Banyuasin	Maize	15.0	*Aspergillus flavus*	JgPWSR	MZ359829
Tanjung Pering. Ogan Ilir	Maize	36.0	*Penicillium citrinum*	JaTpOI(2)	MZ359812
Tanjung Payang. Pagar Alam	Red chilies	689.6	*Metarhizium anisopliae*	CaTpPga	MZ242073

fungal isolates that were proven to be endophytic were used for bioassays. Prior to the bioassay, the isolates were observed for microscopic characteristics for morphological identification, followed by molecular identification.

Morphological and molecular identification of fungal species

Macroscopic and microscopic observation

Morphological observations were carried out based on the macroscopic characteristics of the endophytic fungi that included colony color and shape, shape, and size of conidia and conidiophores, following the method of Herlinda et al. (2020a).

DNA extraction and PCR amplification

The used endophytic fungal DNA extraction method refers to the Swibawa et al. (2020) method. DNA extraction was carried out on fungal conidia. The conidia were harvested from pure cultures of 7-day-old fungus, which was cultured in sterile Petri dishes with a diameter of 9 cm. As much as 10 ml of sterile water was placed into a Petri dish containing fungal cultures and slowly harvested, using a Drigalski. The obtained conidia suspension was transferred to a centrifuge tube (volume 30 ml) and centrifuged using CF15RXII (Hitachi, Japan) for 10 min at a speed of 14,000 rpm. After being centrifuged, 1 ml of 70% ethanol was added to the centrifuge tube and then centrifuged again for 10 min. After that, the supernatant was removed and 1 ml of extraction buffer was added with a composition of 0.5 ml Tris HCl, 1 mL SDS 1% + 2.8 mL NaCl, 0.2 ml Mercaptho Ethanol, 2 ml EDTA, 3.5 ml sterile water, and then homogenized. The suspension was transferred to a sterile mortal and incubated at -40 °C for 24 h. After that, the frozen suspension was ground until it was smooth and melted. A total of 500 µl of suspension was then transferred into a 1.5 ml tube. A total of 400 µl of 2% cetyltrimethylammonium bromide (CTAB) was then added to the tube, homogenized, and then incubated at 65 °C for 1 h using a water bath (Brookfield TC 550 MX-230, USA). After the incubation, 500 µl of Phenol Chloroform Isoamyl alcohol (PCI) (25:24:1) was added, homogenized, and centrifuged (Microspin12; Biosan, Latvia) at 14,000 rpm for 10 min. A total of 600 µl of supernatant was taken and transferred to a new 1.5 ml tube. A total of 600 µl Chloroform Isoamyl Alcohol (CI) (24:1) was added to the tube, homogenized, and centrifuged (Microspin12; Biosan, Latvia) at 14,000 rpm for 10 min. A total of 400 µl of supernatant was then transferred to a new 1.5 ml tube, and 400 µl of cold isopropanol was added, homogenized, and incubated at − 40 °C for 20 min. After the incubation, it was centrifuged (Microspin12; Biosan, Latvia) at 14,000 rpm for 15 min. The supernatant was then discarded. After the supernatant was removed, 500 µl of 70% cold ethanol was added and centrifuged (Microspin12; Biosan, Latvia) at 14,000 rpm for 5 min. The supernatant was then

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discarded, and the pellets obtained were dried at room temperature for 24 h. After drying, the tube was then added as much as 50 µl 1 × Tris–HCL EDTA (TE) pH 8.0 (1st Base Malaysia).

PCR amplification was carried out using the Sensoquest Thermal Cycler (Germany) PCR machine on the internal transcribed spacer (ITS) using ITS1 and ITS4 primers (White et al. 1990). The PCR was carried out with a total volume of 25 µl consisting of a mixture of Master Mix (Red Mix) (bioline) as much as 12.5 µl, 10 µM of primer ITS 1 (5′TCC GTA GGT GAA CCT TGC GG 3′) and ITS 4 (5′TCC TCC GCT TAT TGA TAT GC 3′) 1 µL each, 1 L of template DNA, and 9.5 µl of sterile water. The DNA amplification stage consisted of 1 initiation cycle at 95 °C for 5 min, followed by 30 cycles consisting of denaturation at 95 °C for 1 min, primer attachment at 52 °C for 1 min, primer extension at 72 °C for 1 min, and 1 elongation cycle at 72 °C for 5 min. The PCR results were then electrophoresed, using 0.5% agarose in 20 ml of 1 × Tris-Boric Acid-EDTA (TBE) buffer (1st Base Malaysia), and added 1 µl of ethidium bromide (EtBr 10 mg/ml). The electrophoresis was carried out in 1 × TBE buffer solution at 50 V for 70 min. Electrophoresis results were visualized using a DigiDoc UV transilluminator (UVP, USA).

Sequencing and analysis of the results

The PCR results were then sent to 1st Base Malaysia for the sequencing process. The results of the sequencing were analyzed, using Bio Edit ver. 7.2.6 for windows. Then, the results were submitted to the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to find out the genus or species that had the greatest homology or similarity and molecularly. The phylogeny tree was created using the Mega 7 for Windows program (Kumar et al. 2016), using the UPGMA (jukes and cantor model) method. The ITS region sequences for several strains used as a reference in this study were obtained from NCBI (https://www.ncbi.nlm.nih.gov/).

Calculation of conidial density and viability

All the isolates of endophytic fungi were counted for their conidial density and viability. The conidial density of the 7-old-day fungi was calculated according to the method of Sumikarsih et al. (2019), using a hemocytometer and observed with a light microscope at 40 × magnification. The viability was observed by growing 1 ml of conidia fungal suspension (1×10^6 conidia ml^{-1}) onto 2% agar–water medium, and then, the culture was incubated for 1 × 24 and 2 × 24 h under a light microscope at 40 × magnification.

Mass-rearing of *S. frugiperda*

The mass-rearing of *S. frugiperda* was carried out, following the method of Herlinda et al. (2020a). The larvae were collected from the maize plants grown around the campus (3°13′25″S 104°39′51″E) and were reared in the laboratory for more than 5 generations to obtain the larvae with relatively homogeneous genetics and to eliminate the effects of exposure to synthetic insecticides from the fields. In the laboratory, the larvae of *S. frugiperda* were kept individually in a porous plastic cup (Ø 6.5 cm, height 4.6 cm) because the larvae were cannibals. The larvae were fed daily by maize leaves (measuring 2 cm × 5 cm). Prepupae were transferred to a plastic container (Ø 15 cm, height 25 cm) on which its bottom had a sterile soil (5 cm thick). The plastic container containing the prepupae was placed in a wire mesh cage ($30 \times 30 \times 30$ cm^3) and inside this cage placed also fresh maize leaves for the adults to lay eggs, which were changed daily. The one-day-old 2nd instar larvae were used for the bioassay.

Bioassay of endophytic fungi against larvae of *S. frugiperda*

The bioassay of endophytic fungi followed the method of Zea et al. (2019). The young maize (seedling) already inoculated with the endophytic fungi and not-inoculated (control) aged 10 days old was given to the 2nd instar larvae of *S. frugiperda*. Each larva was given one maize seedling whose cotyledons were already removed. The larvae were allowed to feed on the leaves and stems of the young maize for 6 h. in a sterile room (a laminar air flow cabinet), and each replication was tested for 25 individuals of *S. frugiperda* larvae. Then, the larvae were transferred to a porous plastic cup (Ø 6.5 cm, height 4.6 cm) and fed on healthy non-inoculated leaves measuring 2 cm × 5 cm per day per larvae. The experiment was designed, using completely randomized designs with treatments of 20 isolates, 3 replications per treatment. The dead larvae were recorded daily for 12 days, following the method of Herlinda et al. (2020b). The dead larvae were grown in the agar–water medium to confirm whether the infection was caused by the endophytic fungi or not. The number of dead larvae was used to calculate the mortality.

Data analysis

The differences in the mortality data of *S. frugiperda* larvae were analyzed using analysis of variance (ANOVA). Tukey's honestly significant difference (HSD) test (Tukey's test) was employed to test for the significant differences among the treatments (isolates) at $P = 0.05$. All data were calculated using software of SAS University Edition 2.7 9.4 M5.

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Results

Colonization of endophytic fungi on maize tissues

All fungal isolates, isolated from the leaves, shoots, and roots of the maize, bananas, ridged gourd, and red chilies were found to be 124 isolates. However, after assessing for the fungal colonization in young maize (seedlings) tissue, only 20 isolates of the fungi were confirmed as endophytic fungi (Fig. 1). Evidence of fungal colonization that entered the maize seedlings tissue was that mycelia fungi were seen entering and growing spread throughout the stem and leaf tissue. Mycelia endophytic fungi were able to invade the stem and leaf of the maize tissue, the

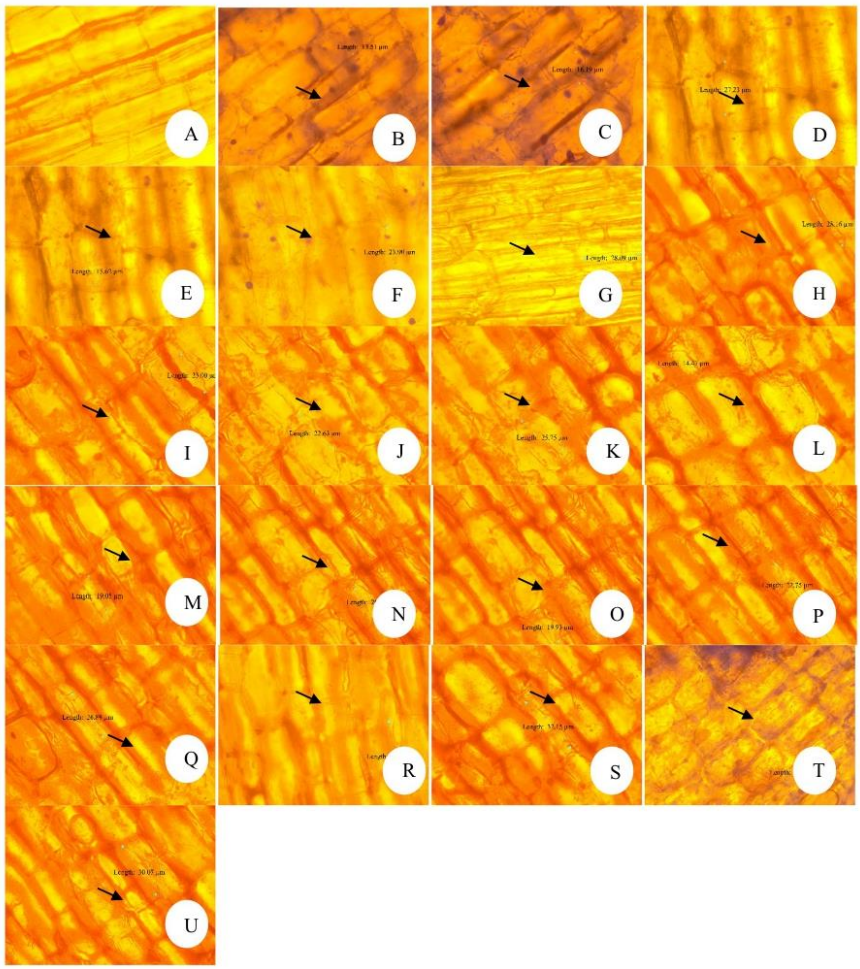

Fig. 1 Young maize tissues colonized by endophytic fungal species: Control (**A**); *Chaetomium* sp. isolates of GaTpeOi (**B**), JgTjPr (**E**), PiCrPga (**G**), and CaCjPga (**O**); *Aspergillus niger* isolates of PsgTjPr (**C**), JgByU (**P**), and JaBuBys (**R**); *Beauveria bassiana* isolates of JgSPK (**D**), JaGiP (**F**) JaSpkPGA(2) (**K**), JgCrJr (**L**), and JaTpOi(1) (**M**); *Curvularia lunata* isolates of JaGiPRB (**H**), CMTJP (**I**), JaMsBys (**J**), JaSpkPga(3) (**N**), and JgTgSr (**Q**); *Aspergillus flavus* isolate of JgPWSR (**S**); *Penicillium citrinum* isolate of JaTpOi(2) (**T**); and *Metarhizium anisopliae* isolate of CaTpPga (**U**)

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mycelia appeared to be branched. The size of mycelia varied daily from 13.51 to 34.25 µm. In the maize tissue, which their seeds were not inoculated by the endophytic fungi (control), the cells in the maize tissue were clean and clear without fungal colonization.

Identification results of the endophytic fungal isolates

The colony morphology of 20 isolates of the endophytic fungi showed different colors (Fig. 2) and likewise the shapes of hyphae and conidia of each isolate showed their own characteristics (Fig. 3). The colony isolates of GaTpeOi, JgTjPr, PiCrPga, and CaCjPga grown on SDA media showed similarities; namely, the colony was pale gray, hyphae and mycelia were septate, and the conidia were in the shape of letter D (asymmetric/elliptical) and non-septation. Based on the phylogenetic tree, these 4 isolates were placed within the group of *Chaetomium* sp. (Fig. 4). The isolates were deposited in the GenBank with the accession number MZ359734 (GaTpeOi), MZ359736 (JgTjPr), MZ359735 (PiCrPga), MZ359737 (CaCjPga) (Table 1).

The colony isolates of PsgTjPr, JgByU, and JaBuBys showed similarities, their colonies were black, the hyphae and mycelia were also black, and the conidia were non-septate, globose in shape. The 3 isolates were placed within the group of *Aspergillus niger* (L.) Van Tieghem (Fig. 4). The isolates were deposited in the GenBank with the accession number MZ242060 (PsgTjPr), MZ242059 (JgByU), and MZ242058 (JaBuBys) (Table 1).

The isolates of JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, and JaTpOi (1) had a white colony, white hyphae and mycelia, and the globose conidia, which were non-septate conidia. The isolates were placed within the group of *B. bassiana* (Fig. 4). The isolates were deposited in the GenBank with the accession number MZ356494 (JgSPK), MZ356495 (JaGiP), MZ356496 (JaSpkPGA(2)), MZ356497 (JgCrJr), and MZ356498 (JaTpOi1) (Table 1).

The isolates of JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr had a black colony, black hyphae and mycelia, two-septate boomerang-shaped conidia. All the isolates were placed within the group of *Curvularia lunata* (Wakker) Boed. (Fig. 4). The isolates were deposited in the GenBank with the accession number MZ359815 (JaGiPRB), MZ359816 (CMTJP), MZ359819 (JaMsBys), MZ359818 (JaSpkPga(3)), and MZ359817 (JgTgSr) (Table 1).

The isolate of JgPWSR had a green colony, green hyphae and mycelia, non-septate globose conidia. The JgPWSR isolate was placed within the group of *Aspergillus flavus* Link. (Fig. 4). The isolate was deposited in the GenBank with the accession number MZ359829 (JgPWSR) (Table 1).

The color of the colony JaTpOi(2) isolate was initially white; as the fungus got older, it turned bluish green with white margin, rounded conidia. The isolate was placed within the group of *Penicillium citrinum* Thom F 1539 (Fig. 4). The isolate was deposited in the GenBank with the accession number MZ359812 (JaTpOi (2)) (Table 1).

The isolate of CaTpPGA had a white colony, and as the fungus got older, it turned greenish white to dark green, green hyphae and mycelia, and the conidia are clear, cylindrical, and non-septation. The isolate was placed within the group of *M. anisopliae* (Fig. 4). The isolate was deposited in the GenBank with the accession number MZ242073 (CaTpPGA) (Table 1).

The result of BLAST search revealed that the isolates of GaTpeOi, JgTjPr, PiCrPga, and CaCjPga had a similarity (100%) with the *Chaetomium* sp. isolate A13 (Acc. No. MH34803.1), isolate MJ51 (Acc No. KM203618.1), and isolate CP-2009 (Acc. No. AB50680.1). The isolates of PsgTjPr, JgByU, and JaBuBys showed 100% of similarity with *A. niger* NBRC: 105649 (Acc. No LC573609.1), strain WM04.470 (Acc No. AJ853742.1), and isolate IFM61597 (Acc. No. LC602036.1). The isolates of JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, and JaTpOi (1) showed (100%) similarity to the *B. bassiana* isolate GZMS-28 (Acc. No. KT715480.1), strain TF6-1B (Acc No. JX122736.1), and isolate BSwTd4 (Acc. No. MT4487322.1). The isolates of JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr showed 99.81% of similarity to *C. lunata* strain D25A (Acc. No MH010917.1), strain AN 2 (Acc No. KY859790.1), and isolate 1z -7 (Ac. No. MN213745.1). The isolate of JgPWSR showed 100% of similarity to *A. flavus* strain GFRS9 (Acc. No MT447477.1), strain GFRS06 (Acc. No MT447474.1), and strain aT3 (Acc. No KU561920.1). The isolate of JaTpOi(2) showed 100% of similarity to *P. citrinum* strain yx-001 (Acc. No. MN826202.1), strain AKF2-KU (Acc No. MN879404.1), and strain J (Acc. No. MK791668.1). The isolate of CaTpPGA had a similarity (99.29%) to *M. anisopliae* isolate MSwTp3 (Acc. No MT448733.1), strain STBMa-001 (Acc No. KF766520.1), and isolate C1 (Acc. No. KX809520.1).

There were 7 species from the 20 isolates of the endophytic fungi found in this study. The 4 isolates (GaTpeOi, JgTjPr, PiCrPga, and CaCjPga) were in the group of *Chaetomium* sp., the three isolates (PsgTjPr, JgByU, and JaBuBys) were in the group of *Aspergillus niger*, the 5 isolates (JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, and JaTpOi (1)) were placed within the group of *B. bassiana*, the 5 isolates (JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr) were in the group of *C. lunata*, an isolate (JgPWSR) was in the group of *A. flavus*, an isolate (JaTpOi(2)) was in the group of *P. citrinum*, and an isolate (CaTpPGA) was in the group of *M. anisopliae*.

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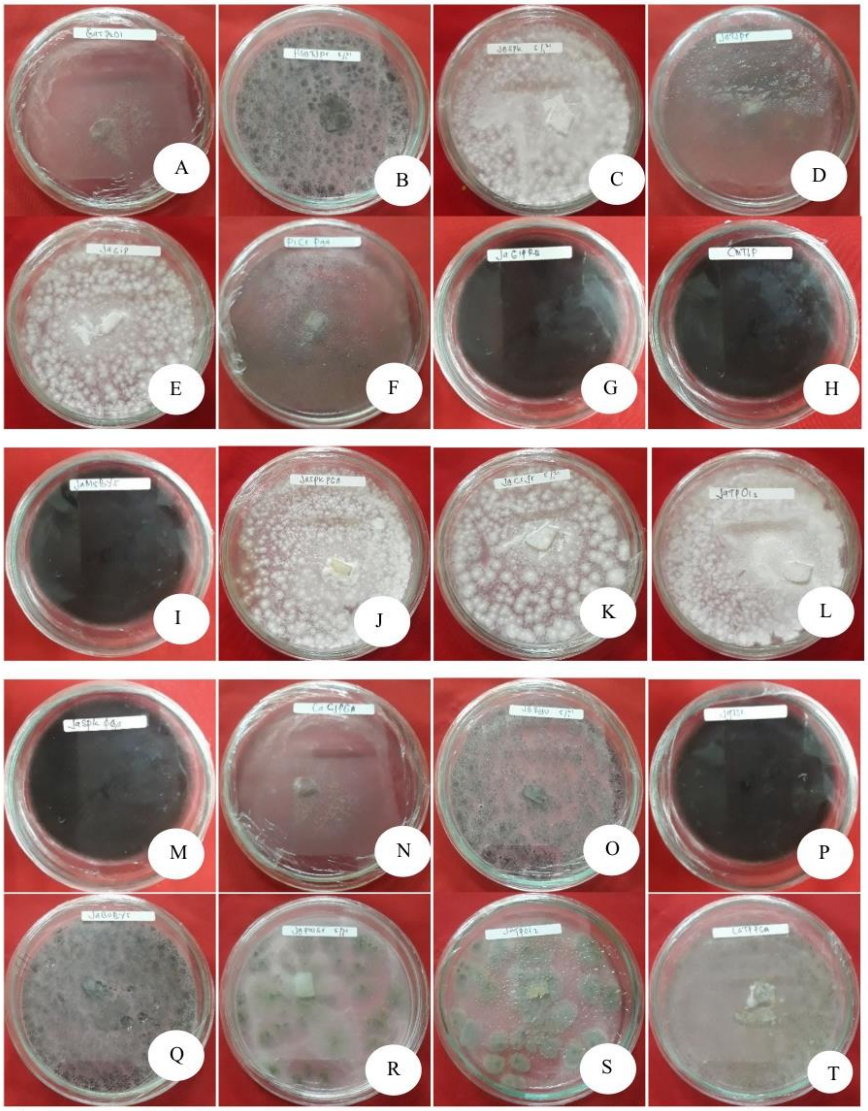

Fig. 2 Colony morphology of endophytic fungal species: *Chaetomium* sp. isolates of GaTpeOi (**A**), JgTJPr (**D**), PiCrPga (**F**), and CaCjPga (**M**); *Aspergillus niger* isolates of PsgTJPr (**B**), JgByU (**O**), and JaBuBys (**Q**); *Beauveria bassiana* isolates of JgSPK (**C**), JaGiP (**D**), JaSpkPGA(2) (**J**), JgCrJr (**K**), and JaTpOi(1) (**L**); *Curvularia lunata* isolates of JaGiPRB (**G**), CMTJP (**H**), JaMsBys (**I**), JaSpkPga(3) (**M**), and JgTgSr (**P**); *Aspergillus flavus* isolate of JgPWSR (**R**); *Penicillium citrinum* isolate of JaTpOi(2) (**S**); and *Metarhizium anisopliae* isolate of CaTpPga (**T**)

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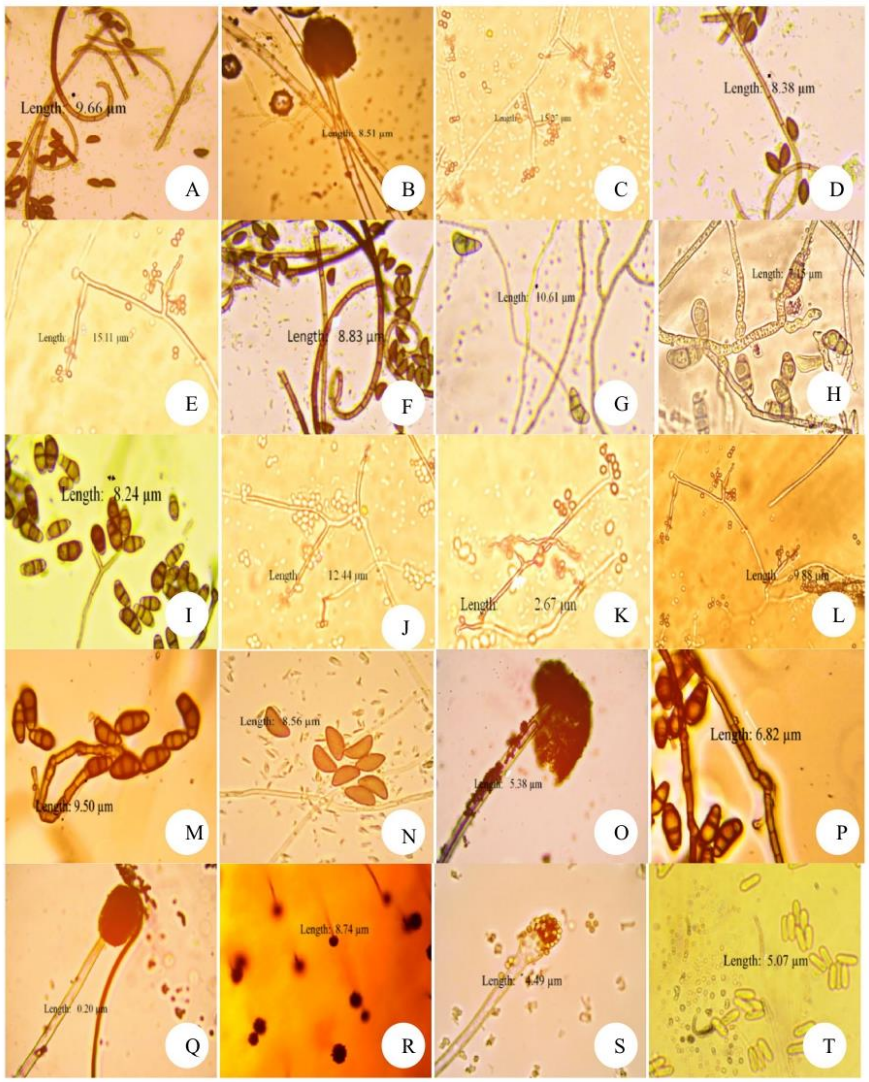

Fig. 3 Conidial and hyphal morphology of endophytic fungal species: *Chaetomium* sp. isolates of GaTpeOi (**A**), JgTjPr (**D**), PiCrPga (**F**), and CaCjPga (**M**); *Aspergillus niger* isolates of PsgTjPr (**B**), JgByU (**O**), and JaBuBys (**Q**); *Beauveria bassiana* isolates of JgSPK (**C**), JaGiP (**D**), JaSpkPGA(2) (**J**), JgCrJr (**K**), and JaTpOi(1) (**L**); *Curvularia lunata* isolates of JaGiPRB (**G**), CMTJP (**H**), JaMsBys (**I**), JaSpkPga(3) (**M**), and JgTgSr (**P**); *Aspergillus flavus* isolate of JgPWSR (**R**); *Penicillium citrinum* isolate of JaTpOi(2) (**S**); and *Metarhizium anisopliae* isolate of CaTpPga (**T**)

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Fig. 4 Phylogenetic tree based on the sequencing of internal transcribed spacer (ITS) region using UPGMA method (Jukes and cantor model) performed with Mega 7 program for windows (Kumar et al., 2016). This research found 20 isolates of entomopathogenic fungi: 4 isolates placed within the group of *Chaetomium* sp., 3 isolates placed within the group of *Aspergillus niger*, 5 isolates placed within the group of *Beauveria bassiana*, 5 isolates placed within the group of *Curvularia lunata*, 1 Isolate placed within the group of *Aspergillus flavus*, 1 isolate placed within the group of *Penicillium citrinum*, and 1 isolate placed within the group of *Metarhizium anisopliae*. = fungal isolates obtained from this research

Pathogenicity of the endophytic fungi against *S. frugiperda* larvae

Out of the 7 species of the 20 isolates of the endophytic fungi found in this study, there were 6 isolates that were the most pathogenic against *S. frugiperda* larvae (Table 2). The 4 isolates (JgSPK, JaGiP, JgCrJr, and JaTpOi1) resulted to mortality rates of 22.67, 21.3%, 21.33, and 17.33% consecutively were *B. bassiana*. In addition, the JaSpkPga(3) isolate of *C. lunata* caused a fairly high mortality (18.67%) and was non-significantly different than the mortality caused by the isolates of JgSPK, JaGiP, JgCrJr, JaTpOi1. The CaTpPga isolate of *M. anisopliae* caused (8%) larval mortality and was non-significantly different than the mortality caused by JaSpkPga(3) isolate. The *B. bassiana* and *C. lunata* isolates were isolated from maize, while the *M. anisopliae* isolate was isolated from red chili. The JaSpkPGA(2) isolate of *B. bassiana* AQ1 resulting a mortality rate of only (2.67%). Likewise, *P. citrinum*, *Chaetomium* sp., *A. niger,* and *A. flavus* caused the lowest larval mortality of less than (8%).

The larvae of *S. frugiperda* that died because of feeding on leaves colonized by the endophytic fungi had the characteristics, *i.e.*, dull and dark integument, shriveled body, dry, and odorless. The feces excreted by sick larvae were wetter than those of untreated larvae (control). The larvae that died as a result of treatment with the endophytic fungi when grown in SDA media and their cadavers were not overgrown with mycelia fungi.

Discussion

The isolates of the endophytic fungi found in this study had an rDNA sequence similarity value of more than 99% to the reference species (BLAST), meaning that the isolates had a high phylogenetic relationship and were in the same species. Bich et al. (2021) stated that ribosomal DNA sequences were used to determine the phylogenetic relationships of organisms to taxa species. Based on the similarity value, it is stated that if the similarity value is 100%, it means that the isolates are the same strain, whereas if the similarity value of 99% indicates that the isolates are the same species, and if the similarity value of the isolates is 89–99%, that means they are the same genus (Henry et al. 2000). An organism is declared the same species when the difference in DNA sequences is between 0.2 and 1% (Shenoy et al. 2007).

The results of identification based on the morphological characters of several species of fungi showed the similarities; for example, the morphology of the colony and the shape of the conidia isolates of *B. bassiana* were similar to that of *P. citrinum*, but after the molecular identification, they showed them different species. According to Minarni et al. (2021), proper species identification is very important in determining a species as an entomopathogen.

The fungi in this study proved to be endophytic based on the characteristics of maize stalks and leaves colonized by the fungal mycelia. According to Barra-Bucarei et al. (2020), endophytic fungus (*B. bassiana*) had a systemic mode of action ability to colonize their host plant (tomato). The endophytic fungi can still be found on roots, stems, and leaves of tomato up to 30 days after inoculation (Carolina et al. 2020). Endophytic *B. bassiana* colonized 100% of leaves, 80% of stems, and 60% of roots of maize 7 days after foliar spray and caused significant reductions in *S. frugiperda* growth and reproduction (Russo et al. 2020). The research results of Shikano (2018) showed the endophytic fungi were able to live in the tissue for several months depending on the age of the host plant.

Out of the 7 species of 20 isolates of the endophytic fungi found in this study, there were only 6 pathogenic isolates against the larvae of *S. frugiperda*. The isolates JgSPK, JaGiP, JgCrJr, JaTpOi1 belong to *B. bassiana*, and the JaSpkPga(3) and CaTpPga isolates belong to *C. lunata* and *M. anisopliae*, respectively. First report of *B. bassiana* and *C. lunata* isolated from maize, and *M. anisopliae* isolated from red chili in Indonesia were found as entomopathogenic endophytic fungi against *S. frugiperda*. *C. lunata* infected and killed grain insect pests, such as *Tribolium castaneum* (Herbst.) (Coleoptera: Tenebrionidae), *Trogoderma granarium* (Everts) (Coleoptera: Dermestidae), *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae), and *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Cucujidae) (Wakil et al. 2014). The endophytic *M. anisopliae* effectively killed the larvae of *S. frugiperda* (Ramos et al. 2020).

The endophytic fungi species of *B. bassiana* (JgSPK, JaGiP, JgCrJr, JaTpOi1 isolates), *C. lunata* (JaSpkPga(3) isolate), and *M. anisopliae* (CaTpPga isolate) had potential as entomopathogens of *S. frugiperda* larvae. *S. frugiperda* larvae that died from consuming plants

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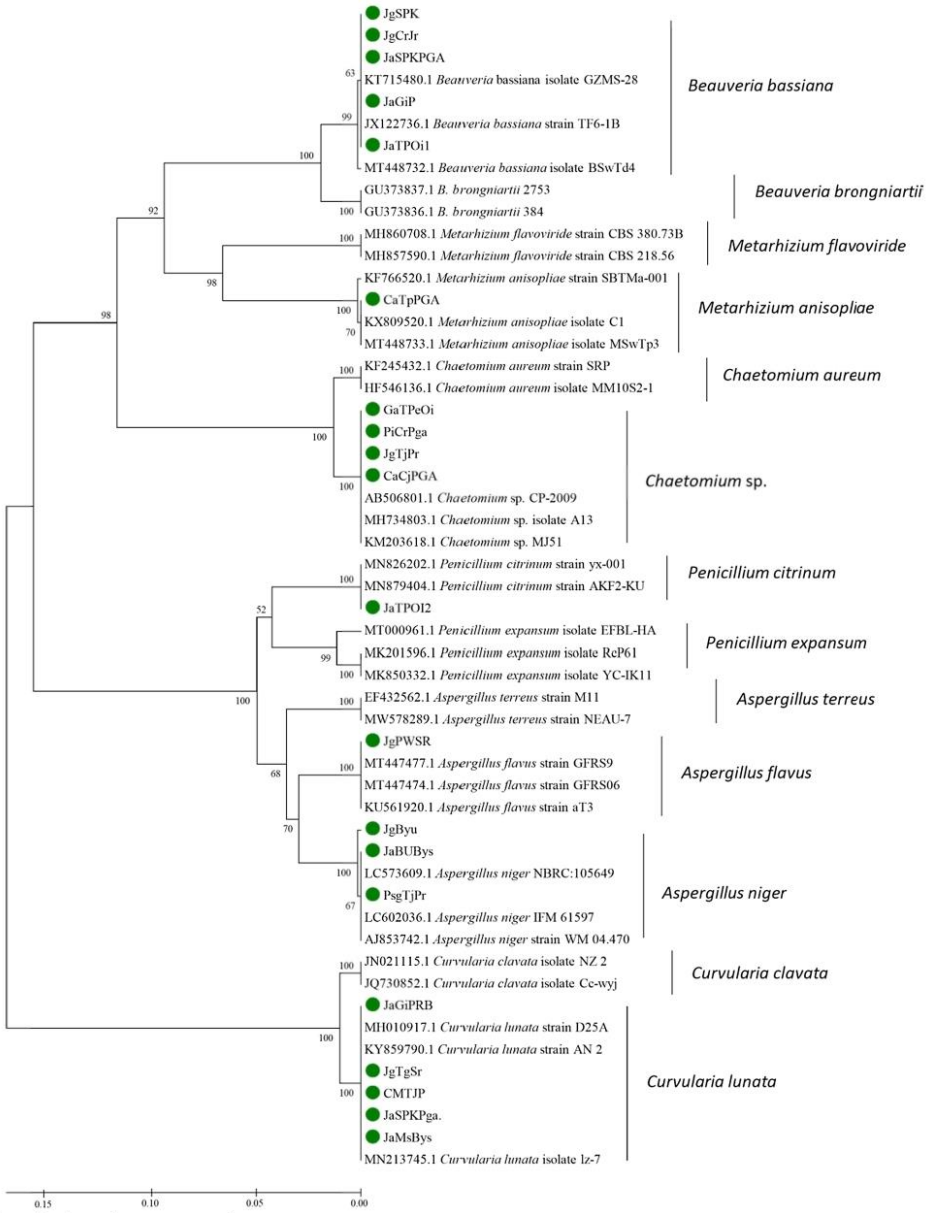

Fig. 4 (See legend on previous page.)

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Table 2 Mean mortality of *Spodoptera frugiperda* larvae treated with endophytic fungi (1×10^6 conidia ml^{-1}), and conidial density and viability of endophytic fungi

Fungal isolate code	Fungal species	Mortality (%)	Conidial viability (%)for 1 × 24 h	Conidial viability (%) for 2 × 24 h
Control	–	0.00a	–	–
GaTpeOI	*Chaetomium* sp.	4.00bcd	45.17bcd	47.00a
PsgTjPr	*Aspergillus niger*	2.67abc	38.20ab	51.63abcd
JgSPK	*Beauveria bassiana*	22.67f	47.59efgh	55.23cde
JgTjPr	*Chaetomium* sp.	2.67abcd	35.74a	55.76de
JaGiP	*Beauveria bassiana*	21.33f	42.86abc	52.91abcd
PiCrPga	*Chaetomium* sp.	5.33cd	44.59cde	47.99abc
JaGiPRB	*Curvularia lunata*	4.00bcd	45.18bcd	52.75abcd
CMTJP	*Curvularia lunata*	2.67abcd	45.25efg	54.94bcde
JaMsBys	*Curvularia lunata*	6.67d	49.98gh	58.60de
JaSpkPGA(2)	*Beauveria bassiana*	2.67abcd	47.20cde	48.15abc
JgCrJr	*Beauveria bassiana*	21.33f	46.50cd	52.63abcd
JaTpOI (1)	*Beauveria bassiana*	18.67f	54.02efg	56.55de
JaSpkPga(3)	*Curvularia lunata*	17.33ef	50.85def	56.93de
CaCjPga	*Chaetomium* sp.	2.67abcd	37.24a	47.77ab
JgByU	*Aspergillus niger*	1.33ab	58.84gh	69.57g
JgTgSr	*Curvularia lunata*	4.00bcd	55.33efgh	61.24ef
JaBuBys	*Aspergillus niger*	2.67abcd	58.45gh	76.50h
JgPWSR	*Aspergillus flavus*	4.00bcd	62.54h	68.39g
JaTpOI(2)	*Penicillium citrinum*	6.67cd	57.44fgh	65.98fg
CaTpPga	*Metarhizium anisopliae*	8.00de	50.76def	68.75f
F value	7.27*	9.05*	15.67*	
P value	0.00	0.00	0.00	
HSD value	8.8	4.27	4.07	

* = significantly different; values within a column followed by the same letters were not significantly different at $P < 0.05$ according to Tukey's HSD test

colonized by the endophytic fungi showed that the fungi could be applied through seed treatment, especially for maize seeds. The seed treatment causes the endophytic fungi to colonize plants (Gustianingtyas et al. 2021), and if *S. frugiperda* larvae eat them, it can cause them die (Ramos et al. 2020).

The *B. bassiana* of JaSpkPGA(2) isolate and all isolates of *P. citrinum*, *Chaetomium* sp., *A. niger*, and *A. flavus* showed no potential as entomopathogens of *S. frugiperda* with their resulting mortality rates of less than 8%. In contrast to the research, results of Gustianingtyas et al. (2021) reported that the endophytic fungi species (*Aspergillus* sp. and *Chaetomium* sp.) applied topically (contact) caused mortality rates of *S. frugiperda* larvae as many as 18.67 and 14.67%, respectively, and the mortality rate caused by *Beauveria* sp. reached 29%. This difference in the rates was due to different ways of application of the fungi; in this study, the larvae of *S. frugiperda* consumed the plants that had been colonized by the endophytic fungi which were not applied topically.

The larvae of *S. frugiperda* that were sick and died by feeding on the leaves colonized by the endophytic fungus had different characteristics from the larvae that were sick and died due to the topical suspension of the endophytic fungus. The sick larvae of *S. frugiperda* in this study had wetter feces, and the cadavers were not overgrown and covered by the fungal mycelia, while the sick larvae caused by contact with suspension of the endophytic fungi according to Gustianingtyas et al. (2021) had dry feces and the cadavers were covered with the fungal mycelia. The dead larvae treated by the endophytic fungi were not overgrown by mycelia fungi because the cadavers were too dry. According to El-Ghany (2015), the success of a fungus in germinating was affected by several external factors, such as humidity (more than 98%). So, if the cadavers were too dry, the fungal conidia could not germinate on the cadavers.

Conclusions

Molecular identifications recorded 7 species of the endophytic fungi, namely *Chaetomium* sp. (GaTpeOi, JgTjPr, PiCrPga, and CaCjPga isolates), *Aspergillus niger*

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(PsgTjPr, JgByU, and JaBuBys), *B. bassiana* (JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, and JaTpOi (1) isolates), *Curvularia lunata* (JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr isolates), *Aspergillus flavus* (JgPWSR isolate), *Penicillium citrinum* (JaTpOi(2) isolate), and *Metarhizium anisopliae* (CaTpPGA isolate). The endophytic fungal species of *B. bassiana* (JgSPK isolate) caused up to (22.67%) mortality, *C. lunata* (JaSpkPga(3) isolate) caused (17.33%) mortality, and *M. anisopliae* (CaTpPga isolate) caused (8%) mortality against *S. frugiperda* larvae. First report of *B. bassiana* and *C. lunata* isolated from maize, and *M. anisopliae* isolated from red chili in Indonesia were found as entomopathogenic endophytic fungi against *S. frugiperda* larvae. The endophytic fungi species of *B. bassiana*, *C. lunata*, and *M. anisopliae* had potentials as entomopathogens of *S. frugiperda*.

Abbreviations

ANOVA: Analysis of variance; BLAST: Basic Local Alignment Search Tool; CTAB: Cetyltrimethylammonium bromide; DNA: Deoxyribonucleic acid; EtOH: Ethyl alcohol; HSD: Tukey's honestly significant difference; ITS: Internal transcribed spacer; MEA: The malt extract agar; NaOCl: Sodium hypochlorite; SDA: Sabouraud dextrose agar; TBE: Tris-Boric Acid-EDTA.

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Authors' contributions

SH performed research concept and design, writing the article, and final approval of article. MG performed collection and assembly of data. SS prepared and performed morphological identification and critical revision of the article. RS performed molecular identification and data analysis and interpretation. JMPS performed collection and assembly of data. RPL performed collection and assembly of data. All the authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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.

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Endophytic fungi confirmed as entomopathogens of the new invasive pest, the fall armyworm, *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae), infesting maize in South Sumatra, Indonesia

ArticleCategory : Research

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Abstract [OutputMedium: All]

Background

The fall armyworm, *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae), is a new invasive pest in Indonesia causing financial losses. *S. frugiperda* larvae hide in the leaf midrib all day. To overcome the hidden larvae, pathogenic endophytic fungi are needed. The objective of this research was to molecularly identify the endophytic fungal species from South Sumatra and determine the most pathogenic species against *S. frugiperda* larvae. Endophytic fungal identification was based on morphological and molecular characteristics. The molecular identification was based on gene sequential analysis of Intergenic Transcribed Spacer (ITS) 1 and ITS 4. Bioassay of the endophytic fungal species was treated against the 2nd larval instar of *S. frugiperda.*

Result

The results of molecular identification revealed endophytic fungal species consisted of *Chaetomium* sp. (GaTpeOi, JgTjPr, PiCrPga, and CaCjPga isolates), *Aspergillus niger* (L.) Van Tieghem (PsgTjPr, JgByU, and JaBuBys), *Beauveria bassiana* (Balsamo) Vuillemin (JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, and JaTpOi (1) isolates), *Curvularia lunata* (Wakker) Boed. (JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr isolates), *Aspergillus flavus* Link. (JgPWSR isolate), *Penicillium citrinum* Thom F 1539 (JaTpOi(2) isolate), and *Metarhizium anisopliae* (Metschn.) Sorokin (CaTpPGA isolate). All fungal

isolates were deposited in the GenBank. The endophytic fungal species of *B. bassiana* (JgSPK isolate) caused up to (22.67%) mortality, *C. lunata* (JaSpkPga(3) isolate) caused (17.33%) mortality, and *M. anisopliae* (CaTpPga isolate) caused (8%) mortality of the pest's larvae. First report of *B. bassiana* and *C. lunata* isolated from maize (*Zea mays* L.), while *M. anisopliae* was isolated from red chili (*Capsicum annuum* L.) as entomopathogenic endophytic fungi against *S. frugiperda* larvae in Indonesia.

Conclusion

The endophytic fungi species of *B. bassiana*, *C. lunata*, and *M. anisopliae* had potentials as entomopathogens of *S. frugiperda*.

Keywords: Endophytic fungi, Invasive pest, *Spodoptera frugiperda*, Morphological and molecular identifications, Indonesia

Abbreviations

ANOVA	Analysis of variance
BLAST	Basic Local Alignment Search Tool
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
EtOH	Ethyl alcohol
HSD	Tukey's ~~Honestly Significant Difference~~honestly significant difference
ITS	Internal ~~Transcribed Spacer~~transcribed spacer
MEA	The malt extract agar
NaOCl	Sodium hypochlorite
SDA	Sabouraud ~~Dextrose Agar~~dextrose agar
TBE	Tris-Boric Acid-EDTA

Background

The fall armyworm, *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae), is a new invasive pest, originating from South America (Otim et al. **2018**). Currently, it has spread throughout the world and its first record in Indonesia was in March 2019 in West Sumatra (Sartiami et al. **2020**) and in July 2019 in South Sumatra (Hutasoit et al. **2020**). Now, it has been spread all over Indonesia. The pest attacks maize (*Zea mays* L.) and various other plant species by means of its larval stage, eating leaves, stems, flowers, fruits, growing points, and the whole maize plant until it becomes bare (Montezano et al. **2018**). Larvae are found on the surface of leaves or maize stalks in the morning and then hide in the leaves' midribs (Gustianingtyas et al. **2021**). Such behavior makes it difficult to be controlled.

Laboratory experiments of Herlinda et al. (**2020a**) showed that the entomopathogenic fungus (EPF), *Metarhizium* sp., could kill (78%) of *S. frugiperda* larvae. *Beauveria bassiana* (Balsamo) Vuillemin, *Metarhizium anisopliae* (Metschn.) Sorokin, and *M. rileyi* (Farlow) Samson also killed more than (80%) *S. frugiperda* larvae (Ramanujam et al. **2020**). Their treatments were applied topically (direct contact) with the larvae exposed. In the field, the presence of hiding larvae in the midribs all the day makes the contacted EPF less effective (Gustianingtyas et al. **2021**). To overcome such behavior, EPF ~~that~~ found in plant tissues (endophytic fungi) ~~are~~ is needed (Ramos et al. **2020**). The endophytic fungi systemically colonize plant tissues and associate mutually their host plants (Lira et al. **2020**). The results of previous studies have proven that 8 isolates of endophytic EPF could topically kill *S. frugiperda* larvae (Gustianingtyas et al. **2021**)~~,~~; however, the endophytic fungi found in their studies were only isolated from the roots and their molecular identification had not yet been carried out.

In this study, the fungi isolated from leaves, shoots, and roots of maize plants from South Sumatra (Indonesia) were identified morphologically and molecularly and their pathogenicity to *S. frugiperda* larvae was evaluated.

Methods

Purification, identification, and bioassay of fungi were conducted from January to March 2021. The endophytic fungal species were identified based on the molecular analysis carried out at a laboratory accredited according to the ISO 17025 standard. Bioassay of the fungi was carried out in an incubator at controlled and constant temperature and relative humidity, respectively, 25 °C and 97%.

Exploration, isolation, and purification of fungi

Exploration of endophytic fungi was carried out by collecting leaves, shoots, and roots of maize and other crop plants around it such as bananas (*Musa* spp.), ridged gourd (*Luffa acutangula* (Roxb.) L.), and red chilies (*Capsicum annuum* L.). Selection of individual crop plant samples to obtain the endophytic fungi followed the method of Kasambala et al. (**2018**). Samples of the plant parts were placed into an ice box and then taken to the laboratory. The exploration of endophytic fungi was carried out from the lowlands to the highlands of South Sumatra, Indonesia, namely in Banyuasin District (2.8833°S 104.3831°E), Ogan Ilir District (3.43186°S 104.62727°E), Prabumulih City (3.4328°S 104.2356°E), Pagar Alam City (3°52′43.8″S 103°21′30″E) (Table **1**).

Table 1 Origin of isolates of endophytic fungi from South Sumatra, Indonesia

Location (village, district/city)	Isolate origin	Altitude (m)	Fungal species	Fungal isolate code	GenBank acc. no
Tanjung Pering. Ogan Ilir	Ridged gourd	36.0	*Chaetomium* sp.	GaTpeOi	MZ359734
Tanjung Pering. Ogan Ilir	Bananas	36.0	*Aspergillus niger*	PsgTjPr	MZ242060
Simpang Padang Karet. Pagar Alam	Maize	797.7	*Beauveria bassiana*	JgSPK	MZ356494
Tanjung Pering. Ogan Ilir	Maize	36.0	*Chaetomium* sp.	JgTjPr	MZ359736
Gunung Ibul. Prabumulih	Maize	53.0	*Beauveria bassiana*	JaGiP	MZ356495
Curup Jare. Pagar Alam	Bananas	806.0	*Chaetomium* sp.	PiCrPga	MZ359735
Gunung Ibul. Prabumulih	Maize	53.0	*Curvularia lunata*	JaGiPRB	MZ359815
Tanjung Payang. Pagar Alam	Red chilies	689.6	*Curvularia lunata*	CMTJP	MZ359816
Mulia Sari. Banyuasin	Maize	19.0	*Curvularia lunata*	JaMsBys	MZ359819
Simpang Padang Karet. Pagar Alam	Maize	789.5	*Beauveria bassiana*	JaSpkPGA(2)	MZ356496
Curup Jare. Pagar Alam	Maize	806.7	*Beauveria bassiana*	JgCrJr	MZ356497
Tanjung Pering. Ogan Ilir	Maize	36.0	*Beauveria bassiana*	JaTpOi (1)	MZ356498
Simpang Padang Karet. Pagar Alam	Maize	797.7	*Curvularia lunata*	JaSpkPga(3)	MZ359818
Curup Jare. Pagar Alam	Red	806.0	*Chaetomium* sp.	CaCjPga	MZ359737

Location (village, district/city)	Isolate origin	Altitude (m)	Fungal species	Fungal isolate code	GenBank acc. no.
Banyuurip. Banyuasin	chilies Maize	15.0	*Aspergillus niger*	JgByU	MZ242059
Telang Sari. Banyuasin	Maize	15.0	*Curvularia lunata*	JgTgSr	MZ359817
Banyuurip. Banyuasin	Maize	20.0	*Aspergillus niger*	JaBuBys	MZ242058
Purwosari. Banyuasin	Maize	15.0	*Aspergillus flavus*	JgPWSR	MZ359829
Tanjung Pering. Ogan Ilir	Maize	36.0	*Penicillium citrinum*	JaTpOi(2)	MZ359812
Tanjung Payang. Pagar Alam	Red chilies	689.6	*Metarhizium anisopliae*	CaTpPga	MZ242073

The leaves, shoots, and roots of the sample plants were cleaned and washed aseptically with running tap water. Then, the plant parts were surface sterilized using the method of Elfita et al. (2019) with 70% EtOH (~~Ethyl~~ ethyl alcohol) and 1% NaOCl (~~Sodium~~ sodium hypochlorite) ~~,~~ and then rinsed 3 times. The last rinse water was used to check whether the microorganisms causing surface contamination were still present and carried out by growing them onto ~~Sabouraud Dextrose Agar~~ sabouraud dextrose agar (SDA) (Russo et al. 2020). Obtaining the endophytic fungi isolates started with growing the plant parts that sterilized earlier onto a media. The roots were grown on a specific medium, the malt extract agar (MEA), following the method of Silva et al. (2018). The shoots and leaves of the sample plants were grown on SDA media. The plant parts were grown as many as 5 pieces per Petri dish with a length of 5 mm and a diameter of 1–5 mm (depending on the diameter of the leaves, shoots, and roots). Fungi growing from the leaves, shoots, and roots were purified to be one isolate per sample. The isolates were then observed for their microscopic characteristics and continued with the fungal colonization test into the maize seed tissue.

Assess endophytic fungal colonization

Fungal inoculation on maize seeds was carried out to observe the colonization of fungi into the maize seedling tissue and to further ensure that the isolates found were truly endophytic. The 15 seeds of maize were surface sterilized, using 70% EtOH (~~Ethyl~~ ethyl alcohol) and 1% NaOCl (~~Sodium~~ sodium hypochlorite) (Elfita et al. 2019). The seeds were immersed in 10 ml of fungal suspension, with a concentration of 1×10^6 conidia ml^{-1} for 6 h, while for the control, the sterilized seeds were only immersed in 10 ml of ~~destilled~~ distilled water. Then, the seeds were grown in a sterile glass bottle (250 ml volume) with a sterile filter paper (~~whatman~~ Whatman no. 42) at the bottom, which was moistened with 1 ml ~~destilled~~ distilled water and incubated for 10 days. All the treatments in this experiment were repeated 3 times. After the maize seedlings were 10 days old, the stems and leaves were sliced crosswise and longitudinally with a thickness of 0.02 mm each. The slices were observed with a light microscope at 40 × magnification with 0.05% lactofenol trypan blue dye. Mycelia endophytic fungi found in the maize seedling tissue were documented. The fungal isolates that were proven to be endophytic were used for bioassays. Prior to the bioassay, the isolates were observed for microscopic characteristics for morphological identification, followed by molecular identification.

Morphological and molecular identification of fungal species

Macroscopic and microscopic observation

Morphological observations were carried out based on the macroscopic characteristics of the endophytic fungi that included colony color and shape, shape, and size of conidia and conidiophores, following the method of Herlinda et al. (2020a).

DNA extraction and PCR amplification

The used endophytic fungal DNA extraction method refers to the Swibawa et al. (2020) method. DNA extraction was carried out on fungal conidia. The conidia were harvested from pure cultures of ~~7 days~~ 7-

day-old fungus, which was cultured in sterile Petri dishes with a diameter of 9 cm. As much as 10 ml of sterile water was placed into a Petri dish containing fungal cultures and slowly harvested, using a ~~drigalski~~Drigalski. The obtained conidia suspension was transferred to a centrifuge tube (volume 30 ml) and centrifuged using CF15RXII (Hitachi, Japan) for 10 min at a speed of 14,000 rpm. After being centrifuged, 1 ml of 70% ethanol was added to the centrifuge tube and then centrifuged again for 10 min. After that, the supernatant was removed and 1 ml of extraction buffer was added with a composition of 0.5 ml Tris HCl, 1 mL SDS 1% + 2.8 mL NaCl, 0.2 ml Mercaptho Ethanol, 2 ml EDTA, 3.5 ml sterile water, and then homogenized. The suspension was transferred to a sterile mortal and incubated at -40 °C for 24 h. After that, the frozen suspension was ground until it was smooth and melted. A total of 500 µl of suspension was then transferred into a 1.5 ml tube. A total of 400 µl of 2% cetyltrimethylammonium bromide (CTAB) was then added to the tube, homogenized, and then incubated at 65 °C for 1 h using a water bath (Brookfield TC 550 MX-230, USA). After the incubation, 500 µl of Phenol Chloroform Isoamyl alcohol (PCI) (25:24:1) was added, homogenized, and centrifuged (Microspin12; Biosan, Latvia) at 14,000 rpm for 10 min. A total of 600 µl of supernatant was taken and transferred to a new 1.5 ml tube. A total of 600 µl Chloroform Isoamyl Alcohol (CI) (24:1) was added to the tube, homogenized, and centrifuged (Microspin12; Biosan, Latvia) at 14,000 rpm for 10 min. A total of 400 µl of supernatant was then transferred to a new 1.5 ml tube, and 400 µl of cold isopropanol was added, homogenized, and incubated at − 40 °C for 20 min. After the incubation, it was centrifuged (Microspin12; Biosan, Latvia) at 14,000 rpm for 15 min. The supernatant was then discarded. After the supernatant was removed, 500 µl of 70% cold ethanol was added and centrifuged (Microspin12; Biosan, Latvia) at 14,000 rpm for 5 min. The supernatant was then discarded, and the pellets obtained were dried at room temperature for 24 h. After drying, the tube was then added as much as 50 µl 1 × Tris–HCL EDTA (TE) pH 8.0 (1st Base Malaysia).

PCR amplification was carried out using the Sensoquest Thermal Cycler (Germany) PCR machine on the ~~Internal Transcribed Spacer~~ internal transcribed spacer (ITS) using ITS1 and ITS4 primers (White et al. **1990**). The PCR was carried out with a total volume of 25 µl consisting of a mixture of Master Mix (Red Mix) (bioline) as much as 12.5 µl, 10 µM of primer ITS 1 (5′TCC GTA GGT GAA CCT TGC GG 3′) and ITS 4 (5′TCC TCC GCT TAT TGA TAT GC 3′) 1 µL each, 1 L of template DNA , and 9.5 µl of sterile water. The DNA amplification stage consisted of 1 initiation cycle at 95 °C for 5 min, followed by 30 cycles consisting of denaturation at 95 °C for 1 min, primer attachment at 52 °C for 1 min, primer extension at 72 °C for 1 min, and 1 elongation cycle at 72 °C for 5 min. The PCR results were then electrophoresed, using 0.5% agarose in 20 ml of 1 × Tris-Boric Acid-EDTA (TBE) buffer (1st Base Malaysia), and added 1 µl of ~~Ethidium Bromide~~ ethidium bromide (EtBr 10 mg/ml). The electrophoresis was carried out in 1 × TBE buffer solution at 50 V for 70 min. Electrophoresis results were visualized using a DigiDoc UV transilluminator (UVP, USA).

Sequencing and analysis of the results

The PCR results were then sent to 1st Base Malaysia for the sequencing process. The results of the sequencing were analyzed, using Bio Edit ver. 7.2.6 for windows. Then, the results were submitted to the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to find out the genus or species that had the greatest homology or similarity and molecularly. The phylogeny tree was created using the Mega 7 for Windows program (Kumar et al. **2016**), using the UPGMA (jukes and cantor model) method. The ITS region sequences for several strains used as a reference in this study were obtained from NCBI (https://www.ncbi.nlm.nih.gov/).

Calculation of conidial density and viability

All the isolates of endophytic fungi were counted for their conidial density and viability. The conidial density of the 7-old ~~days~~ -day fungi was calculated according to the method of Sumikarsih et al. (**2019**), using a ~~haemocytometer~~ hemocytometer and observed with a light microscope at 40 × magnification. The viability was observed by growing 1 ml of conidia fungal suspension (1×10^6 conidia ml^{-1}) onto 2% ~~agar-water~~ agar–water medium, and then, the culture was incubated for 1 × 24 and 2 × 24 h under a light microscope at 40 × magnification.

Mass-rearing of S. frugiperda

The mass-rearing of *S. frugiperda* was carried out, following the method of Herlinda et al. (**2020a**). The larvae were collected from the maize plants grown around the campus (3°13′25″S 104°39′51″E) ~~,~~ and were reared in the laboratory for more than 5 generations to obtain the larvae with relatively homogeneous genetics and to eliminate the effects of exposure to synthetic insecticides from the fields. In the laboratory, the larvae of *S. frugiperda* were kept individually in a porous plastic cup (Ø 6.5 cm, height 4.6 cm) because the larvae were cannibals. The larvae were fed daily by maize leaves (measuring 2 cm × 5 cm). Prepupae were transferred to a plastic container (Ø 15 cm, height 25 cm) on which its bottom had a sterile soil (5 cm thick). The plastic container containing the prepupae was placed in a wire mesh cage (30 × 30 × 30 cm^3) and inside this cage placed also fresh maize leaves for the adults to lay eggs, which were changed daily. The one-day-old 2nd instar larvae were used for the bioassay.

Bioassay of endophytic fungi against larvae of S. frugiperda

The bioassay of endophytic fungi ~~,~~ followed the method of Zea et al. (**2019**). The young maize (seedling) already inoculated with the endophytic fungi and not-inoculated (control) aged 10 days old ~~were~~ was given to the 2nd instar larvae of *S. frugiperda*. Each larva was given one maize seedling whose cotyledons were already removed. The larvae were allowed to feed on the leaves and stems of the young maize for 6 h. in a sterile room (a laminar air flow cabinet), and each replication was tested for 25 individuals of *S. frugiperda* larvae. Then, the larvae were transferred to a porous plastic cup (Ø 6.5 cm, height 4.6 cm) and fed on healthy non-inoculated leaves measuring 2 cm × 5 cm per day per larvae. The experiment was designed, using completely randomized designs with treatments of 20 isolates, 3 replications per treatment. The dead larvae were recorded daily for 12 days, following the method of Herlinda et al. (**2020b**). The dead larvae were grown in the ~~agar-water~~ agar–water medium to confirm whether the infection was caused by the endophytic fungi or not. The number of dead larvae was used to calculate the mortality.

Data analysis

The differences in the mortality data of *S. frugiperda* larvae were analyzed using analysis of variance (ANOVA). Tukey's ~~Honestly Significant Difference~~ honestly significant difference (HSD) test (Tukey's test) was employed to test for the significant differences among the treatments (isolates) at $P = 0.05$. All data were calculated using software of SAS University Edition 2.7 9.4 M5.

Results

Colonization of endophytic fungi on maize tissues

All fungal isolates, isolated from the leaves, shoots, and roots of the maize, bananas, ridged gourd, and red chilies were found to be 124 isolates. However, after assessing for the fungal colonization in young maize (seedlings) tissue, only 20 isolates of the fungi were confirmed as endophytic fungi (Fig. **1**). Evidence of fungal colonization that entered the maize seedlings tissue was that mycelia fungi were seen entering and growing spread throughout the stem and leaf tissue. Mycelia endophytic fungi were able to invade the stem and leaf of the maize tissue, the mycelia appeared to be branched. The size of mycelia varied daily from 13.51 to 34.25 µm. In the maize tissue, which their seeds were not inoculated by the endophytic fungi (control), the cells in the maize tissue were clean and clear without fungal colonization.

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Fig. 1 Young maize tissues colonized by endophytic fungal species: Control (**A**); *Chaetomium* sp. isolates of GaTpeOi (**B**), JgTjPr (**E**), PiCrPga (**G**), and CaCjPga (**O**); *Aspergillus niger* isolates of PsgTjPr (**C**), JgByU (**P**), and JaBuBys (**R**); *Beauveria bassiana* isolates of JgSPK (**D**), JaGiP (**F**) JaSpkPGA(2) (**K**), JgCrJr (**L**), and JaTpOi(1) (**M**); *Curvularia lunata* isolates of JaGiPRB (**H**), ~~CMTjP~~ CMTJP (**I**), JaMsBys

(**J**), JaSpkPga(3) (**N**), and JgTgSr (**Q**); *Aspergillus flavus* isolate of JgPWSR (**S**); *Penicillium citrinum* isolate of JaTpOi(2) (**T**); and *Metarhizium anisopliae* isolate of CaTpPga (**U**)

Identification results of the endophytic fungal isolates

The colony morphology of 20 isolates of the endophytic fungi showed different colors (Fig. 2) and likewise the shapes of hyphae and conidia of each isolate showed their own characteristics (Fig. 3). The colony isolates of GaTpeOi, JgTjPr, PiCrPga, and CaCjPga grown on SDA media showed similarities; namely, ~~namely~~ the colony was pale gray, hyphae and mycelia were septate, and the conidia were in the shape of letter D (asymmetric/elliptical) and non-septation. Based on the phylogenetic tree, these 4 isolates were placed within the group of *Chaetomium* sp. (Fig. 4). The isolates were deposited in the GenBank with the accession number MZ359734 (GaTpeOi), MZ359736 (JgTjPr), MZ359735 (PiCrPga), MZ359737 (CaCjPga) (Table 1).

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Fig. 2 Colony morphology of endophytic fungal species: *Chaetomium* sp. isolates of GaTpeOi (**A**), JgTjPr (**D**), PiCrPga (**F**), and CaCjPga (**M**); *Aspergillus niger* isolates of PsgTjPr (**B**), JgByU (**O**), and JaBuBys (**Q**); *Beauveria bassiana* isolates of JgSPK (**C**), JaGiP (**D**), JaSpkPGA(2) (**J**), JgCrJr (**K**), and JaTpOi(1) (**L**); *Curvularia lunata* isolates of JaGiPRB (**G**), ~~CMTjP~~ CMTJP (**H**), JaMsBys (**I**), JaSpkPga(3) (**M**), and JgTgSr (**P**); *Aspergillus flavus* isolate of JgPWSR (**R**); *Penicillium citrinum* isolate of JaTpOi(2) (**S**); and *Metarhizium anisopliae* isolate of CaTpPga (**T**)

41938_2021_470_Fig3_print.png
Fig. 3 Conidial and hyphal morphology of endophytic fungal species: *Chaetomium* sp. isolates of GaTpeOi (**A**), JgTjPr (**D**), PiCrPga (**F**), and CaCjPga (**M**); *Aspergillus niger* isolates of PsgTjPr (**B**), JgByU (**O**), and JaBuBys (**Q**); *Beauveria bassiana* isolates of JgSPK (**C**), JaGiP (**D**), JaSpkPGA(2) (**J**), JgCrJr (**K**), and JaTpOi(1) (**L**); *Curvularia lunata* isolates of JaGiPRB (**G**), ~~CMTjP~~ CMTJP (**H**), JaMsBys (**I**), JaSpkPga(3) (**M**), and JgTgSr (**P**); *Aspergillus flavus* isolate of JgPWSR (**R**); *Penicillium citrinum* isolate of JaTpOi(2) (**S**); and *Metarhizium anisopliae* isolate of CaTpPga (**T**)

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Fig. 4 Phylogenetic tree based on the sequencing of ~~Internal Transcribed Spacer~~ internal transcribed spacer (ITS) region using UPGMA method (jukes and cantor model) performed with Mega 7 program for windows (Kumar et al., 2016). This research found 20 isolates of entomopathogenic fungi: 4 isolates placed within the group of *Chaetomium* sp., 3 isolates placed within the group of *Aspergillus niger*, 5 isolates placed within the group of *Beauveria bassiana*, 5 isolates placed within the group of *Curvularia lunata*, 1 isolate placed within the group of *Aspergillus flavus*, 1 isolate placed within the group of *Penicillium citrinum*, and 1 isolate placed within the group of *Metarhizium anisopliae.* = fungal isolates obtained from this research

The colony isolates of PsgTjPr, JgByU, and JaBuBys showed similarities, their colonies were black, the hyphae and mycelia were also black, and the conidia were non-septate, globose in shape. The 3 isolates were placed within the group of *Aspergillus niger* (L.) Van Tieghem (Fig. 4). The isolates were deposited in the GenBank with the accession number MZ242060 (PsgTjPr), MZ242059 (~~JgByu~~JgByU), and MZ242058 (~~JaBUBys~~JaBuBys) (Table 1).

The isolates of JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, and JaTpOi (1) had a white colony, white hyphae and mycelia, and the globose conidia, which were non-septate conidia. The isolates were placed within the group of *B. bassiana* (Fig. 4). The isolates were deposited in the GenBank with the accession number MZ356494 (JgSPK), MZ356495 (JaGiP), MZ356496 (JaSpkPGA(2)), MZ356497 (JgCrJr), and MZ356498 (JaTpOi1) (Table 1).

The isolates of JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr had a black colony, black hyphae and mycelia, two-septate boomerang-shaped conidia. All the isolates were placed within the group of

Curvularia lunata (Wakker) Boed. (Fig. 4). The isolates were deposited in the GenBank with the accession number MZ359815 (JaGiPRB), MZ359816 (CMTJP), MZ359819 (JaMsBys), MZ359818 (JaSpkPga(3)), and MZ359817 (JgTgSr) (Table 1).

The isolate of JgPWSR had a green colony, green hyphae and mycelia, non-septate globose conidia. The ~~JgPwSr~~ JgPWSR isolate was placed within the group of *Aspergillus flavus* Link. (Fig. 4). The isolate was deposited in the GenBank with the accession number MZ359829 (JgPWSR) (Table 1).

The color of the colony JaTpOi(2) isolate was initially white~~,~~; as the fungus got older, it turned bluish green with white margin, rounded conidia. The isolate was placed within the group of *Penicillium citrinum* Thom F 1539 (Fig. 4). The isolate was deposited in the GenBank with the accession number MZ359812 (JaTpOi (2)) (Table 1).

The isolate of CaTpPGA had a white colony, and as the fungus got older, it turned greenish white to dark green, green hyphae and mycelia, and the conidia are clear, cylindrical, and non-septation. The isolate was placed within the group of *M. anisopliae* (Fig. 4). The isolate was deposited in the GenBank with the accession number MZ242073 (CaTpPGA) (Table 1).

The result of BLAST search revealed that the isolates of GaTpeOi, JgTjPr, PiCrPga, and CaCjPga had a similarity (100%) with the *Chaetomium* sp. isolate A13 (Acc. No. MH34803.1), isolate MJ51 (Acc No. KM203618.1), and isolate CP-2009 (Acc. No. AB50680.1). The isolates of PsgTjPr, JgByU, and JaBuBys showed 100% of similarity with *A. niger* NBRC: 105649 (Acc. No LC573609.1), strain WM04.470 (Acc No. AJ853742.1), and isolate IFM61597 (Acc. No. LC602036.1). The isolates of JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, and JaTpOi (1) showed (100%) similarity to the *B. bassiana* isolate GZMS-28 (Acc. No. KT715480.1), strain TF6-1B (Acc No. JX122736.1), and isolate BSwTd4 (Acc. No. MT4487322.1). The isolates of JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr showed 99.81% of similarity to *C. lunata* strain D25A (Acc. No MH010917.1), strain AN 2 (Acc No. KY859790.1), and isolate 1z -7 (Ac. No. MN213745.1). The isolate of JgPWSR showed 100% of similarity to *A. flavus* strain GFRS9 (Acc. No MT447477.1), strain GFRS06 (Acc. No MT447474.1), and strain aT3 (Acc. No KU561920.1). The isolate of JaTpOi(2) showed 100% of similarity to *P. citrinum* strain yx-001 (Acc. No. MN826202.1), strain AKF2-KU (Acc No. MN879404.1), and strain J (Acc. No. MK791668.1). The isolate of CaTpPGA had a similarity (99.29%) to *M. anisopliae* isolate MSwTp3 (Acc. No MT448733.1), strain STBMa-001 (Acc No. KF766520.1), and isolate C1 (Acc. No. KX809520.1).

There were 7 species from the 20 isolates of the endophytic fungi found in this study. The 4 isolates (GaTpeOi, JgTjPr, PiCrPga, and CaCjPga) were in the group of *Chaetomium* sp., the three isolates (PsgTjPr, JgByU, and JaBuBys) were in the group of *Aspergillus niger*, the 5 isolates (JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, and JaTpOi (1)) were placed within the group of *B. bassiana*, the 5 isolates (JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr) were in the group of *C. lunata*, an isolate (JgPWSR) was in the group of *A. flavus*, an isolate (JaTpOi(2)) was in the group of *P. citrinum*, and an isolate (CaTpPGA) was in the group of *M. anisopliae*.

Pathogenicity of the endophytic fungi against S. frugiperda larvae

Out of the 7 species of the 20 isolates of the endophytic fungi found in this study, there were 6 isolates that were the most pathogenic against *S. frugiperda* larvae (Table 2). The 4 isolates (JgSPK, JaGiP, JgCrJr, and JaTpOi1) resulted to mortality rates of 22.67, 21.3%, 21.33, and 17.33% consecutively ~~was~~ were *B. bassiana*. In addition, the ~~isolate~~ JaSpkPga(3) isolate of *C. lunata* caused a fairly high mortality (18.67%) and was non-significantly different than the mortality caused by the isolates of JgSPK, JaGiP, JgCrJr, JaTpOi1. The CaTpPga isolate of *M. anisopliae* caused (8%) larval mortality and was non-significantly different than the mortality caused by JaSpkPga(3) isolate. The *B. bassiana* and *C. lunata* isolates were isolated from maize, while the *M. anisopliae* isolate was isolated from red chili. The JaSpkPGA(2) isolate of *B. bassiana* resulting a mortality rate of only (2.67%).Query ID="Q1" Text="Please check the clarity of the sentence ‘The JaSpkPGA(2) isolate of...’." Likewise, *P. citrinum*, *Chaetomium* sp.,

A. niger, and *A. flavus* caused the lowest larval mortality of less than (8%).

Table 2 Mean mortality of *Spodoptera frugiperda* larvae treated with endophytic fungi (1×10^6 conidia ml^{-1}), and conidial density and viability of endophytic fungi

Fungal isolate code	Fungal species	Mortality (%)	Conidial viability (%)for 1 × 24 h	Conidial viability (%) for 2 × 24 h
Control	–	0.00a	–	–
GaTpeOi	*Chaetomium* sp.	4.00bcd	45.17bcd	47.00a
PsgTjPr	*Aspergillus niger*	2.67abc	38.20ab	51.63abcd
JgSPK	*Beauveria bassiana*	22.67f	47.59efgh	55.23cde
JgTjPr	*Chaetomium* sp.	2.67abcd	35.74a	55.76de
JaGiP	*Beauveria bassiana*	21.33f	42.86abc	52.91abcd
PiCrPga	*Chaetomium* sp.	5.33cd	44.59cde	47.99abc
JaGiPRB	*Curvularia lunata*	4.00bcd	45.18bcd	52.75abcd
CMTJP	*Curvularia lunata*	2.67abcd	45.25efg	54.94bcde
JaMsBys	*Curvularia lunata*	6.67d	49.98gh	58.60de
JaSpkPGA(2)	*Beauveria bassiana*	2.67abcd	47.20cde	48.15abc
JgCrJr	*Beauveria bassiana*	21.33f	46.50cd	52.63abcd
JaTpOi (1)	*Beauveria bassiana*	18.67f	54.02efg	56.55de
JaSpkPga(3)	*Curvularia lunata*	17.33ef	50.85def	56.93de
CaCjPga	*Chaetomium* sp.	2.67abcd	37.24a	47.77ab
JgByU	*Aspergillus niger*	1.33ab	58.84gh	69.57g
JgTgSr	*Curvularia lunata*	4.00bcd	55.33efgh	61.24ef
JaBuBys	*Aspergillus niger*	2.67abcd	58.45gh	76.50h
JgPWSR	*Aspergillus flavus*	4.00bcd	62.54h	68.39g
JaTpOi(2)	*Penicillium citrinum*	6.67cd	57.44fgh	65.98fg
CaTpPga	*Metarhizium anisopliae*	8.00de	50.76def	68.75f
F value	7.27*	9.05*	15.67*	
P value	0.00	0.00	0.00	
HSD value	8.8	4.27	4.07	

* = significantly different; values within a column followed by the same letters were not significantly different at $P < 0.05$ according to Tukey's HSD test

The larvae of *S. frugiperda* that died because of feeding on leaves colonized by the endophytic fungi had the characteristics, *i.e.*, dull and dark integument, shriveled body, dry, and odorless. The feces excreted by sick larvae were wetter than those of untreated larvae (control). The larvae that died as a result of

treatment with the endophytic fungi when grown in SDA media ~~,~~ and their cadavers were not overgrown with mycelia fungi.

Discussion

The isolates of the endophytic fungi found in this study had an rDNA sequence similarity value of more than 99% to the reference species (BLAST), meaning that the isolates had a high phylogenetic relationship and were in the same species. Bich et al. (2021) stated that ribosomal DNA sequences were used to determine the phylogenetic relationships of organisms to taxa species. Based on the similarity value, it is stated that if the similarity value is 100%, it means that the isolates are the same strain, whereas if the similarity value of 99% indicates that the isolates are the same species, and if the similarity value of the isolates is 89–99%, that means they are the same genus (Henry et al. 2000). An organism is declared the same species when the difference in DNA sequences is between 0.~~2–1%~~2 and 1% (Shenoy et al. 2007).

The results of identification based on the morphological characters of several species of fungi showed the similarities~~,~~; for example, the morphology of the colony and the shape of the conidia isolates of *B. bassiana* were similar to that of *P. citrinum*, but after the molecular identification, they showed them different species. According to Minarni et al. (2021), proper species identification is very important in determining a species as an entomopathogen.

The fungi in this study proved to be endophytic based on the characteristics of maize stalks and leaves colonized by the fungal mycelia. According to Barra-Bucarei et al. (2020), endophytic fungus (*B. bassiana*) had a systemic mode of action ability to colonize their host plant (tomato). The endophytic fungi can still be found on roots, stems, and leaves of tomato up to 30 days after inoculation (Carolina et al. 2020). Endophytic *B. bassiana* colonized 100% of leaves, 80% of stems, and 60% of roots of maize 7 days after foliar spray and caused significant reductions in *S. frugiperda* growth and reproduction (Russo et al. 2020). The research results of Shikano (2018) showed the endophytic fungi were able to live in the tissue for several months depending on the age of the host plant.

Out of the 7 species of 20 isolates of the endophytic fungi found in this study, there were only 6 pathogenic isolates against the larvae of *S. frugiperda.* The isolates JgSPK, JaGiP, JgCrJr, JaTpOi1 belong to *B. bassiana*, and the JaSpkPga(3) and CaTpPga isolates belong to *C. lunata* and *M. anisopliae*, respectively. First report of *B. bassiana* and *C. lunata* isolated from maize, and *M. anisopliae* isolated from red chili in Indonesia were found as entomopathogenic endophytic fungi against *S. frugiperda*. *C. lunata* infected and killed grain insect pests, such as *Tribolium castaneum* (Herbst.) (Coleoptera: Tenebrionidae), *Trogoderma granarium* (Everts) (Coleoptera: Dermestidae), *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae), and *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Cucujidae) (Wakil et al. 2014). The endophytic *M. anisopliae* effectively killed the larvae of *S. frugiperda* (Ramos et al. 2020).

The endophytic fungi species of *B. bassiana* (JgSPK, JaGiP, JgCrJr, JaTpOi1 isolates), *C. lunata* (JaSpkPga(3) isolate), and *M. anisopliae* (CaTpPga isolate) had potential as entomopathogens of *S. frugiperda* larvae. *S. frugiperda* larvae that died from consuming plants colonized by the endophytic fungi showed that the fungi could be applied through seed treatment, especially for maize seeds. The seed treatment causes the endophytic fungi to colonize plants (Gustianingtyas et al. 2021), and if *S. frugiperda* larvae eat them, it can cause them die (Ramos et al. 2020).

The *B. bassiana* of JaSpkPGA(2) isolate and all isolates of *P. citrinum, Chaetomium* sp., *A. niger*, and *A. flavus* showed no potential as entomopathogens of *S. frugiperda* with their resulting mortality rates of less than 8%. In contrast to the research, results of Gustianingtyas et al. (2021) reported that the endophytic fungi species (*Aspergillus* sp. and *Chaetomium* sp.) applied topically (contact) caused mortality rates of *S. frugiperda* larvae as many as 18.67 and 14.67%, respectively, and the mortality rate caused by *Beauveria* sp. reached 29%. This difference in the rates was due to different ways of application of the fungi~~,~~; in this study, the larvae of *S. frugiperda* consumed the plants that had been colonized by the endophytic fungi

which were not applied topically.

The larvae of *S. frugiperda* that were sick and died by feeding on the leaves colonized by the endophytic fungus had different characteristics from the larvae that were sick and died due to the topical suspension of the endophytic fungus. The sick larvae of *S. frugiperda* in this study had wetter feces, and the cadavers were not overgrown and covered by the fungal mycelia, while the sick larvae caused by contact with suspension of the endophytic fungi according to Gustianingtyas et al. (**2021**) had dry feces and the cadavers were covered with the fungal mycelia. The dead larvae treated by the endophytic fungi were not overgrown by mycelia fungi because the cadavers were too dry. According to El-Ghany (**2015**), the success of a fungus in germinating was affected by several external factors, such as humidity (more than 98%). So, if the cadavers were too dry, the fungal conidia could not germinate on the cadavers.

Conclusions

Molecular identifications recorded 7 species of the endophytic fungi, namely *Chaetomium* sp. (GaTpeOi, JgTjPr, PiCrPga, and CaCjPga isolates), *Aspergillus niger* (PsgTjPr, JgByU, and JaBuBys), *B. bassiana* (JgSPK, JaGiP, JaSpkPGA(2), JgCrJr, and JaTpOi (1) isolates), *Curvularia lunata* (JaGiPRB, CMTJP, JaMsBys, JaSpkPga(3), and JgTgSr isolates), *Aspergillus flavus* (JgPWSR isolate), *Penicillium citrinum* (JaTpOi(2) isolate), and *Metarhizium anisopliae* (CaTpPGA isolate). The endophytic fungal species of *B. bassiana* (JgSPK isolate) caused up to (22.67%) mortality, *C. lunata* (JaSpkPga(3) isolate) caused (17.33%) mortality, and *M. anisopliae* (CaTpPga isolate) caused (8%) mortality against *S. frugiperda* larvae. First report of *B. bassiana* and *C. lunata* isolated from maize, and *M. anisopliae* isolated from red chili in Indonesia were found as entomopathogenic endophytic fungi against *S. frugiperda* larvae. The endophytic fungi species of *B. bassiana*, *C. lunata*, and *M. anisopliae* had potentials as entomopathogens of *S. frugiperda.*

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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.

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