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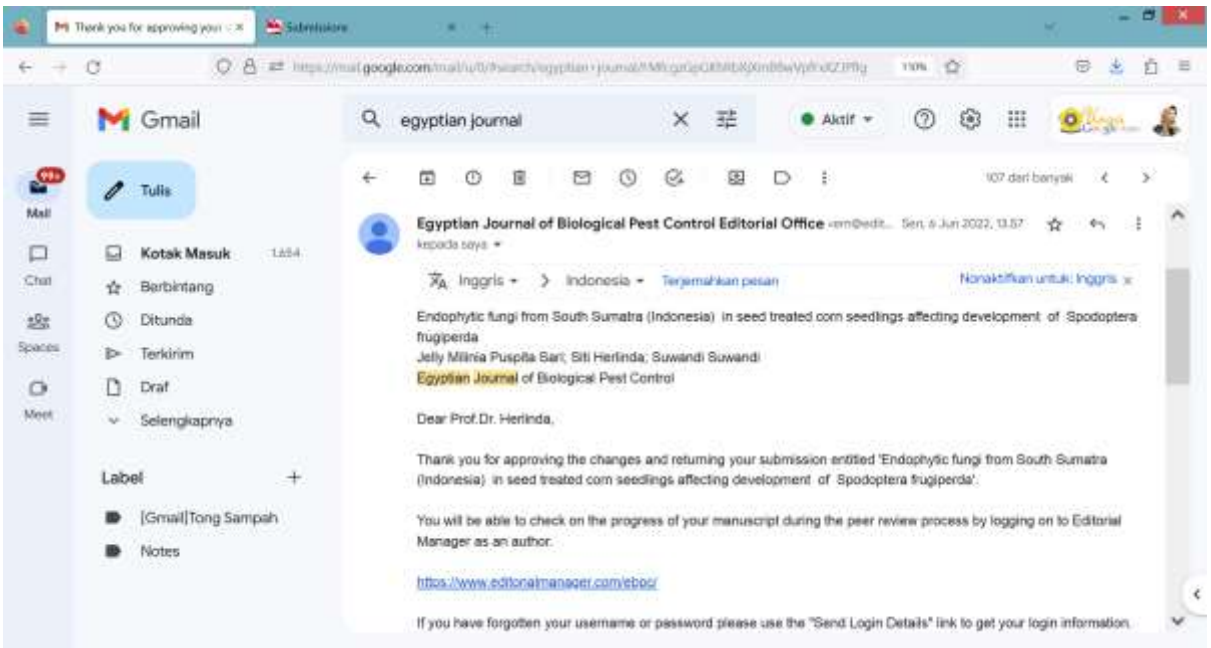

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Endophytic fungi from South Sumatra (Indonesia) in seed treated corn seedlings affecting development of Spodoptera frugiperda

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Abstract:	Background: Topical application of the entomopathogenic fungi against the Spodoptera frugiperda larvae is less effective due to larvae hiding in the corn midribs in the field. To control the larvae, the fungi colonize in plant tissues or endophytic fungi are needed. There is no information on the pathogenicity of the endophytic fungi from Indonesia on the development of S. frugiperda. The endophytic fungi isolated from infected-host cadavers from South Sumatra (Indonesia) were identified morphologically and molecularly and the effect of seed treated corn seedlings with the fungi on S. frugiperda development was evaluated. The fungal identification was based on morphological and molecular characteristics. Bioassay of the endophytic fungal species in seed treated young maize was performed against the neonate larvae (hatching within 24 hrs) of first instar and their development were observed. Results: The results of molecular identification showed that the fungal species found were Beauveria bassiana of five fungal isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) and Metarhizium anisopliae of an isolate (WTTJC260521B). The lifespan of S. frugiperda fed on leaves of fungal colonized maize was significantly longer compared to those fed on leaves of non-colonized maize. The fungal colonized young maize significantly increased mortality of all instar larvae compared to non-colonized one. The last instar mortality treated with B. bassiana (JGTP240521A isolates) (51.33%) was the highest among other treatments and did not significantly differ from each of the B. bassiana of WTTJC260521A and WTTJC290521A isolates (45.33% and 44.67%, respectively). Feeding on leaves of fungal colonized maize significantly decreased the percentage of the last instar becoming pupal stage, the adult emergence, the eggs laid, and the percentage of hatched eggs. This is first report that the B. bassiana and M. anisopliae from South Sumatra (Indonesia) in seed treated corn seedlings have negative effects on development of S. frugiperda. Conclusions: Finally, these results highlight the promising potential of endophytic entomopathogenic fungi to protect corn plants against S. frugiperda. Keywords: Beauveria bassiana, endophyte, entomopathogen, fall armyworm, Metarhizium anisopliae
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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

Chief Editor
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Dear Editor,
We wish to submit an article entitled, "Endophytic fungi from South Sumatra (Indonesia) in seed treated corn seedlings affecting development of *Spodoptera frugiperda*" for intended publication in Egyptian Journal of Biological Pest Control for your kind consideration. Our manuscript deals with biological pest control (non-chemical control) in line with aims and scope of the Egyptian Journal of Biological Pest Control.

We declare that this manuscript has not been published and is not under consideration for publication to any other journal or any other type of publication (including web hosting) either by me or any of my co-authors. All authors have been read and agreed to the Ethical Guidelines. I also certify that all the authors have approved the paper for release and are in agreement with its content. All authors have agreed to the manuscript submission to the Egyptian Journal of Biological Pest Control.

This study highlights a finding that first report of *Beauveria bassiana* and *Metarhizium anisopliae* isolated from *Spodoptera frugiperda* and other Lepidoptera from South Sumatra Indonesia in seed treated corn seedlings have negative effects on development of *S. frugiperda*.

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

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Thank you for your consideration of this manuscript.

Sincerely,

Prof. Dr. Siti Herlinda

Endophytic fungi from South Sumatra (Indonesia) in seed treated corn seedlings affecting development of *Spodoptera frugiperda*

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

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Endophytic fungi from South Sumatra (Indonesia) in seed treated corn seedlings affecting development of *Spodoptera frugiperda*

Abstract

Background: Topical application of the entomopathogenic fungi against the *Spodoptera frugiperda* larvae is less effective due to larvae hiding in the corn midribs in the field. To control the larvae, the fungi colonize in plant tissues or endophytic fungi are needed. There is no information on the pathogenicity of the endophytic fungi from Indonesia on the development of *S. frugiperda*. The endophytic fungi isolated from infected-host cadavers from South Sumatra (Indonesia) were identified morphologically and molecularly and the effect of seed treated corn seedlings with the fungi on *S. frugiperda* development was evaluated. The fungal identification was based on morphological and molecular characteristics. Bioassay of the endophytic fungal species in seed treated young maize was performed against the neonate larvae (hatching within 24 hrs) of first instar and their development were observed.

Results: The results of molecular identification showed that the fungal species found were *Beauveria bassiana* of five fungal isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) and *Metarhizium anisopliae* of an isolate (WTTJC260521B). The lifespan of *S. frugiperda* fed on leaves of fungal colonized maize was significantly longer compared to those fed on leaves of non-colonized maize. The fungal colonized young maize significantly increased mortality of all instar larvae compared to non-colonized one. The last instar mortality treated with *B. bassiana* (JGTP240521A isolates) (51.33%) was the highest among other treatments and did not significantly differ from each of the *B. bassiana* of WTTJC260521A and WTTJC290521A isolates (45.33% and 44.67%, respectively). Feeding on leaves of fungal colonized maize significantly decreased the percentage of the last instar becoming pupal stage, the adult emergence, the eggs laid, and the percentage of hatched eggs. This is first report that the *B. bassiana* and *M. anisopliae* from South Sumatra (Indonesia) in seed treated corn seedlings have negative effects on development of *S. frugiperda.*

Conclusions: Finally, these results highlight the promising potential of endophytic entomopathogenic fungi to protect corn plants against *S. frugiperda.*

Keywords: *Beauveria bassiana*, endophyte, entomopathogen, fall armyworm, *Metarhizium anisopliae*

Background

Spodoptera frugiperda J.E. Smith (Lepidoptera: Noctuidae) or fall armyworm (FAW) is the most important noctuid pests of corn in the world. The FAW is a migratory and polyphagous pest and can attack 353 host plant species from 76 plant families (Montezano *et al.* 2018). The percent of infested maize fields by FAW in East Africa range from 80% to 100% in Ethiopia and 82.2% to 100% in Kenya (Sisay *et al.* 2019) and cause losses of about a third of the annual maize production or about 1 million tonnes in Kenya (De Groote *et al.* 2020), and 18 million tons/year in 12 African countries and the losses reach US $ 13 millions (Harrison *et al.* 2019). The pest is native to the neotropics of the Americas and has spread throughout the world (Otim *et al.* 2018). More recently, the FAW become a new invasive pest in many parts of Africa (Goergen *et al.* 2016; Niassy *et al.* 2021) and Asia (Lamsal *et al.* 2020), including Indonesia (Herlinda *et al.* 2021). This pest commonly controls using synthetic insecticides (Kumela *et al.* 2018), however the resistances to many insecticides have occured in *S. frugiperda*, such as pyrethroid, spinosad, and organophosphorus insecticides (Zhang *et al.* 2021). In addition, the insecticide application negatively affects human health and the environment (Harrison *et al.*

2019). An alternative more sustainable and eco-friendly control methods against *S. frugiperda* is urgently needed.

The preferred control option for FAW is biological control based on utilizing entomopathogenic fungi (Mantzoukas and Eliopoulos 2020). Laboratory experiment showed that topical application (direct contact) of the entomopathogenic fungus, *Beauveria bassiana* (Balsamo) Vuillemin killed more than 80% *S. frugiperda* larvae (Ramanujam *et al.* 2020). *Metarhizium* sp. could kill 78% of *S. frugiperda* larvae (Herlinda *et al.* 2020). However, in the field, larvae were occured on the surface of leaves or maize stalks only in the morning but at daylight up to night they hide in the corn midribs (Herlinda *et al.* 2021). So, topical application of the fungus against the *S. frugiperda* larvae is less effective (Gustianingtyas *et al.* 2021). To control such hiding larvae in the field, the fungi colonize in plant tissues or endophytic fungi are needed (Ramos *et al.* 2020). The endophytic fungi associate mutually their host plants (Lira *et al.* 2020) and can stimulate the plant growth but suppress the insect pest growth (Russo *et al.* 2020).

Previous studies have demonstrated that the endophytic fungal *B. bassiana* from corn root from Indonesia applied topically caused the FAW larval mortality of 29.33% (Gustianingtyas *et al.* 2021) and the endophytic fungal *B. bassiana* used as seed treatment caused up to 22.67% of the FAW larval mortality (Herlinda *et al.* 2021). An experiment has previously also reported that endophytic *B. bassiana* sprayed on leaves has high ability to colonize corn plants and the fungus caused significant reductions in the growth and develoment of *S. frugiperda* (Russo *et al.* 2020). There is no information on the pathogenicity of the endophytic fungi from Indonesia on the development of *S. frugiperda*. In addition, the potential of the fungi isolated from the infected-host cadaver as endophytic entomopathogens needs to be investigated. In this study, the fungi isolated from infected-host cadavers from South Sumatra (Indonesia) were identified morphologically and molecularly and the effect of seed treated corn seedlings with the fungi on *S. frugiperda* development was evaluated.

Methods

Exploration of the fungi was performed by collecting infected-host insect cadavers from crops in South Sumatra, Indonesia from may until June 2021. Purification and isolation of the fungi were carried out from June to July 2021. The morphological identification was carried out in the Laboratory of Entomology, Faculty of Agriculture, Universitas Sriwijaya in July 2021 and the molecular identification was performed from August to December 2021 at the Laboratory of Agricultural Biotechnology (accredited according to the ISO/IEC 17025 standard), Department of Plant Protection, Faculty of Agriculture, Universitas Lampung, Indonesia.

Fungal Exploration, isolation, and purification

Fungal exploration from the infected-host cadavers using the method of Ab Majid *et al.* (2015) by collecting infected-host insects or cadaver infected with the fungi from the fields. The exploration was carried out in Tanjung Pering, Ogan Ilir, South Sumatra (3°13′23″S104°38′27″E), Tanjung Cermin, Pagar Alam, South Sumatra (4°02′23″S103°13″14″E), and Nendagung, Pagar Alam, South Sumatra (3°56′22″S103°12′15″E) (Table 1). The infected insects or cadavers were first surface sterilized with 70% EtOH (Ethyl alcohol) and 1% NaOCl (Sodium hypochlorite), then rinsed 3 times (Elfita *et al.* 2019). After that, the sample cadavers were cultured aseptically onto SDA (Sabouraud Dextrose Agar) medium (Russo *et al.* 2020). The fungal culture was purified to make an isolate per sample. The fungal macroscopic and microscopic characteristics, such as the colonial color and shape, the conidial shape and size, and the conidiophores were observed (Herlinda *et al.* 2021) and then molecular identification was performed.

DNA extraction, PCR amplification, and sequencing

DNA was extracted according to the method of Swibawa *et al.* (2020) and carried out on fungal conidia of 7 days old fungus. As much as 10 ml of conidia suspension was centrifuged using CF15RXII for 10 min at a speed of 14,000 rpm. Then, 1 ml of 70% ethanol was added to the centrifuge tube and centrifuged again for 10 min. The supernatant was removed and 1 ml of extraction buffer (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) was added. The suspension was incubated at -40 °C for 24 hours. The frozen suspension was crushed until pulverized. A total of 500 µl of pellet suspension was put into a 1.5 ml tube and 400 µl of 2% CTAB (cetyltrimethylammonium bromide) was added, homogenized and heated at 65 °C for an hour using a water bath (Brookfield TC 550 MX-230, USA). After the incubation, 500 µl of PCI (Phenol Chloroform Isoamyl alcohol) (25: 24: 1) was added, homogenized and centrifuged at 14,000 rpm for 10 minutes at 14,000 rpm for 10 min. A total of 600 µL supernatant was transferred to a new 1.5 mL tube, and 600 µL Chloroform Isoamyl Alcohol (24:1) was added, homogenized, and centrifuged (Microspin12; Biosan, Latvia) again at 14,000 rpm for 10 minutes. A total of 400 µl of supernatant was then put into to a new 1.5 ml tube and 400 µl of cold isopropanol was homogenized and incubated at -40 ºC for 20 minutes. Then, the suspension was centrifuged at 14,000 rpm for 15 min. The supernatant was then discarded, and the pellet was added with 500 µl of 70% cold ethanol and centrifuged at 14,000 rpm for 5 minutes. The supernatant was then discarded and the pellets obtained were incubated at room temperature for 24 hours to dry. After drying, the pellets were 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 ITS (the Internal Transcribed Spacer) using ITS1 and ITS4 primers (White *et al.* 1990). The DNA amplification stage consisted of 1 initiation cycle at 95 ºC for 5 min, 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. Then, the PCR results were 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 under taken in 1x TBE buffer solution at 50 volts for 70 min, and the results were visualized using a DigiDoc UV transilluminator (UVP, USA).

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

Mass-rearing of *Spodoptera frugiperda*

The mass-rearing of *S. frugiperda* was performed using the method of Herlinda *et al.* (2020). The eggs of *S. frugiperda* were obtained from Laboratory of Entomology, Faculty of Agriculture, Universitas Sriwijaya. They were reared in laboratory for more than 5 generations at 28–29°C temperature, and 82–83% RH and the lighting set to to photoperiod 12:12 (L:D) h. In the laboratory, the larvae of *S. frugiperda* were maintaned individually due to cannibal behaviors and reared using fresh maize leaves. The prepupae and pupae were replaced 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. Emerged adults were used for bioassays.

Assessing endophytic fungal colonization

Fungal inoculation for maize seeds treated was carried out to assess the ability of the fungal colonization into the maize seedling tissue and to ensure that the fungi used in this experiment were truly endophytic. All the isolates used were grown in SDA medium incubated for 14 days, then the SDA fungal culture was transferred to the broth medium (SDB, Sabouraud Dextrose Broth) following the method of Gustianingtyas *et al.* (2020) and incubated for 7 days on the shaker and 7 days unshaken position. The 45 corn seeds for an isolate were surface sterilized by using (Russo *et al.* 2020) method. The seeds were immersed in 10 ml of fungal suspension (1 x 10^{10} conidia ml^{-1} for 24 hrs, while for the control only 10 ml of sterilized water was treated for the seeds. Then, the seeds were grown in the hydroponic medium following method of (Novianti *et al.* 2020) and incubated for 14 days and this treatment was repeated 3 times for each isolate. The tip leaves of 14-day old maize seedlings (young maize) were cut of 5 x 5 mm^2 to be grown onto the SDA medium to detect the mycelia of the endophytic fungi. The leaf materials were first surface-sterilized by using method of (Russo *et al.* 2020) before grown onto the SDA medium. The leaf material surface-sterilized was carried out by immersion in 70% ethanol, then followed by sodium hypochlorite for 2 minutes, and rinsed twice in steril distilled water and the final rinse water was grown onto SDA and incubated for 10 days. The rest or remaining leaves were used for bioassays as described below.

The bioassay for assessing effect of corn seed treatment on *S. frugiperda* development

The bioassay for assessing the effect of corn seed treatment on *S. frugiperda* growth and development, followed the method of Zea *et al.* (2019). The 14-day old maize seedlings already inoculated with the endophytic fungi as described above were given to be consumed to the first instar neonate larvae of *S. frugiperda*, while for control treatment, the larvae were provided the non-inoculated maize seedlings and this experiment was repeated three times. The 50 neonate larvae (hatching within 24 hrs) of first instar were allowed to feed on the treated young maize and untreated ones (control) for 6 hrs or until the leaves eaten up and this treatment was replicated three times for each isolate. Then, the larvae were individually kept in 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 and replaced with new ones everyday. The treatments of this experiment consisted of six fungal isolates and control (water) and used the completely randomized block designs. The variables were recorded were development stages (egg, larval, pupal, and adult), mortality of each stage. The larval and pupal mortality were recorded daily, and the adults emerging were observed everyday. The sex of adults emerged were recorded and they were placed in the wire mesh cage for copulation with fresh maize leaves inside to allow egg laying. Egg collection and 10% honey bee solution replacement for adults were carried out everyday. The adult longevity was also observed until the adult death.

Data analysis

The differences in the length of different stages (egg, larval, pupal, and adult), mortality of each stage, adult longevity, eggs laid, and sex ratio of each treatment 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

Identification results of the endophytic fungal isolates

Five fungal isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) had the same macroscopic and microscopic characteristics, while an isolate (WTTJC260521B) had different characteristics (Fig. 1). The WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A isolates had a white

colonial, hyphal, and mycelia color, and non-septate and globose conidia or spores. These characteristics were placed the isolates within the group of *B. bassiana* (Fig. 2). The isolates were deposited in the GenBank with the accession number ON631784 (JGTP240521A isolate), ON631780 (WTTJC260521A isolate), ON631783 (WTTJC290521A isolate), ON631782 (WTTJC290521B isolate), ON631778 (JGNT300521 isolate) (Table 1). The isolate of WTTJC260521B had a white young colony, then the older colony turning greenish white to dark green, and the isolate had the green hyphae and mycelia, the isolate had non-septation, clear, and cylindrical conidia. The isolate was placed within the group of *M. anisopliae* (Fig. 2). The WTTJC260521B isolate was deposited in the GenBank with the accession number ON631793 (WTTJC260521B isolate) (Table 1).

The result of BLAST search revealed that five fungal isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) share 100% of similarity each other as well as with JgSPK (Acc. No. MZ356494.1). All the isolates shared 99.80% of similarity with JGNT300521 as well as with BSwTd4 (Acc. No. MT448732.1). JGNT300521 isolate shared 99.61% of similarity with BSwTd4. All the isolates shared 98.83% of similarity with B1 UNILA (=NKPT) (Acc. No. LC413808.1) and type strain on *B. bassiana* (ARSEF 1564.T., Acc. No. HQ880761.1). Isolate of WTTJC260521B shared 99.992% of similarity with reference isolate of IPPM010202 (Acc. No. KY437678.1), 99.994% of similarity with strain MSwTp3 (Acc. No. MT448733.1), and 99.985% of similarity with the type strain of *M. anisopliae* ARSEF 7487.T (Acc. No. HQ331446.1). So, there were 2 species from the 6 investigated isolates of the endophytic fungi found in this study. The 5 isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) were in the group of *B. bassiana*, the one isolate (WTTJC260521B) were in the group of *M. anisopliae.*

The development of *Spodoptera frugiperda* fed on young maize colonized and non-colonized by fungi

Six fungal isolates found and used in this experiment were able to colonize young maize (seedling) when inoculated by seed immersion treatment. They were confirmed as endophytic fungi. The seed immersion treatment resulted the leaves of treated young maize grown on to the SDA medium were overgrown with the fungal isolates. No fungal growth was found on the leaves of untreated seedling and on the last rinse water. This corfirmed that the fungal isolates used in this study were endophytic fungi and it also showed that the surface-sterilization of maize tissues eliminated the epiphytic microorganisms and the fungi growing out of the leaf surface were the endophytic fungi originating from within the maize tissues.

Feeding on leaves of fungal colonized maize significantly increased development time of the second instar ($P<0.0001$), third instar ($P<0.0001$), fourth instar ($P<0.0001$), fifth instar ($P<0.0001$), sixth instar ($P<0.0001$) (Table 2). However, no significant difference in the development time of first instar of treated and untreated maize (control). This fungal colonized maize also increased egg and pupal development time, but decreased female and male adult longevity. The lifespan of *S. frugiperda* fed on leaves of fungal colonized maize was significantly longer compared to those fed on leaves of non-colonized maize (Table 3). The longest lifespan of *S. frugiperda* occurred on insects feeding on leaves of *B. bassiana* colonized maize.

The fungal colonized young maize significantly increased mortality of all instar larvae compared to non-colonized one (Table 4). The last instar mortality treated with *B. bassiana* (JGTP240521A isolates) (51.33%) was the highest among other treatments and did not significantly differ from each of the *B. bassiana* (WTTJC260521A and WTTJC290521A isolates) (45.33% and 44.67%, respectively). Feeding on leaves of fungal colonized maize

significantly decreased the percentage of the last instar becoming pupal stage and adult emergence (Table 5). The young maize colonized with fungi significantly reduced eggs laid by the adults (fecundity), but did not effect the sex ratio of *S. frugiperda*. Percentage of hatched eggs significantly decreased on the treatment of *B. bassiana* (JGTP240521A, WTTJC260521A, WTTJC290521A, and WTTJC290521B isolates) (Table 5).

Discussion

The results of identification based on the morphological characters of five isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) showed that they have similar morphology of the colony, hypha, and mycelia, and the conidial shape. They belong to species of *B. bassiana*. These characters match to *B. bassiana* described by Herlinda *et al.* (2021). The isolate of WTTJC260521B belongs to species of *M. anisopliae*. The isolate morphology of the colony, and the hyphal, mycelia, and conidial of shape matches to *M. anisopliae* described by Herlinda *et al.* (2020).

The five fungal isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) found in this study had an rDNA sequence similarity value of 100% to the reference species (BLAST), JgSPK (Acc. No. MZ356494.1) an endophytic fungi isolated from maize (Herlinda *et al.* 2021) except one isolate (JGNT300521). If the similarity value is 100%, It means that they are the same strain (Henry *et al.* 2000). These isolates shared 99.80% of similarity with JGNT300521 as well as with BSwTd4 (Acc. No. MT448732.1) which was isolated from oil palm rhizosphere (Herlinda *et al.* 2020). JGNT300521 isolate shared 99.61% of similarity with BSwTd4. All the isolates shared 98.83% of similarity with B1 UNILA (=NKPT) (Acc. No. LC413808.1) which was isolated from maize rhizosphere (Fitriana *et al.* 2021) and type strain on *B. bassiana* (ARSEF 1564.T., Acc. No. HQ880761.1). The isolate of WTTJC260521B had an rDNA sequence similarity value of more than 99% to the BLAST (reference species). The similarity value of 99-100% indicates that the isolates are the same species (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). If the similarity value of the isolates is 89–99%, it means that the isolates are the same genus (Henry *et al.* 2000).

All fungal isolates of the *B. bassiana* and *M. anisopliae* tested in this study were able to colonize leaves of young maize when inoculated by seed immersion treatment. From the leaves of young maize treated by the fungi were overgrown with the fungal mycelia, but no fungal mycelia were found on the leaves of untreated seeds. Our finding showed that the both species of fungi from seed treatment are able to colonize the leaves. In addition to, the fungi of *B. bassiana* and *M. anisopliae* not only can colonize maize by seed treatment, but they also can colonize maize by foliar spray and root deeping and the fungi can systemically colonized leaves, stems, and roots of plants (Russo *et al.* 2020). *B. bassiana* inoculated by foliar spray can penetrate the leaf surface and move within the maize vascular (Wagner and Lewis 2000). *M. anisopliae* often reported to be restrited to plant roots (Russo *et al.* 2020), however our study reported that the strain of *M. anisopliae* in this study is able to colonize the leaves of young maize. The extent and persistence of plant colonization by the fungi were influenced by fungal species/strain, inoculation method, and host plant species (Russo *et al.* 2020). The fungi used for seed treatment in this study is easier to be applied by soaking the seeds in fungal suspension before planting, or when the seeds stored, dry conidia of the fungi can be covered on the seeds.

Our other finding is that *B. bassiana* and *M. anisopliae* from South Sumatra (Indonesia) in seed treated corn seedlings have negative effects on development of *S. frugiperda.* This is first report that the fungi as an endophyte could decrease the female and male adult longevity of *S. frugiperda* and increased the larval mortality. The young maize colonized with the *B. bassiana* and *M. anisopliae* also reduced the percentage of the last instar becoming pupal stage and adult

emergence, and decreased the egg laid by the adults and the percentage of hatched eggs. Previous study reported that *B. bassiana* and *M. anisopliae* in foliar treated caused adverse effects on *S. frugiperda* development and survival (Russo *et al.* 2020). These adverse effects of endophytic fungi against *S. frugiperda* were caused by fungal production of secondary metabolites and mycosis (Vidal and Jaber 2015). The fungal secondary metabolites are produced by blastospores in insect hemolymph and disrupted the normal cell metabolism (Mancillas-Paredes *et al.* 2019) and then the toxins produced by the metabolites kill the insects (El-Ghany 2015). Our previous study showed that the endophytic fungi could decrease the leaf consumption by the *S. frugiperda* larvae resulting in larval weight loss and low survival (Gustianingtyas *et al.* 2021). The reduction in leaf area consumed by the larvae treated with the endophytes is caused by antifeedant or deterrent properties of *in planta*-produced *B. bassiana* metabolites (Russo *et al.* 2020). The corn plants colonized with *B. bassiana* may enhance levels of terpenoid defense compounds against *S. frugiperda* (Russo *et al.* 2020). The endophytic fungi could produce secondary metabolites *in planta* resulting antibiosis and feeding deterrence for the insects (Jaber and Ownley 2018).

These adverse effects of endophytic fungi against *S. frugiperda* were also caused by mycosis (Vidal and Jaber 2015). This research found that the mycosis found on the cadavers of *S. frugiperda* treated with the fungi. The mycosis was evidenced by fungal mycelia and spores emerging from the cadavers of treated insects. However, no fungal mycelia and spores were found on the cadavers of untreated insects. Some previous studies have similar reported insect mycosis feeding on fungal-endophytically colonized plants by *S. frugiperda* (Russo *et al.* 2020; Herlinda *et al.* 2021).

This study also showed that the fungal colonized young maize increased egg, larvae, pupal development time, and lifespan of *S. frugiperda.* In contrast to the previous study of Russo *et al.* (2020) that these fungal species could decreased the development time of *S. frugiperda*. However, our findings are in agreement with previous study of Hussain *et al.* (2009) showing that the lepidopteran, *Ocinara varians* treated with *B. bassiana* and *M. anisopliae* extended the developmental time of treated insects as compared to untreated ones (control) and the conversion of digested food and ingested food declined in treated insects compared to untreated insects, and stimulated the larvae to develop more slowly.

Conclusions

The results of molecular identification showed that the fungal species found were *B. bassiana* of five fungal isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) and *M. anisopliae* of an isolate (WTTJC260521B). Feeding on leaves of fungal colonized maize significantly decreased the percentage of the last instar becoming pupal stage and the adult emergence and the eggs laid, and the percentage of hatched eggs and increased the larval mortality. This is first report that the *B. bassiana* and *M. anisopliae* from South Sumatra (Indonesia) in seed treated corn seedlings have negative effects on development of *S. frugiperda.* Finally, these results highlight the promising potential of endophytic entomopathogenic fungi to protect corn plants against *S. frugiperda.*

List of abbreviations

ANOVA: analysis of variance; BLAST: Basic Local Alignment Search Tool; CTAB: cetyltrimethylammonium bromide; DNA: Deoxyribonucleic acid; EtOH: Ethyl alcohol; FAW: fall armyworm; 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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Table 1 Origin of isolates of endophytic-entomopathogenic 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	*Spodoptera frugiperda*	35.0	*Beauveria bassiana*	JGTP240521A	ON631784
Tanjung Cermin, Pagar Alam	Lepidoptera	817.2	*Beauveria bassiana*	WTTJC260521A	ON631780
Tanjung Cermin, Pagar Alam	Lepidoptera	817.2	*Metarhizium anisopliae*	WTTJC260521B	ON631793
Tanjung Cermin, Pagar Alam	Lepidoptera	817.2	*Beauveria bassiana*	WTTJC290521A	ON631783
Tanjung Cermin, Pagar Alam	Lepidoptera	817.2	*Beauveria bassiana*	WTTJC290521B	ON631782
Nendagung, Pagar Alam	*Spodoptera frugiperda*	802.6	*Beauveria bassiana*	JGNT300521	ON631778

Table 2 Length of different developmental stages of instar larvae of *Spodoptera frugiperda* fed on leaves of endophytic fungi colonized (seed treated) and non-colonized (control) young maize

		Length of different developmental stages (days)					
Isolate	Species	1st larvae	2nd larvae	3rd larvae	4th larvae	5th larvae	6th larvae
Control	-	2.67	3.34c	2.36d	2.27c	3.26b	3.23b
JGTP240521A	*Beauveria bassiana*	2.71	3.66b	5.70a	4.45a	3.95a	3.86ab
WTTJC260521A	*Beauveria bassiana*	2.59	3.71b	4.00b	4.60a	2.99b	3.76ab
WTTJC260521B	*Metarhizium anisopliae*	2.63	3.68b	2.66d	3.71b	3.65ab	3.51ab
WTTJC290521A	*Beauveria bassiana*	2.63	3.71b	3.63a	4.46a	3.69ab	4.28a
WTTJC290521B	*Beauveria bassiana*	2.60	4.28a	5.46a	3.79b	3.28ab	3.13b
JGNT300521	*Beauveria bassiana*	2.65	3.64b	3.62c	2.27c	3.37ab	3.57ab
F-value		2.37^{ns}	23.40^{*}	292.73^{*}	296.38^{*}	4.26^{*}	5.22^{*}
P-value		0.10	<0.0001	<0.0001	<0.0001	0.02	0.007
HSD value		-	0.07	0.90	0.07	0.22	0.21

Note: ns = not significantly differen * = 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

Tabel 3 Length of different developmental stages of pupae and adults of *Spodoptera frugiperda* fed on leaves of endophytic fungi colonized (seed treated) and non-colonized (control) young maize

		Length of different developmental stages (days)					
Isolate	Species	Prepupae	Pupae	Female adult	Male adult	Egg	Total lifespan
Control	-	3.61	6.95b	4.82a	4.40a	2.59c	32.51d
JGTP240521A	*Beauveria bassiana*	3.68	9.71a	4.37ab	3.28b	3.22abc	41.62a
WTTJC260521A	*Beauveria bassiana*	3.11	10.24a	4.21ab	3.40b	2.74bc	39.21ab
WTTJC260521B	*Metarhizium anisopliae*	3.65	7.43b	4.52ab	3.63b	2.84bc	35.44c
WTTJC290521A	*Beauveria bassiana*	3.79	9.85a	3.99b	4.34a	3.39ab	40.05a
WTTJC290521B	*Beauveria bassiana*	3.22	10.58a	4.23ab	3.52b	3.27abc	40.57a
JGNT300521	*Beauveria bassiana*	3.71	9.49a	4.93a	4.58a	3.65a	37.25bc
F-value		0.95^{ns}	22.43^{*}	5.12^{*}	49.86^{*}	6.39^{*}	34.57^{*}
P-value		0.50	<0.0001	0.008	<0.0001	0.003	<0.0001
HSD value		-	1.47	0.17	0.09	0.19	0.22

Note: ns = not significantly differen * = 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

Table 4 Mean of mortality of different instar larvae of *Spodoptera frugiperda* fed on leaves of endophytic fungi colonized (seed treated) and non-colonized (control) young maize

Isolate	Species	Mean of mortality of different instar larvae (%)

		1st larvae	2nd larvae	3rd larvae	4th larvae	5th larvae	6th larvae
Control	-	2.67	6.00b	6.67e	6.67c	6.67c	6.67e
JGTP240521A	*Beauveria bassiana*	14.67	24.00a	43.33a	48.67a	51.33a	51.33a
WTTJC260521A	*Beauveria bassiana*	8.00	21.33a	36.67ab	39.33ab	42.00ab	45.33ab
WTTJC260521B	*Metarhizium anisopliae*	2.67	5.33b	8.00de	12.00c	15.33c	24.67d
WTTJC290521A	*Beauveria bassiana*	7.33	11.33ab	20.00cd	30.67b	36.00b	44.67ab
WTTJC290521B	*Beauveria bassiana*	8.67	16.67ab	22.67bc	27.33b	29.33b	38.67bc
JGNT300521	*Beauveria bassiana*	8.00	16.00ab	21.33bc	26.67b	28.00b	32.67c
F-value		2.93^{ns}	7.74^{*}	22.96^{*}	27.02^{*}	35.02^{*}	176.07^{*}
P-value		0.053	0.001	<0.0001	<0.0001	<0.0001	<0.0001
HSD value		-	10.95	10.00	9.64	8.74	3.90

Note: ns = not significantly differen * = 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

Table 5 Mean of percentage of pupae and adults emergence, sex ratio, egg laid, and viable eggs of *Spodoptera frugiperda* fed on leaves of endophytic fungi colonized (seed treated) and non-colonized (control) young maize

Isolates	Fungal species	Pupae emergence (%)	Adult emergence (%)	Sex ratio	Eggs laid per female	Viable (hatched) eggs (%)
Control		93.33a	93.33a	0.56a	68.28a	99.92a
JGTP240521A	*Beauveria bassiana*	46.00e	42.00e	0.83a	15.91c	88.86d
WTTJC260521A	*Beauveria bassiana*	52.67de	48.00de	0.84a	15.31c	90.93cd
WTTJC260521B	*Metarhizium anisopliae*	74.67b	71.33b	0.47a	42.89b	99.72a
WTTJC290521A	*Beauveria bassiana*	54.00d	47.33de	0.74a	27.86bc	95.91b
WTTJC290521B	*Beauveria bassiana*	59.33cd	54.00cd	0.87a	17.50c	92.98bc
JGNT300521	*Beauveria bassiana*	65.33c	58.67c	0.51a	39.36b	99.31a
F-value		134.80^{*}	95.08^{*}	3.67^{*}	34.26^{*}	75.47^{*}
P-value		<0.0001	<0.0001	0.026	<0.0001	<0.0001
HSD value		6.85	9.03	0.19	1.37	4.16

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

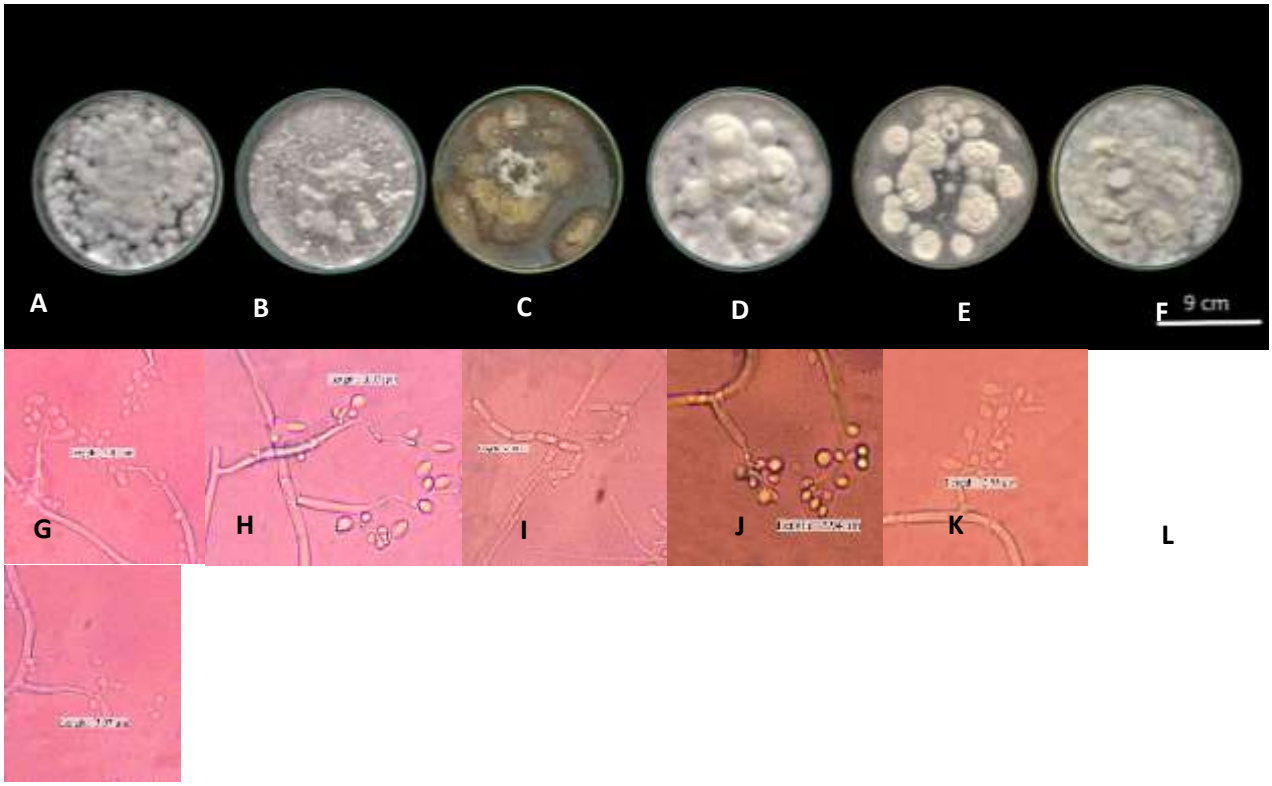


Fig. 1. Colony morphology of endophytic fungi cultured on PDA media (above) and the conidial and hyphal morphology (below) of the fungi: *Beauveria bassiana* of JGTP240521 isolate (A and G) and WTTJC260521A isolate (B and H), *Metarhizium anisoplae* WTTJC260521B isolate (C and I), *Beauveria bassiana* of WTTJC290521A isolate (D and J), WTTJC290521B isolate (E and K), and JGNT300521 isolate (F and L)

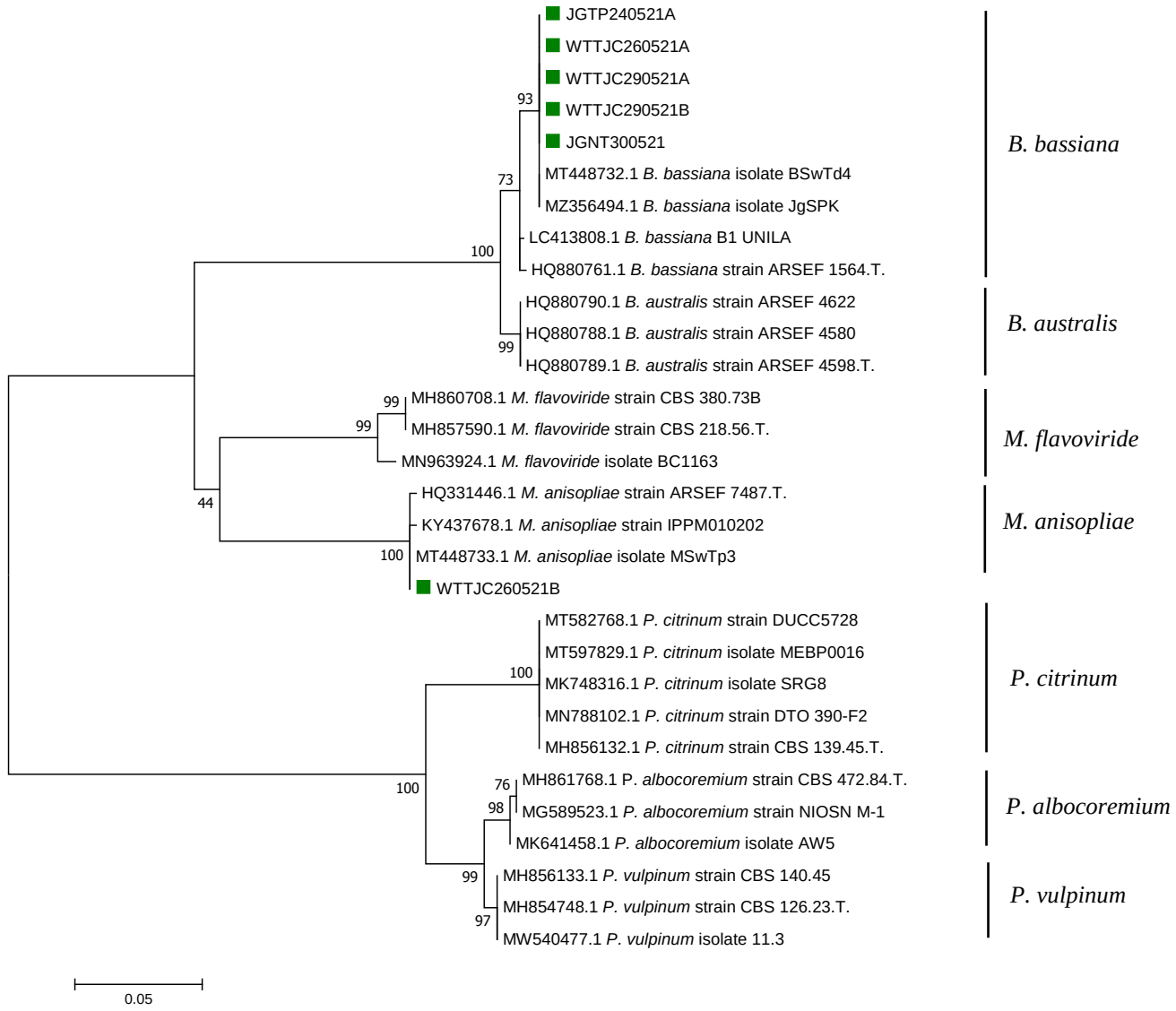
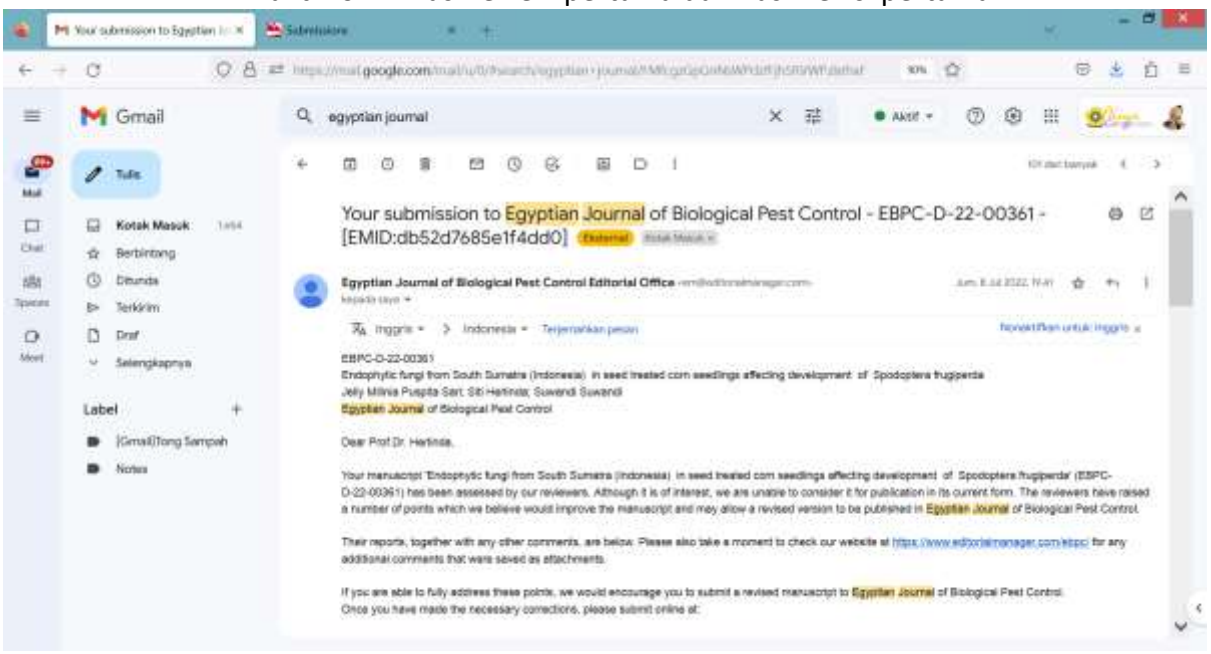
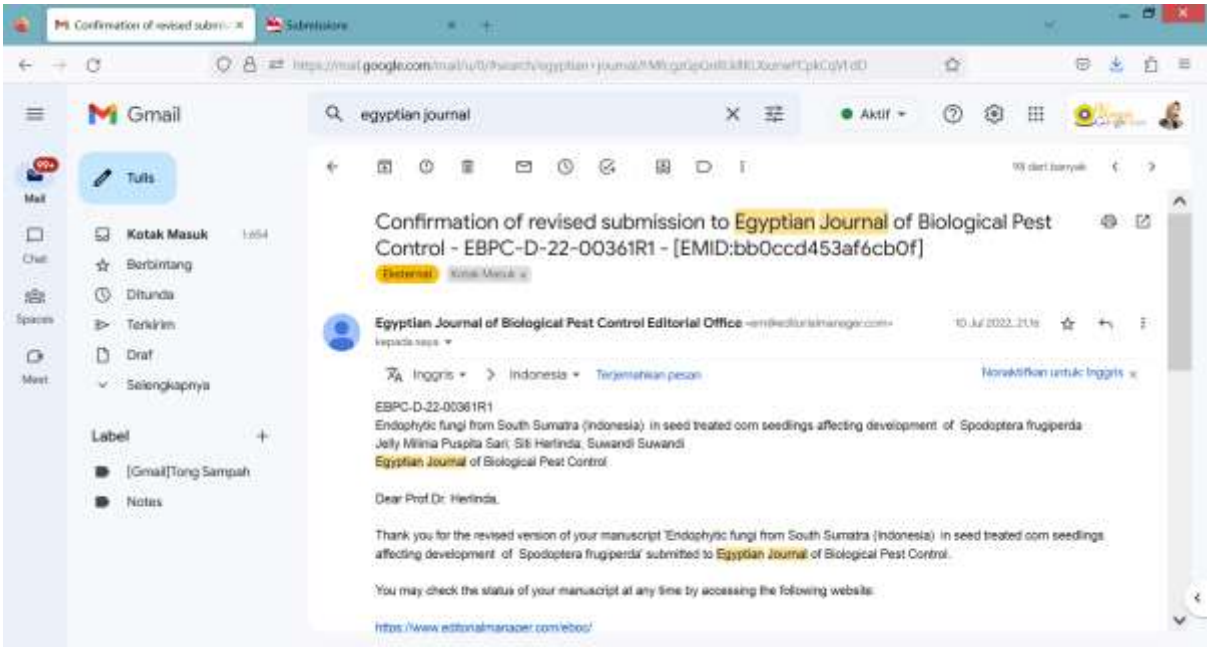


Fig. 2. Phylogenetic tree developed based on Internal Transcribed Spacer (ITS) region by Maximum Likelihood (Tamura-Nei model) using Mega7 for windows (Kumar *et al.* 2016). The six investigated isolates were placed within group of *Beauveria bassiana* (5 isolates) and *Metarhizium anisopliae* (1 isolate). T= Type isolate

2. Bukti konfirmasi review pertama dan hasil revisi pertama





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Endophytic fungi from South Sumatra (Indonesia) in seed treated corn seedlings affecting development of Spodoptera frugiperda

--Manuscript Draft--

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Abstract:	Background: Topical application of the entomopathogenic fungi against the S podoptera frugiperda larvae is less effective due to larvae hiding in the corn midribs in the field. To control the larvae, the fungi colonize in plant tissues or endophytic fungi are needed. There is no information on the pathogenicity of the endophytic fungi from Indonesia on the development of S. frugiperda . The endophytic fungi isolated from infected-host cadavers from South Sumatra (Indonesia) were identified morphologically and molecularly and the effect of seed treated corn seedlings with the fungi on S. frugiperda development was evaluated. The fungal identification was based on morphological and molecular characteristics. Bioassay of the endophytic fungal species in seed treated young maize was performed against the neonate larvae (hatching within 24 hrs) of first instar and their development were observed. Results: The results of molecular identification showed that the fungal species found were Beauveria bassiana of five fungal isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) and Metarhizium anisopliae of an isolate (WTTJC260521B). The lifespan of S. frugiperda fed on leaves of fungal colonized maize was significantly longer compared to those fed on leaves of non-colonized maize. The fungal colonized young maize significantly increased mortality of all instar larvae compared to non-colonized one. The last instar mortality treated with B. bassiana (JGTP240521A isolates) (51.33%) was the highest among other treatments and did not significantly differ from each of the B. bassiana of WTTJC260521A and WTTJC290521A isolates (45.33% and 44.67%, respectively). Feeding on leaves of fungal colonized maize significantly decreased the percentage of the last instar becoming pupal stage, the adult emergence, the eggs laid, and the percentage of hatched eggs. This is first report that the B. bassiana and M. anisopliae from South Sumatra (Indonesia) in seed treated corn seedlings have negative effects on development of S . frugiperda. Conclusions: Finally, these results highlight the potential of endophytic entomopathogenic fungi to protect corn plants against S . frugiperda. Keywords: Beauveria bassiana , endophyte, entomopathogen, fall armyworm, Metarhizium anisopliae
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Response to Reviewers:	Reviewer reports: Reviewer #1: Interesting paper with quite original data which look properly analyzed. The general english form need to be revised in many sentences. Notes are in the attached files Author Response to Reviewers #1: 1.All revisions for reviewer #1 in the manuscript have been highlighted with yellow colour. 2.For the last comment of reviewer about some authors missing in the "References", we have check and no authors missing in the "References" because when we wrote the references we used Mendeley Reviewer reports: Reviewer #2: The authors may be asked to provide the clarifications for the above points and the revised manuscript shall be considered for further consideration to publish in the journal. Response to Reviewers #2: 1. Reviewer comments: The authors use the term 'endophytic' in many places in the article including the title. But the information available in abstract, introduction and materials and methods reveals that the entomopathogenic isolates were isolated from infected cadavers. If we isolate fungi from plants by repeatedly doing sterility checks at different dilutions, then only they can be called endophytic fungi. If these isolates were screened for entomo-pathogenicity, then only they can be called endophytic entomopathogen. Author Response: we assessed the ability of the fungal colonization into the maize tissue and we found that six fungal isolates in this experiment were able to colonize maize. We have the data or evidence to confirm that they are endophytic fungi: 1) fungal colony of the corresponding isolates in the leaves of plants, 2) larvae that died after feeding on the leaves and re-isolation of the same isolates from the cadavers has been carried out, 3) number of leaves colonized by the fungal isolates. The data or evidences to confirm the endophytic fungi have been presented in this revised manuscript (Table 2 and Fig. 3 and 4). 2. Reviewer comments: Since, in the present study, the entomopathogen isolated from infected cadaver were used, while assaying the endophytic nature of the isolates (by isolating from leaves after treating the seeds), the sterility checks should have been carried out to prove the endophytic nature of the isolates. Author Response: The sterility checks have been carried out to prove the endophytic nature of the isolates. 3.Reviewer comments: In materials and methods, the common protocols followed shall be written briefly. If any modifications were there, then only details are required. Author Response: The revisions in the manuscript have been highlighted with green colour. 4. Reviewer comments: The data on the efficacy of the seed treatments with entomopathogenic fungi are given. However, the supporting evidence are required in terms of (i) number of colony forming unit of the corresponding isolates in the leaves of plants where seed treatment was given (ii) re-isolation of the same isolates from the larvae that died after feeding on the leaves of the plants where seed treatment was given. Without pieces of evidences, the cause & effect can not be scientifically correlated. Author Response: We have data or evidence about: (i) conidial density from colony of the corresponding isolates in the leaves of plants where seed treatment was given, (ii) larvae that died after feeding on the leaves and re-isolation of the same isolates from the cadavers has been carried out, (iii) number of leaves colonized by the fungal

	isolates. The data or evidences to confirm the endophytic fungi have been presented in this revised manuscript (Table 2 and Fig. 3 and 4). The revisions in the manuscript have been highlighted with green colour. 5. Reviewer comments: If there has been significant mortality due to seed treatment with entomopathogenic fungi, (up to 51% reported), how was the sampling carried out for the results obtained in table 2 & 3. There might not have been uniformity. Probably the larvae escaped from entomopathogens (i.e.) feeding on un-colonized leaves only were collected. Clarification is required in this regard. Author Response: The 50 neonate (hatching within 24 hours) larvae of first instar were allowed to feed on the colonized leaves (or treated) for 6 hours or until the leaves eaten up so that no larvae escaped from entomopathogens. For the control (or untreated) the larva were provided the un-colonized leaves. Then, the larvae (treated and control) were individually kept in a porous plastic cup and fed on healthy non-inoculated leaves during larvae stage. 6. Reviewer comments: As it is, the highest mortality of 51.3% by isolate JGTP240521A isolates cannot be termed as promising particularly for a pest like FAW, which has capability of destroying crop. Author Response: The mortality of 51.3% by the endophytic fungi is a potential candidate for biocontrol agents of FAW larvae which always hide in the corn midribs (at daylight up to night) because the larvae are able to consume the colonized leaves or stems, but if the fungi are not endophytic which should be applied by contact (topical application), it cannot be termed as promising to protect corn plants against the such hiding S. frugiperda larvae. Our finding also highlighted that the maize colonized with the fungi not only reduced the percentage of the last instar becoming pupal stage (or increased the mortality of larvae), but also decreased the adult emergence, the egg laid by the adults, and the percentage of hatched eggs. So, based on our data, the fungi can be termed as promising for the FAW. 7. Reviewer comments: English needs improvement. It has to be edited by professional language editors. Author Response: We have improve the English and edited by professional language editor, Bambang A. Loeneto, M.A., Ph.D. All revisions by him have been highlighted with green colour.
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 from South Sumatra (Indonesia) in seed treated corn seedlings affecting development of *Spodoptera frugiperda*

Abstract

Background: Topical application of the entomopathogenic fungi against the *Spodoptera frugiperda* larvae is less effective due to larvae hiding in the corn midribs in the field. To control the larvae, the fungi colonize in plant tissues or endophytic fungi are needed. There is no information on the pathogenicity of the endophytic fungi from Indonesia on the development of *S. frugiperda*. The endophytic fungi isolated from infected-host cadavers from South Sumatra (Indonesia) were identified morphologically and molecularly and the effect of seed treated corn seedlings with the fungi on *S. frugiperda* development was evaluated. The fungal identification was based on morphological and molecular characteristics. Bioassay of the endophytic fungal species in seed treated young maize was performed against the neonate larvae (hatching within 24 hrs) of first instar and their development were observed.

Results: The results of molecular identification showed that the fungal species found were *Beauveria bassiana* of five fungal isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) and *Metarhizium anisopliae* of an isolate (WTTJC260521B). The lifespan of *S. frugiperda* fed on leaves of fungal colonized maize was significantly longer compared to those fed on leaves of non-colonized maize. The fungal colonized young maize significantly increased mortality of all instar larvae compared to non-colonized one. The last instar mortality treated with *B. bassiana* (JGTP240521A isolates) (51.33%) was the highest among other treatments and did not significantly differ from each of the *B. bassiana* of WTTJC260521A and WTTJC290521A isolates (45.33% and 44.67%, respectively). Feeding on leaves of fungal colonized maize significantly decreased the percentage of the last instar becoming pupal stage, the adult emergence, the eggs laid, and the percentage of hatched eggs. This is first report that the *B. bassiana* and *M. anisopliae* from South Sumatra (Indonesia) in seed treated corn seedlings have negative effects on development of *S. frugiperda*.
Conclusions: Finally, these results highlight the potential of endophytic entomopathogenic fungi to protect corn plants against *S. frugiperda*.

Keywords: *Beauveria bassiana*, endophyte, entomopathogen, fall armyworm, *Metarhizium anisopliae*

Background

Spodoptera frugiperda J.E. Smith (Lepidoptera: Noctuidae) or fall armyworm (FAW) is the most important noctuid pest of corn in the world. The FAW is a migratory and polyphagous pest and can attack 353 host plant species from 76 plant families (Montezano *et al.* 2018). The percent of infested maize fields by FAW in East Africa ranges from 80% to 100% in Ethiopia and 82.2% to 100% in Kenya (Sisay *et al.* 2019) and the pest causes losses of about a third of the annual maize production or about 1 million tonnes in Kenya (De Groote *et al.* 2020), and 18 million tons/year in 12 African countries and the losses reach US $ 13 millions (Harrison *et al.* 2019). The pest is native to the neotropics of the Americas and has spread throughout the world (Otim *et al.* 2018). More recently, the FAW becomes a new invasive pest in many parts of Africa (Goergen *et al.* 2016; Niassy *et al.* 2021) and Asia (Lamsal *et al.* 2020), including Indonesia (Herlinda *et al.* 2021). This pest is commonly controled using synthetic insecticides (Kumela *et al.* 2018), however the resistances of the FAW to many insecticides, such as pyrethroid, spinosad, and organophosphorus insecticides have occured (Zhang *et al.* 2021). In addition, the insecticide application negatively affects the human health and the environment (Harrison *et al.* 2019). An alternative more sustainable and eco-friendly control methods against *S. frugiperda* is urgently needed.

The preferred control option for FAW is biological control based on utilizing entomopathogenic fungi (Mantzoukas and Eliopoulos 2020). Topical application (direct contact) of the entomopathogenic fungus, *Beauveria bassiana* (Balsamo) Vuillemin (Deuteromycotina: Hypomycetes) killed more than 80% *S. frugiperda* larvae (Ramanujam *et al.* 2020). *Metarhizium anisopliae* (Metsch.) Sorok. (Deuteromycotina: Hypomycetes) could kill 75% of *S. frugiperda* larvae (Ramos *et al.* 2020). However, in the field, the larvae were occured on the surface of leaves or maize stalks only in the morning but at daylight up to night, they hide in the corn midribs (Herlinda *et al.* 2021). So, topical application of the fungus against the *S. frugiperda* larvae is less effective (Gustianingtyas *et al.* 2021). To control such hiding larvae in the field, the fungi colonizing in plant tissues or endophytic fungi are needed (Ramos *et al.* 2020). The endophytic fungi associate mutually with their host plants (Lira *et al.* 2020) and can stimulate the plant growth but suppress the insect pest growth (Russo *et al.* 2020).

Previous studies have demonstrated that the endophytic fungal *B. bassiana* from corn root from Indonesia applied topically caused 29.33% of the FAW larval mortality (Gustianingtyas *et al.* 2021). The endophytic fungal *B. bassiana* used as seed treatment caused up to 22.67% of the FAW larval mortality (Herlinda *et al.* 2021). An experiment has previously also reported that endophytic *B. bassiana* sprayed on leaves has high ability to colonize corn plants and the fungus caused significant reductions in the growth and development of *S. frugiperda* (Russo *et al.* 2020). There is no information on the pathogenicity of the endophytic fungi from Indonesia on the development of *S. frugiperda*. In addition, the potential of the fungi isolated from the infected-host cadaver as endophytic entomopathogens needs to be investigated. In this study, the fungi isolated from infected-host cadavers from South Sumatra (Indonesia) were identified morphologically and molecularly and the effect of seed treated corn seedlings with the fungi on *S. frugiperda* development was evaluated.

Methods

Exploration of the fungi was performed by collecting infected-host insect cadavers from crops in South Sumatra, Indonesia from may until June 2021. Purification and isolation of the fungi were carried out from June to July 2021. The morphological identification was carried out in the Laboratory of Entomology, Faculty of Agriculture, Universitas Sriwijaya in July 2021 and the molecular identification was performed from August to December 2021 at the Laboratory of Agricultural Biotechnology (accredited according to the ISO/IEC 17025 standard), Department of Plant Protection, Faculty of Agriculture, Universitas Lampung, Indonesia. Experimental design used for bioassay was a completely randomized block designs consisted of seven treatments (six fungal isolates and control), and the experiment was repeated three times.

Fungal Exploration, isolation, and purification

Fungal exploration from the infected-host cadavers using the method of Ab Majid *et al.* (2015) by collecting infected-host insects or cadaver infected with the fungi from the fields. The exploration was carried out in Tanjung Pering, Ogan Ilir, South Sumatra (3°13′23″S104°38′27″E), Tanjung Cermin, Pagar Alam, South Sumatra (4°02′23″S103°13″14″E), and Nendagung, Pagar Alam, South Sumatra (3°56′22″S103°12′15″E) (Table 1). The infected insects or cadavers were first surface sterilized with 70% EtOH (Ethyl alcohol) and 1% NaOCl (Sodium hypochlorite), then rinsed 3 times (Elfita *et al.* 2019). After that, the sample cadavers were cultured aseptically onto SDA (Sabouraud Dextrose Agar) medium (Russo *et al.* 2020). The fungal culture was purified to make an isolate per sample. The fungal macroscopic and microscopic characteristics, such as the colonial color and shape, the conidial shape and size, and the conidiophores were observed (Herlinda *et al.* 2021) and then molecular identification was performed.

DNA extraction, PCR amplification, and sequencing

DNA was extracted according to the method of Swibawa *et al.* (2020) and carried out on fungal conidia of 7 days old fungus. As much as 10 ml of conidia suspension was centrifuged using CF15RXII for 10 min at a speed of 14,000 rpm. Then, 1 ml of 70% ethanol was added to the centrifuge tube and centrifuged again for 10 min. The supernatant was removed and 1 ml of extraction buffer (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) was added. The suspension was incubated at -40 °C for 24 hours. The frozen suspension was crushed until pulverized. A total of 500 µl of pellet suspension was put into a 1.5 ml tube and 400 µl of 2% CTAB (cetyltrimethylammonium bromide) was added, homogenized and heated at 65 °C for an hour using a water bath (Brookfield TC 550 MX-230, USA). After the incubation, 500 µl of PCI (Phenol Chloroform Isoamyl alcohol) (25: 24: 1) was added, homogenized and centrifuged at 14,000 rpm for 10 minutes at 14,000 rpm for 10 min. A

total of 600 µL supernatant was transferred to a new 1.5 mL tube, and 600 µL Chloroform Isoamyl Alcohol (24:1) was added, homogenized, and centrifuged (Microspin12; Biosan, Latvia) again at 14,000 rpm for 10 minutes. A total of 400 µl of supernatant was then put into to a new 1.5 ml tube and 400 µl of cold isopropanol was homogenized and incubated at -40 ºC for 20 minutes. Then, the suspension was centrifuged at 14,000 rpm for 15 min. The supernatant was then discarded, and the pellet was added with 500 µl of 70% cold ethanol and centrifuged at 14,000 rpm for 5 minutes. The supernatant was then discarded and the pellets obtained were incubated at room temperature for 24 hours to dry. After drying, the pellets were 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 ITS (the Internal Transcribed Spacer) using ITS1 and ITS4 primers (White *et al.* 1990). The DNA amplification stage consisted of 1 initiation cycle at 95 ºC for 5 min, 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. Then, the PCR results were 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 under taken in 1x TBE buffer solution at 50 volts for 70 min, and the results were visualized using a DigiDoc UV transilluminator (UVP, USA).

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

Mass-rearing of *Spodoptera frugiperda*

The mass-rearing of *S. frugiperda* was performed using the method of Herlinda *et al.* (2020). The eggs of *S. frugiperda* were obtained from Laboratory of Entomology, Faculty of Agriculture, Universitas Sriwijaya. They were reared in laboratory for more than 5 generations at 28–29°C temperature, and 82–83% RH and the lighting set to to photoperiod 12:12 (L:D) h. In the laboratory, the larvae of *S. frugiperda* were maintaned individually due to cannibal behaviors and reared using fresh maize leaves. The prepupae and pupae were replaced 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. Emerged adults were used for bioassays.

Assessing endophytic fungal colonization

Fungal inoculation for maize seeds treated was carried out to assess the ability of the fungal colonization into the maize seedling tissue and to ensure that the fungi used in this experiment were truly endophytic. All the isolates used were grown in SDA medium incubated for 14 days, then the SDA fungal culture was transferred to the broth medium (SDB, Sabouraud Dextrose Broth) following the method of Gustianingtyas *et al.* (2020) and incubated for 7 days on the shaker and 7 days unshaken position. The 45 corn seeds for an isolate were surface sterilized by using (Russo *et al.* 2020) method. The seeds were immersed in 10 ml of fungal suspension (1×10^{10} conidia ml^{-1}) for 24 hrs, while for the control only 10 ml of sterilized water was treated for the seeds. Then, the seeds were grown in the hydroponic medium following method of Novianti *et al.* (2020) and incubated for 14 days and this treatment was repeated 3 times for each isolate. The tip leaves of 14-day old maize seedlings (young maize) were cut of 5 x 5 mm^2 to be grown onto the SDA medium to detect the mycelia of the endophytic fungi. The

leaf materials were first surface-sterilized by using method of (Russo *et al.* 2020) before grown onto the SDA medium. The leaf material surface-sterilized was carried out by immersion in 70% ethanol, then followed by sodium hypochlorite for 2 minutes, and rinsed twice in steril distilled water and the final rinse water was grown onto SDA and incubated for 10 days. The rest or remaining leaves were used for bioassays as described below.

The bioassay for assessing effect of corn seed treatment on *S. frugiperda* development

The bioassay for assessing the effect of corn seed treatment on *S. frugiperda* growth and development, followed the method of Russo *et al.* (2019). The 14-day old maize seedlings already inoculated with the endophytic fungi as described above were given to be consumed to the first instar neonate larvae of *S. frugiperda*, while for control treatment, the larvae were provided the non-inoculated young maize and this experiment was repeated three times. The 50 neonate larvae (hatching within 24 hrs) of first instar were allowed to feed on the treated young maize and untreated ones (control) for 6 hrs or until the leaves eaten up and this treatment was replicated three times for each isolate and the control. Then, the larvae were individually kept in a porous plastic cup (Ø 6.5 cm, height 4.6 cm) and were fed on healthy non-inoculated leaves measuring 2 cm x 5 cm per day per larvae and replaced with a fresh new one everyday. The treatments of this experiment consisted of the six fungal isolates and the control (water) and used the completely randomized block designs. The variables were recorded were development time of each stage (egg, larval, pupal, and adult), and mortality of each stage. The larval and pupal mortality were recorded daily, and the adults emerging were observed everyday. The sex of adults emerging were recorded and the adults were placed in the wire mesh cage for copulation with fresh maize leaves inside to allow egg-laying. Egg collection and 10% honey bee solution replacement for adults were carried out everyday. The adult longevity was also observed until the adult death.

Data analysis

The differences in the length of different stages (egg, larval, pupal, and adult), mortality of each stage, adult longevity, eggs laid, and sex ratio of each treatment 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

Identification results of the endophytic fungal isolates

Five fungal isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) had the same macroscopic and microscopic characteristics, while an isolate (WTTJC260521B) had different characteristics (Fig. 1). The WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A isolates had a white colonial, hyphal, and mycelia color, and non-septate and globose conidia or spores. These characteristics were placed the isolates within the group of *B. bassiana* (Fig. 2). The isolates were deposited in the GenBank with the accession number ON631784 (JGTP240521A isolate), ON631780 (WTTJC260521A isolate), ON631783 (WTTJC290521A isolate), ON631782 (WTTJC290521B isolate), ON631778 (JGNT300521 isolate) (Table 1). The isolate of WTTJC260521B had a white young colony, then the older colony turned greenish white to dark green, and the isolate had the green hyphae and mycelia, the isolate had the non-septation, clear, and cylindrical conidia. The isolate was placed within the group of *M. anisopliae* (Fig. 2). The WTTJC260521B isolate was deposited in the GenBank with the accession number ON631793 (WTTJC260521B isolate) (Table 1).

The result of BLAST search revealed that five fungal isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) shared 100% of similarity each other as well as with JgSPK (Acc. No. MZ356494.1). All the isolates shared 99.80% of similarity with JGNT300521 as well as with BSwTd4 (Acc. No. MT448732.1). JGNT300521 isolate shared 99.61% of similarity with BSwTd4. All the isolates shared 98.83% of similarity with B1 UNILA (=NKPT) (Acc. No. LC413808.1) and type strain on *B. bassiana* (ARSEF 1564.T., Acc. No. HQ880761.1). Isolate of WTTJC260521B shared 99.992% of similarity with reference isolate of IPPM010202 (Acc. No. KY437678.1), 99.994% of similarity with strain MSwTp3 (Acc. No. MT448733.1), and 99.985% of similarity with the type strain of *M. anisopliae* ARSEF 7487.T (Acc. No. HQ331446.1). So, there were two 2 species from the 6 investigated isolates of the endophytic fungi found in this study. The five isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) were in the group of *B. bassiana*, the one isolate (WTTJC260521B) was in the group of *M. anisopliae.*

The development of *Spodoptera frugiperda* fed on young maize colonized and non-colonized by fungi

Six fungal isolates found and used in this experiment were able to colonize young maize (seedling) when inoculated by seed immersion treatment, and a percentage of leaves colonized by endophytic-entomopathogenic fungi was higher at 14 days than at 7 days after inoculation (Table 2). They were confirmed as endophytic fungi. The seed immersion treatment resulted the leaves of treated young maize grown on to the SDA medium were overgrown with the fungal isolates (Fig. 3). No fungal growth was found on the leaves of untreated maize and on the last rinse water. This confirmed that the fungal isolates used in this study were endophytic fungi and it also showed that the surface-sterilization of maize tissues eliminated the epiphytic microorganisms and the fungi growing out of the leaf surface were the endophytic fungi originating from within the maize tissues.

The larvae that consumed leaves of colonized maize exhibited distinctive symptoms, namely smaller body, shrivels, hardens like a mummy, but the healthy larvae of the control were longer and bigger than treated larvae. The cadavers were covered with mycelia and conidia and their colors depending of the fungal species (Fig. 4). The color of cadavers from larvae that consumed leaves colonized by *B. bassiana* and *M. anisopliae* was white and green, respectively. Re-isolation of the fungus from the cadavers showed that the same fungal isolates found from the larvae that died after feeding on the leaves of the plants where seed treatment was given (Fig 4).

Feeding on leaves of fungal colonized maize significantly increased development time of the second instar ($P<0.0001$), third instar ($P<0.0001$), fourth instar ($P<0.0001$), fifth instar ($P<0.0001$), and sixth instar ($P<0.0001$) (Table 3). However, there is no significant difference in the development time of first instar of treated and untreated maize (control). This fungal colonized maize also increased egg and pupal development time, but decreased female and male adult longevity. The lifespan of *S. frugiperda* fed on leaves of fungal colonized maize was significantly longer compared to those fed on leaves of non-colonized maize (Table 4). The longest lifespan of *S. frugiperda* occurred on insects feeding on leaves of *B. bassiana* colonized maize.

The fungal colonized young maize significantly increased mortality of all instar larvae compared to non-colonized one (Table 5). The last instar mortality treated with *B. bassiana* (JGTP240521A isolates) (51.33%) was the highest among other treatments and did not significantly differ from each of the *B. bassiana* (WTTJC260521A and WTTJC290521A isolates) (45.33% and 44.67%, respectively). Feeding on leaves of fungal colonized maize significantly decreased the percentage of the last instar becoming pupal stage and adult

emergence (Table 6). The young maize colonized with fungi significantly reduced eggs laid by the adults (fecundity), but did not effect the sex ratio of *S. frugiperda*. Percentage of hatched eggs significantly decreased on the treatment of *B. bassiana* (JGTP240521A, WTTJC260521A, WTTJC290521A, and WTTJC290521B isolates) (Table 6).

Discussion

The results of identification based on the morphological characters of five isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) showed that they have similar morphology of the colony, hypha, and mycelia, and the conidial shape. They belong to species of *B. bassiana*. These characters match to *B. bassiana* described by Herlinda *et al.* (2021). The isolate of WTTJC260521B belongs to species of *M. anisopliae*. The isolate morphology of the colony, and the hyphal, mycelia, and conidial of shape matches to *M. anisopliae* described by Herlinda *et al.* (2020).

The five fungal isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) found in this study had an rDNA sequence similarity value of 100% to the reference species (BLAST), JgSPK (Acc. No. MZ356494.1) an endophytic fungi isolated from maize (Herlinda *et al.* 2021) except one isolate (JGNT300521). If the similarity value is 100%, It means that they are the same strain (Henry *et al.* 2000). These isolates shared 99.80% of similarity with JGNT300521 as well as with BSwTd4 (Acc. No. MT448732.1) which was isolated from oil palm rhizosphere (Herlinda *et al.* 2020). JGNT300521 isolate shared 99.61% of similarity with BSwTd4. All the isolates shared 98.83% of similarity with B1 UNILA (=NKPT) (Acc. No. LC413808.1) which was isolated from maize rhizosphere (Fitriana *et al.* 2021) and type strain on *B. bassiana* (ARSEF 1564.T., Acc. No. HQ880761.1). The isolate of WTTJC260521B had an rDNA sequence similarity value of more than 99% to the BLAST (reference species). The similarity value of 99-100% indicates that the isolates are the same species (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). If the similarity value of the isolates is 89–99%, it means that the isolates are the same genus (Henry *et al.* 2000).

All fungal isolates of the *B. bassiana* and *M. anisopliae* tested in this study were able to colonize leaves of young maize when inoculated by seed immersion treatment. From the leaves of young maize treated by the fungi were overgrown with the fungal mycelia, but no fungal mycelia were found on the leaves of untreated seeds. Our finding showed that the both species of fungi from seed treatment are able to colonize the leaves. In addition to, the fungi of *B. bassiana* and *M. anisopliae* not only can colonize maize by seed treatment, but they also can colonize maize by foliar spray and root deeping and the fungi can systemically colonized leaves, stems, and roots of plants (Russo *et al.* 2020). *B. bassiana* inoculated by foliar spray can penetrate the leaf surface and move within the maize vascular (Wagner and Lewis 2000). *M. anisopliae* was often reported to be restrited to plant roots (Russo *et al.* 2020), however our study reported that the strain of *M. anisopliae* in this study is able to colonize the leaves of young maize. The extent and persistence of plant colonization by the fungi were influenced by fungal species/strain, inoculation method, and host plant species (Russo *et al.* 2020). The fungi used for seed treatment in this study is easier to be applied by soaking the seeds in fungal suspension before planting, or when the seeds stored, dry conidia of the fungi can be covered on the seeds.

Our other finding is that *B. bassiana* and *M. anisopliae* from South Sumatra (Indonesia) in seed treated corn seedlings have negative effects on development of *S. frugiperda*. This is first report that the fungi as an endophyte could decrease the female and male adult longevity of *S. frugiperda* and increased the larval mortality. The young maize colonized with the *B. bassiana* and *M. anisopliae* also reduced the percentage of the last instar becoming pupal stage and adult

emergence, and decreased the egg laid by the adults and the percentage of hatched eggs. Previous study reported that *B. bassiana* and *M. anisopliae* in foliar treated caused adverse effects on *S. frugiperda* development and survival (Russo *et al.* 2020). These adverse effects of endophytic fungi against *S. frugiperda* were caused by fungal production of secondary metabolites and mycosis (Vidal and Jaber 2015). The fungal secondary metabolites are produced by blastospores in insect hemolymph and disrupted the normal cell metabolism (Mancillas-Paredes *et al.* 2019) and then the toxins produced by the metabolites kill the insects (El-Ghany 2015). Our previous study showed that the endophytic fungi could decrease the leaf consumption by the *S. frugiperda* larvae resulting in larval weight loss and low survival (Gustianingtyas *et al.* 2021). The reduction in leaf area consumed by the larvae treated with the endophytes is caused by antifeedant or deterrent properties of *in planta*-produced *B. bassiana* metabolites (Russo *et al.* 2020). The corn plants colonized with *B. bassiana* may enhance levels of terpenoid defense compounds against *S. frugiperda* (Russo *et al.* 2020). The endophytic fungi could produce secondary metabolites *in planta* resulting antibiosis and feeding deterrence for the insects (Jaber and Ownley 2018).

These adverse effects of endophytic fungi against *S. frugiperda* were also caused by mycosis (Vidal and Jaber 2015). This research found that the mycosis found on the cadavers of *S. frugiperda* treated with the fungi. The mycosis was evidenced by fungal mycelia and spores emerging from the cadavers of treated insects. However, no fungal mycelia and spores were found on the cadavers of untreated insects. Some previous studies have similar reported insect mycosis feeding on fungal-endophytically colonized plants by *S. frugiperda* (Russo *et al.* 2020; Herlinda *et al.* 2021).

This study also showed that the fungal colonized young maize increased egg, larvae, pupal development time, and lifespan of *S. frugiperda.* In contrast to the previous study of Russo *et al.* (2020) that these fungal species could decreased the development time of *S. frugiperda*. However, our findings are in agreement with previous study of Hussain *et al.* (2009) showing that the lepidopteran, *Ocinara varians* treated with *B. bassiana* and *M. anisopliae* extended the developmental time of treated insects as compared to untreated ones (control) and the conversion of digested food and ingested food declined in treated insects compared to untreated insects, and stimulated the larvae to develop more slowly.

Conclusions

The results of molecular identification showed that the fungal species found were *B. bassiana* of five fungal isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) and *M. anisopliae* of an isolate (WTTJC260521B). The *B. bassiana* and *M. anisopliae* colonized young maize significantly increased mortality of all instar larvae compared to non-colonized one. The larval mortality treated with *B. bassiana* (JGTP240521A isolates) (51.33%) was the highest among other treatments. Feeding on leaves of fungal colonized maize significantly decreased the percentage of the last instar becoming pupal stage and the adult emergence and the eggs laid, and the percentage of hatched eggs and increased the larval mortality. This is first report that the *B. bassiana* and *M. anisopliae* from South Sumatra (Indonesia) in seed treated corn seedlings have negative effects on development of *S. frugiperda.* Finally, these results highlight the potential of endophytic entomopathogenic fungi to protect corn plants against *S. frugiperda.*

List of abbreviations

ANOVA: analysis of variance; BLAST: Basic Local Alignment Search Tool; CTAB: cetyltrimethylammonium bromide; DNA: Deoxyribonucleic acid; EtOH: Ethyl alcohol; FAW: fall armyworm; 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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Table 1 Origin of isolates of endophytic-entomopathogenic 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	*Spodoptera frugiperda*	35.0	*Beauveria bassiana*	JGTP240521A	ON631784
Tanjung Cermin, Pagar Alam	Lepidoptera	817.2	*Beauveria bassiana*	WTTJC260521A	ON631780
Tanjung Cermin, Pagar Alam	Lepidoptera	817.2	*Metarhizium anisopliae*	WTTJC260521B	ON631793
Tanjung Cermin, Pagar Alam	Lepidoptera	817.2	*Beauveria bassiana*	WTTJC290521A	ON631783
Tanjung Cermin, Pagar Alam	Lepidoptera	817.2	*Beauveria bassiana*	WTTJC290521B	ON631782
Nendagung, Pagar Alam	*Spodoptera frugiperda*	802.6	*Beauveria bassiana*	JGNT300521	ON631778

Table 2 Effect of fungal isolates on mean colonization (%) or percentage of leaves colonized by endophytic-entomopathogenic fungi at 7 and 14 days after inoculation

		Mean colonization (%)	
Isolate	Species	7 days after inoculation	14 days after inoculation
Control	-	0.00b	0.00c
JGTP240521A	*Beauveria bassiana*	100.00a	100.00a
WTTJC260521A	*Beauveria bassiana*	93.33a	100.00a
WTTJC260521B	*Metarhizium anisopliae*	26.67b	60.00b
WTTJC290521A	*Beauveria bassiana*	100.00a	100.00a
WTTJC290521B	*Beauveria bassiana*	80.00a	100.00a
JGNT300521	*Beauveria bassiana*	80.00a	100.00a
F-value		26.31^{**}	168.50^{**}
P-value		3.07×10^{-6}	7.16×10^{-11}
HSD value		32.16	13.07

Table 3 Length of different developmental stages of instar larvae of *Spodoptera frugiperda* fed on leaves of endophytic fungi colonized (seed treated) and non-colonized (control) young maize

		Length of different developmental stages (days)					
Isolate	Species	1st larvae	2nd larvae	3rd larvae	4th larvae	5th larvae	6th larvae
Control	-	2.67	3.34c	2.36d	2.27c	3.26b	3.23b
JGTP240521A	*Beauveria bassiana*	2.71	3.66b	5.70a	4.45a	3.95a	3.86ab
WTTJC260521A	*Beauveria bassiana*	2.59	3.71b	4.00b	4.60a	2.99b	3.76ab
WTTJC260521B	*Metarhizium anisopliae*	2.63	3.68b	2.66d	3.71b	3.65ab	3.51ab
WTTJC290521A	*Beauveria bassiana*	2.63	3.71b	3.63a	4.46a	3.69ab	4.28a
WTTJC290521B	*Beauveria bassiana*	2.60	4.28a	5.46a	3.79b	3.28ab	3.13b
JGNT300521	*Beauveria bassiana*	2.65	3.64b	3.62c	2.27c	3.37ab	3.57ab
F-value		2.37^{ns}	23.40^{*}	292.73^{*}	296.38^{*}	4.26^{*}	5.22^{*}
P-value		0.10	<0.0001	<0.0001	<0.0001	0.02	0.007
HSD value		-	0.07	0.90	0.07	0.22	0.21

Note: ns = not significantly differen * = 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

Tabel 4 Length of different developmental stages of pupae and adults of *Spodoptera frugiperda* fed on leaves of endophytic fungi colonized (seed treated) and non-colonized (control) young maize

		Length of different developmental stages (days)					
Isolate	Species	Prepupae	Pupae	Female adult	Male adult	Egg	Total lifespan
Control	-	3.61	6.95b	4.82a	4.40a	2.59c	32.51d
JGTP240521A	*Beauveria bassiana*	3.68	9.71a	4.37ab	3.28b	3.22abc	41.62a

WTTJC260521A	*Beauveria bassiana*	3.11	10.24a	4.21ab	3.40b	2.74bc	39.21ab
WTTJC260521B	*Metarhizium anisopliae*	3.65	7.43b	4.52ab	3.63b	2.84bc	35.44c
WTTJC290521A	*Beauveria bassiana*	3.79	9.85a	3.99b	4.34a	3.39ab	40.05a
WTTJC290521B	*Beauveria bassiana*	3.22	10.58a	4.23ab	3.52b	3.27abc	40.57a
JGNT300521	*Beauveria bassiana*	3.71	9.49a	4.93a	4.58a	3.65a	37.25bc
F-value		0.95^{ns}	22.43^{*}	5.12^{*}	49.86^{*}	6.39^{*}	34.57^{*}
P-value		0.50	<0.0001	0.008	<0.0001	0.003	<0.0001
HSD value		-	1.47	0.17	0.09	0.19	0.22

Note: ns = not significantly differen * = 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

Table 5 Mean of mortality of different instar larvae of *Spodoptera frugiperda* fed on leaves of endophytic fungi colonized (seed treated) and non-colonized (control) young maize

		Mean of mortality of different instar larvae (%)					
Isolate	Species	1st larvae	2nd larvae	3rd larvae	4th larvae	5th larvae	6th larvae
Control	-	2.67	6.00b	6.67e	6.67c	6.67c	6.67e
JGTP240521A	*Beauveria bassiana*	14.67	24.00a	43.33a	48.67a	51.33a	51.33a
WTTJC260521A	*Beauveria bassiana*	8.00	21.33a	36.67ab	39.33ab	42.00ab	45.33ab
WTTJC260521B	*Metarhizium anisopliae*	2.67	5.33b	8.00de	12.00c	15.33c	24.67d
WTTJC290521A	*Beauveria bassiana*	7.33	11.33ab	20.00cd	30.67b	36.00b	44.67ab
WTTJC290521B	*Beauveria bassiana*	8.67	16.67ab	22.67bc	27.33b	29.33b	38.67bc
JGNT300521	*Beauveria bassiana*	8.00	16.00ab	21.33bc	26.67b	28.00b	32.67c
F-value		2.93^{ns}	7.74^{*}	22.96^{*}	27.02^{*}	35.02^{*}	176.07^{*}
P-value		0.053	0.001	<0.0001	<0.0001	<0.0001	<0.0001
HSD value		-	10.95	10.00	9.64	8.74	3.90

Note: ns = not significantly differen * = 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

Table 6 Mean of percentage of pupae and adults emergence, sex ratio, egg laid, and viable eggs of *Spodoptera frugiperda* fed on leaves of endophytic fungi colonized (seed treated) and non-colonized (control) young maize

Isolates	Fungal species	Pupae emergence (%)	Adult emergence (%)	Sex ratio	Eggs laid per female	Viable (hatched) eggs (%)
Control		93.33a	93.33a	0.56a	68.28a	99.92a
JGTP240521A	*Beauveria bassiana*	46.00e	42.00e	0.83a	15.91c	88.86d
WTTJC260521A	*Beauveria bassiana*	52.67de	48.00de	0.84a	15.31c	90.93cd
WTTJC260521B	*Metarhizium anisopliae*	74.67b	71.33b	0.47a	42.89b	99.72a
WTTJC290521A	*Beauveria bassiana*	54.00d	47.33de	0.74a	27.86bc	95.91b
WTTJC290521B	*Beauveria bassiana*	59.33cd	54.00cd	0.87a	17.50c	92.98bc
JGNT300521	*Beauveria bassiana*	65.33c	58.67c	0.51a	39.36b	99.31a
F-value		134.80^{*}	95.08^{*}	3.67^{*}	34.26^{*}	75.47^{*}
P-value		<0.0001	<0.0001	0.026	<0.0001	<0.0001
HSD value		6.85	9.03	0.19	1.37	4.16

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

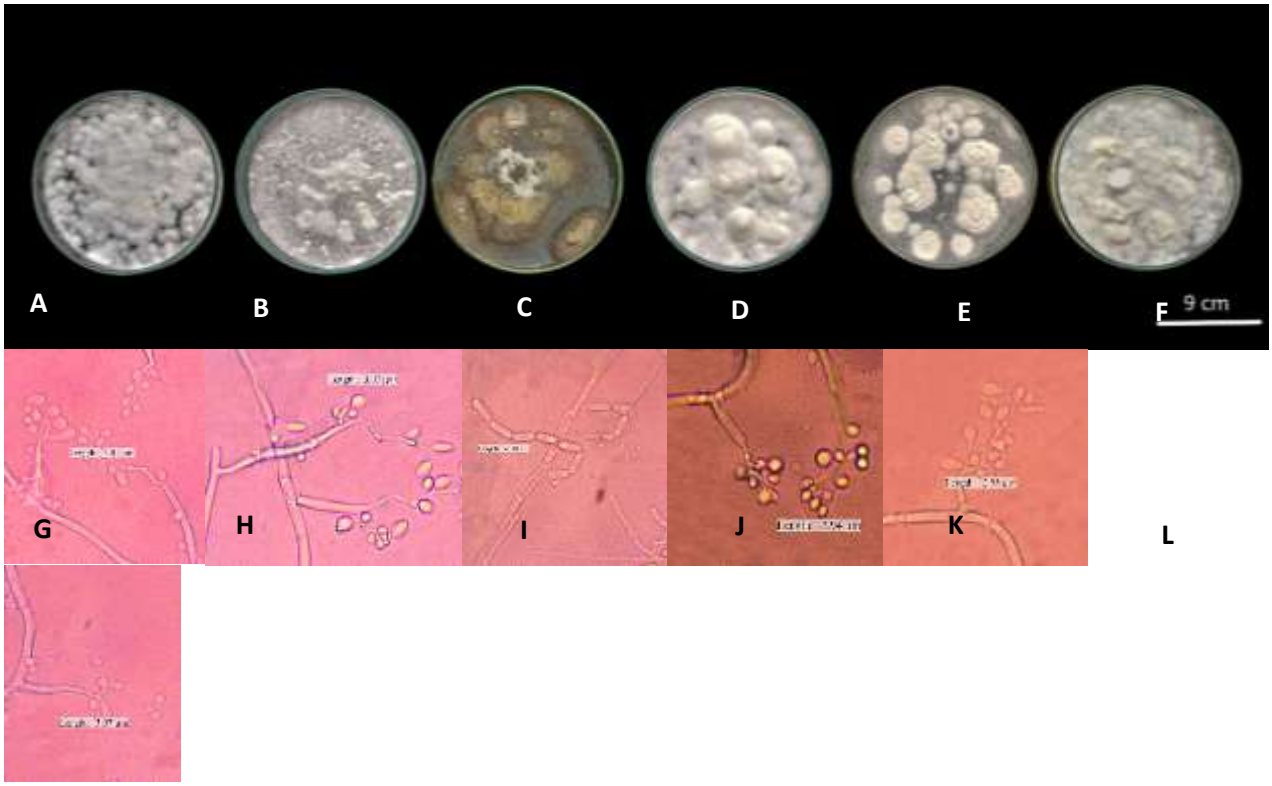


Fig. 1. Colony morphology of endophytic fungi cultured on PDA media (above) and the conidial and hyphal morphology (below) of the fungi: *Beauveria bassiana* of JGTP240521 isolate (A and G) and WTTJC260521A isolate (B and H), *Metarhizium anisoplae* WTTJC260521B isolate (C and I), *Beauveria bassiana* of WTTJC290521A isolate (D and J), WTTJC290521B isolate (E and K), and JGNT300521 isolate (F and L)

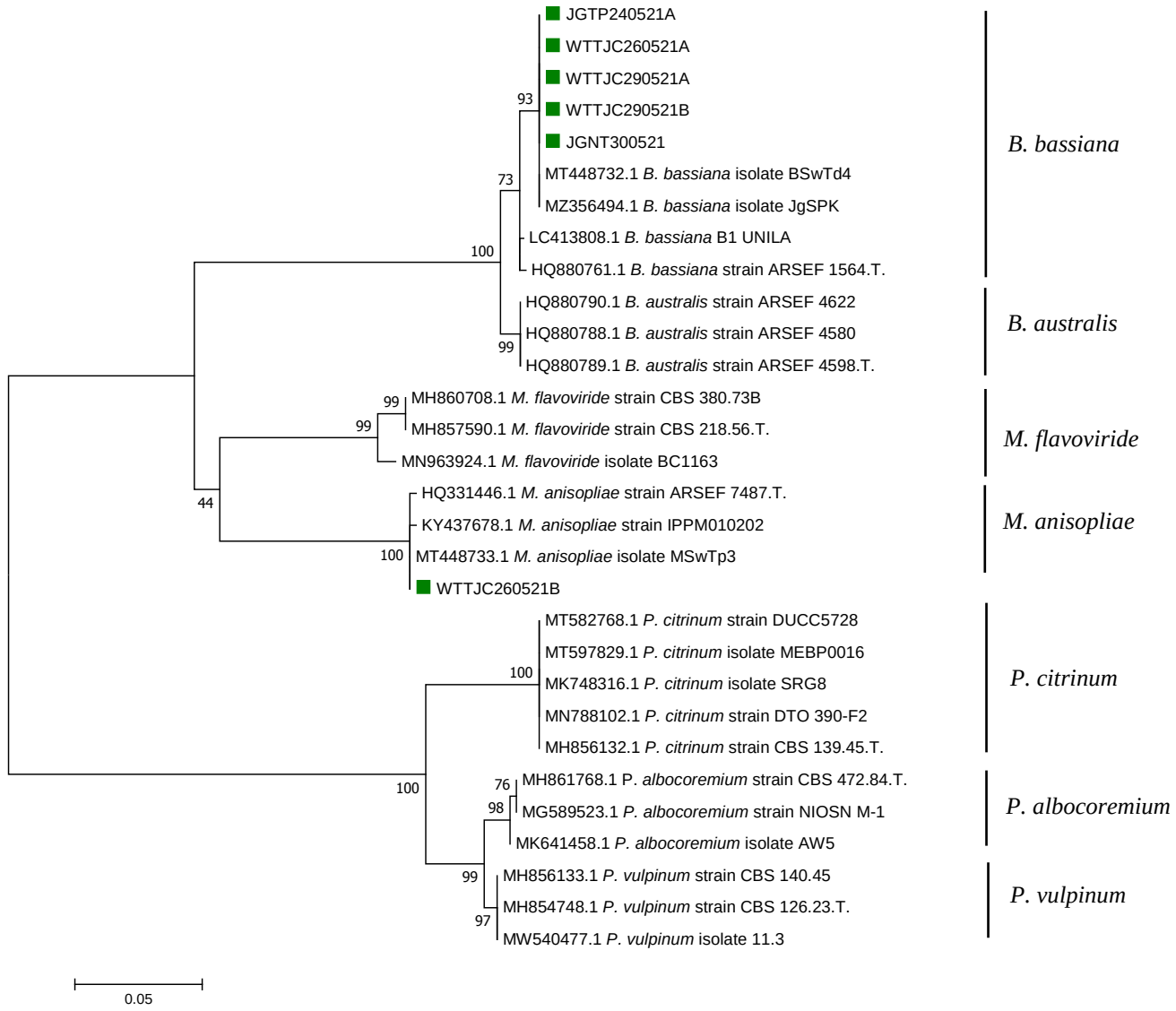


Fig. 2. Phylogenetic tree developed based on Internal Transcribed Spacer (ITS) region by Maximum Likelihood (Tamura-Nei model) using Mega7 for windows (Kumar *et al.* 2016). The six investigated isolates were placed within group of *Beauveria bassiana* (5 isolates) and *Metarhizium anisopliae* (1 isolate). T= Type isolate

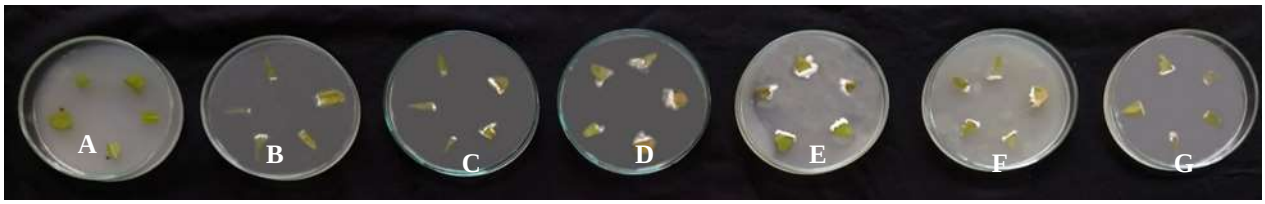
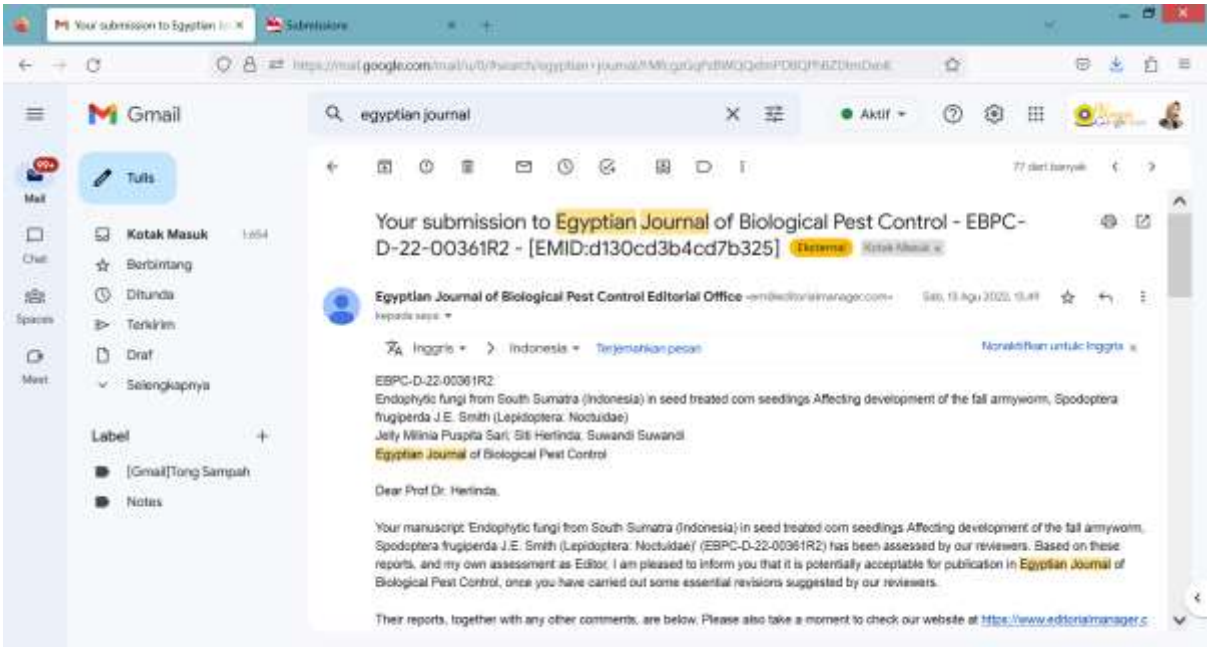
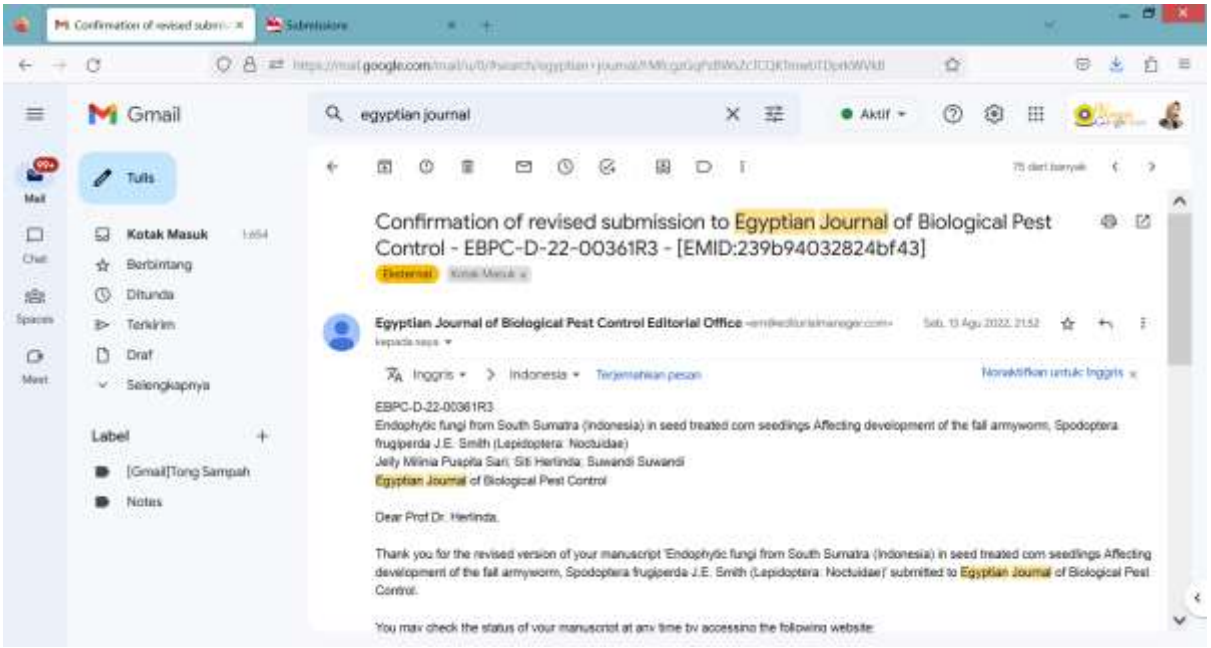


Fig. 3. Colony morphology of endophytic fungi from the leaves of maize where seed treatment was given and control (untreated seeds): Control (A), *Beauveria bassiana* of JGTP240521 isolate (B) and WTTJC260521A isolate (C), *Metarhizium anisoplae* WTTJC260521B isolate (D), *Beauveria bassiana* of WTTJC290521A isolate (E), WTTJC290521B isolate (F), and JGNT300521 isolate (G)

Fig. 4. The morphology of healthy larvae (control) (A), and the cadavers from larvae feeding on leaves colonized by fungi (above) and the conidial and hyphal morphology of fungi from cadaver re-isolation (below): *Beauveria bassiana* of JGTP240521 isolate (B and H) and WTTJC260521A isolate (C and I), *Metarhizium anisoplae* WTTJC260521B isolate (D and J), *Beauveria bassiana* of WTTJC290521A isolate (E and K), WTTJC290521B isolate (F and L), and JGNT300521 isolate (G and M)

3. Bukti konfirmasi review kedua dan hasil revisi kedua





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Endophytic fungi from South Sumatra (Indonesia) in seed treated corn seedlings Affecting development of the fall armyworm, Spodoptera frugiperda J.E. Smith (Lepidoptera: Noctuidae)

--Manuscript Draft--

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Abstract:	Background: Topical application of the entomopathogenic fungi (EPFs) against Spodoptera frugiperda J.E. Smith (Lepidoptera: Noctuidae) larvae is less effective due to larvae hiding in the corn midribs in the field. To control the larvae, the fungi colonize in plant tissues or endophytic fungi are needed. There is no information on the pathogenicity of the endophytic fungi from Indonesia on the development of S. frugiperda. The endophytic fungi isolated from infected-host cadavers from South Sumatra (Indonesia) were identified morphologically and molecularly and the effect of seed treated corn seedlings with the fungi on S. frugiperda development was evaluated. The fungal identification was based on morphological and molecular characteristics. Bioassay of the endophytic fungal species in seed treated young maize was performed against the neonate larvae (hatching within 24 hrs.) of first instar and their development were observed. Results: The results of molecular identification showed that the fungal species were Beauveria bassiana of five fungal isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) and Metarhizium anisopliae of an isolate (WTTJC260521B). The lifespan of S. frugiperda fed on leaves of fungal colonized maize was significantly longer than those fed on leaves of non-colonized maize. The fungal colonized young maize significantly increased mortality rate of all larval instar than non-colonized one. The last instar larvae mortality treated with B. bassiana (JGTP240521A isolates) (51.33%) was the highest among other treatments and did not significantly differ from each of B. bassiana of WTTJC260521A and WTTJC290521A isolates 45.33% and 44.67%, respectively. Feeding on leaves of fungal colonized maize significantly decreased the percentage of the last instar larvae development to the pupal stage, the adult emergence, the eggs laid, and the percentage of hatched eggs. This is first report that B. bassiana and M. anisopliae from South Sumatra (Indonesia) in seed treated corn seedlings had negative effects on development of S. frugiperda. Conclusions: Finally, these results highlight the potential of endophytic EPFs to protect corn plants against S. frugiperda. Keywords: Spodoptera frugiperda, Beauveria bassiana, Metarhizium anisopliae, Endophyte, Entomopathogens
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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 from South Sumatra (Indonesia) in seed treated corn seedlings Affecting development of the fall armyworm, *Spodoptera frugiperda* J.E. Smith (Lepidoptera: Noctuidae)

Abstract

Background: Topical application of the entomopathogenic fungi (EPFs) against *Spodoptera frugiperda* J.E. Smith (Lepidoptera: Noctuidae) larvae is less effective due to larvae hiding in the corn midribs in the field. To control the larvae, the fungi colonize in plant tissues or endophytic fungi are needed. There is no information on the pathogenicity of the endophytic fungi from Indonesia on the development of *S. frugiperda*. The endophytic fungi isolated from infected-host cadavers from South Sumatra (Indonesia) were identified morphologically and molecularly and the effect of seed treated corn seedlings with the fungi on *S. frugiperda* development was evaluated. The fungal identification was based on morphological and molecular characteristics. Bioassay of the endophytic fungal species in seed treated young maize was performed against the neonate larvae (hatching within 24 hrs.) of first instar and their development were observed.

Results: The results of molecular identification showed that the fungal species were *Beauveria bassiana* of five fungal isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) and *Metarhizium anisopliae* of an isolate (WTTJC260521B). The lifespan of *S. frugiperda* fed on leaves of fungal colonized maize was significantly longer than those fed on leaves of non-colonized maize. The fungal colonized young maize significantly increased mortality rate of all larval instar than non-colonized one. The last instar larvae mortality treated with *B. bassiana* (JGTP240521A isolates) (51.33%) was the highest among other treatments and did not significantly differ from each of *B. bassiana* of WTTJC260521A and WTTJC290521A isolates 45.33% and 44.67%, respectively. Feeding on leaves of fungal colonized maize significantly decreased the percentage of the last instar larvae development to the pupal stage, the adult emergence, the eggs laid, and the percentage of hatched eggs. This is first report that *B. bassiana* and *M. anisopliae* from South Sumatra (Indonesia) in seed treated corn seedlings had negative effects on development of *S. frugiperda.*

Conclusions: Finally, these results highlight the potential of endophytic EPFs to protect corn plants against *S. frugiperda.*

Keywords: *Spodoptera frugiperda*, *Beauveria bassiana*, *Metarhizium anisopliae*, Endophyte, Entomopathogens,

Background

Spodoptera frugiperda J.E. Smith (Lepidoptera: Noctuidae) or fall armyworm (FAW) is one of the most important noctuid pests of corn in the world. The FAW is a migratory and polyphagous pest that can attack 353 host plant species from 76 plant families (Montezano *et al.* 2018). The pest is native to the neotropics of the Americas and has spread throughout the world (Otim *et al.* 2018). More recently, the FAW becomes a new invasive pest in many parts of Africa (Niassy *et al.* 2021) and Asia (Lamsal *et al.* 2020), including Indonesia (Herlinda *et al.* 2021). This pest is commonly controled using synthetic insecticides (Kumela *et al.* 2018), however the resistances of the FAW to many insecticides, such as pyrethroid, spinosad, and organophosphorus insecticides have occured (Zhang *et al.* 2021). In addition, the insecticide

application negatively affects the human health and the environment (Harrison *et al.* 2019). An alternative more sustainable and eco-friendly control methods against *S. frugiperda* is urgently needed.

Biological control based on utilizing EPFs is the preferred control option for FAW (Mantzoukas and Eliopoulos 2020). Topical application (direct contact) of the EPF, *Beauveria bassiana* (Balsamo) Vuillemin (Deuteromycotina: Hypomycetes) killed more than 80% of *S. frugiperda* larvae (Ramanujam *et al.* 2020). *Metarhizium anisopliae* (Metsch.) Sorok. (Deuteromycotina: Hypomycetes) could kill 75% of *S. frugiperda* larvae (Ramos *et al.* 2020). However, in the field, the larvae occured on the surface of leaves or maize stalks only in the morning but at daylight up to night, they hide in the corn midribs (Herlinda *et al.* 2021). So, topical application of the fungus against the *S. frugiperda* larvae is less effective (Gustianingtyas *et al.* 2021). To control such hiding larvae in the field, the fungi colonizing in plant tissues or endophytic fungi are needed (Ramos *et al.* 2020). The endophytic fungi associate mutually with their host plants (Lira *et al.* 2020) and can stimulate the plant growth but suppress the insect pest growth (Russo *et al.* 2020).

Previous studies have demonstrated that the endophytic fungal *B. bassiana* from corn root from Indonesia applied topically caused 29.33% of the FAW larval mortality (Gustianingtyas *et al.* 2021). The endophytic fungal *B. bassiana* used as seed treatment caused up to 22.67% of the FAW larval mortality (Herlinda *et al.* 2021). An experiment has previously also reported that endophytic *B. bassiana* sprayed on leaves has a high ability to colonize corn plants and the fungus caused significant reductions in the growth and development of *S. frugiperda* (Russo *et al.* 2020). There is no information on the pathogenicity of the endophytic fungi from Indonesia on the development of *S. frugiperda*. In addition, the potential of the fungi isolated from the infected-host cadaver as endophytic entomopathogens needs to be investigated. In this study, the fungi isolated from infected-host cadavers from South Sumatra (Indonesia) were identified morphologically and molecularly and the effect of seed treated corn seedlings with the fungi on *S. frugiperda* development was evaluated.

Methods

Exploration of the fungi was performed by collecting infected-host insect cadavers from crops in South Sumatra, Indonesia from May until June 2021. Purification and isolation of the fungi were carried out from June to July 2021. The morphological identification was carried out in the Laboratory of Entomology, Faculty of Agriculture, Universitas Sriwijaya in July 2021 and the molecular identification was performed from August to December 2021 at the Laboratory of Agricultural Biotechnology (accredited according to the ISO/IEC 17025 standard), Department of Plant Protection, Faculty of Agriculture, Universitas Lampung, Indonesia. Experimental design used for bioassay was a completely randomized block designs consisted of seven treatments (six fungal isolates and control), and the experiment was repeated three times.

Fungal Exploration, isolation, and purification

Fungal exploration from the infected-host cadavers using the method of Ab Majid *et al.* (2015) by collecting infected-host insects or cadaver infected with the fungi from the fields. The exploration was carried out in Tanjung Pering, Ogan Ilir, South Sumatra (3°13′23″S104°38′27″E), Tanjung Cermin, Pagar Alam, South Sumatra (4°02′23″S103°13″14″E), and Nendagung, Pagar Alam, South Sumatra (3°56′22″S103°12′15″E) (Table 1). The infected

insects or cadavers were first surface sterilized with 70% EtOH (Ethyl alcohol) and 1% NaOCl (Sodium hypochlorite), then rinsed 3 times (Elfita *et al.* 2019). After that, the sample cadavers were cultured aseptically onto SDA (Sabouraud Dextrose Agar) medium (Russo *et al.* 2020). The fungal culture was purified to make an isolate per sample. The fungal macroscopic and microscopic characteristics, such as the colonial color and shape, the conidial shape and size, and the conidiophores were observed (Herlinda *et al.* 2021) and then molecular identification was performed.

DNA extraction, PCR amplification, and sequencing

DNA was extracted according to the method of Swibawa *et al.* (2020) and carried out on fungal conidia of 7 days old fungus. As much as 10 ml of conidia suspension was centrifuged using CF15RXII for 10 min at a speed of 14,000 rpm. Then, 1 ml of 70% ethanol was added to the centrifuge tube and centrifuged again for 10 min. The supernatant was removed and 1 ml of extraction buffer (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) was added. The suspension was incubated at -40 °C for 24 hrs. The frozen suspension was crushed until pulverized. A total of 500 µl of pellet suspension was put into a 1.5 ml tube and 400 µl of 2% CTAB (cetyltrimethylammonium bromide) was added, homogenized and heated at 65 °C for an hour using a water bath (Brookfield TC 550 MX-230, USA). After the incubation, 500 µl of PCI (Phenol Chloroform Isoamyl alcohol) (25: 24: 1) was added, homogenized and centrifuged at 14,000 rpm for 10 minutes at 14,000 rpm for 10 min. A total of 600 µl supernatant was transferred to a new 1.5 ml tube, and 600 µl Chloroform Isoamyl Alcohol (24:1) was added, homogenized, and centrifuged (Microspin12; Biosan, Latvia) again at 14,000 rpm for 10 minutes. A total of 400 µl of supernatant was then put into to a new 1.5 ml tube and 400 µl of cold isopropanol was homogenized and incubated at -40 ºC for 20 minutes. Then, the suspension was centrifuged at 14,000 rpm for 15 min. The supernatant was then discarded, and the pellet was added with 500 µl of 70% cold ethanol and centrifuged at 14,000 rpm for 5 minutes. The supernatant was then discarded and the pellets obtained were incubated at room temperature for 24 hrs to dry. After drying, the pellets were 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 ITS (the Internal Transcribed Spacer) using ITS1 and ITS4 primers (White *et al.* 1990). The DNA amplification stage consisted of 1 initiation cycle at 95 ºC for 5 min, 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. Then, the PCR results were 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 under taken in 1x TBE buffer solution at 50 volts for 70 min, and the results were visualized using a DigiDoc UV transilluminator (UVP, USA).

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

strains used as a reference in this study were obtained from NCBI (https://www.ncbi.nlm.nih.gov/).

Mass-rearing of *Spodoptera frugiperda*

The mass-rearing of *S. frugiperda* was performed using the method of Herlinda *et al.* (2020). The eggs of *S. frugiperda* were obtained from the Laboratory of Entomology, Faculty of Agriculture, Universitas Sriwijaya. They were reared in laboratory for more than 5 generations at 28–29°C temperature, and 82–83% RH and the lighting set to photoperiod 12:12 (L:D) hrs. In the laboratory, the larvae of *S. frugiperda* were maintained individually due to cannibal behaviours and reared using fresh maize leaves. The prepupae and pupae were replaced 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. Emerged adults were used for bioassays.

Assessing endophytic fungal colonization

Fungal inoculation for maize seeds treated was carried out to assess the ability of the fungal colonization into the maize seedling tissue and to ensure that the fungi used in this experiment were truly endophytic. All the isolates used were grown in SDA medium incubated for 14 days, then the SDA fungal culture was transferred to the broth medium (SDB, Sabouraud Dextrose Broth) following the method of Gustianingtyas *et al.* (2020) and incubated for 7 days on the shaker and 7 days unshaken position. The 45 corn seeds for an isolate were surface sterilized by using (Russo *et al.* 2020) method. The seeds were immersed in 10 ml of fungal suspension (1 x 10^{10} conidia ml^{-1}) for 24 hrs, while for the control only 10 ml of sterilized water was treated for the seeds. Then, the seeds were grown in the hydroponic medium, following the method of Novianti *et al.* (2020) and incubated for 14 days and this treatment was repeated 3 times for each isolate. The tip leaves of 14-day old maize seedlings (young maize) were cut of 5 x 5 mm^2 to be grown onto the SDA medium to detect the mycelia of the endophytic fungi. The leaf materials were first surface-sterilized by using method of (Russo *et al.* 2020) before grown onto the SDA medium. The leaf material surface-sterilized was carried out by immersion in 70% ethanol, then followed by sodium hypochlorite for 2 minutes, and rinsed twice in sterile distilled water and the final rinse water was grown onto SDA and incubated for 10 days. The rest or remaining leaves were used for bioassays as described below.

Bioassay for assessing effect of corn seed treatment on *S. frugiperda* development

The bioassay for assessing the effect of corn seed treatment on *S. frugiperda* growth and development, followed the method of Russo *et al.* (2020). The 14-day old maize seedlings already inoculated with the endophytic fungi as described above were given to be consumed to the first instar neonate larvae of *S. frugiperda*, while for control treatment, the larvae were provided the non-inoculated young maize and this experiment was repeated three times. The 50 neonate larvae (hatching within 24 hrs.) of first larvae instar were allowed to feed on the treated young maize and untreated ones (control) for 6 hrs. or until the leaves eaten up and this treatment was replicated three times for each isolate and the control. Then, the larvae were individually kept in a porous plastic cup (Ø 6.5 cm, height 4.6 cm) and were fed on healthy non-inoculated leaves measuring 2 cm x 5 cm per day per larvae and replaced with a fresh new one every day. The treatments of this experiment consisted of the six fungal isolates and the control

(water) and used the completely randomized block designs. The variables were recorded were development time of each stage (egg, larval, pupal, and adult), and mortality of each stage. The larval and pupal mortality were recorded daily, and the adults emerging were observed every day. The sex of adults emerged were recorded and the adults were placed in the wire mesh cage for copulation with fresh maize leaves inside to allow egg-laying. Egg collection and 10% honey bee solution replacement for adults were carried out every day. The adult longevity was also observed until the adult death.

Data analysis

The differences in the length of different stages (egg, larval, pupal, and adult), mortality of each stage, adult longevity, eggs laid, and sex ratio of each treatment were analyzed by analysis 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

Identification of the endophytic fungal isolates

Five fungal isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) had the same macroscopic and microscopic characteristics, while the isolate (WTTJC260521B) had different characteristics (Fig. 1). The WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A isolates had a white colonial, hyphal, and mycelia color, and non-septate and globose conidia or spores. These characteristics were placed the isolates within the group of *B. bassiana* (Fig. 2). The isolates were deposited in the GenBank with the accession number ON631784 (JGTP240521A isolate), ON631780 (WTTJC260521A isolate), ON631783 (WTTJC290521A isolate), ON631782 (WTTJC290521B isolate), ON631778 (JGNT300521 isolate) (Table 1). The isolate of WTTJC260521B had a white young colony, then the older colony turned greenish white to dark green, and the isolate had the green hyphae and mycelia, the isolate had the non-septation, clear, and cylindrical conidia. The isolate was placed within the group of *M. anisopliae* (Fig. 2). The WTTJC260521B isolate was deposited in the GenBank with the accession number ON631793 (WTTJC260521B isolate) (Table 1).

The result of BLAST search revealed that five fungal isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) shared 100% of similarity each other as well as with JgSPK (Acc. No. MZ356494.1). All the isolates shared 99.80% of similarity with JGNT300521 as well as with BSwTd4 (Acc. No. MT448732.1). JGNT300521 isolate shared 99.61% of similarity with BSwTd4. All the isolates shared 98.83% of similarity with B1 UNILA (=NKPT) (Acc. No. LC413808.1) and type strain on *B. bassiana* (ARSEF1564.T., Acc. No. HQ880761.1). Isolate of WTTJC260521B shared 99.992% of similarity with reference isolate of IPPM010202 (Acc. No. KY437678.1), 99.994% of similarity with strain MSwTp3 (Acc. No. MT448733.1), and 99.985% of similarity with the type strain of *M. anisopliae* ARSEF 7487.T (Acc. No. HQ331446.1). So, there were two 2 species from the 6 investigated isolates of the endophytic fungi found in this study. The five isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) were in the group of *B. bassiana* and one isolate (WTTJC260521B) was in the group of *M. anisopliae.*

Table 1: Origin of isolates of endophytic-entomopathogenic 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	*Spodoptera frugiperda*	35.0	*Beauveria bassiana*	JGTP240521A	ON631784
Tanjung Cermin, Pagar Alam	Lepidoptera	817.2	*Beauveria bassiana*	WTTJC260521A	ON631780
Tanjung Cermin, Pagar Alam	Lepidoptera	817.2	*Metarhizium anisopliae*	WTTJC260521B	ON631793
Tanjung Cermin, Pagar Alam	Lepidoptera	817.2	*Beauveria bassiana*	WTTJC290521A	ON631783
Tanjung Cermin, Pagar Alam	Lepidoptera	817.2	*Beauveria bassiana*	WTTJC290521B	ON631782
Nendagung, Pagar Alam	*Spodoptera frugiperda*	802.6	*Beauveria bassiana*	JGNT300521	ON631778

Development of *Spodoptera frugiperda* fed on young maize colonized and non-colonized by fungi

Six fungal isolates found and used in this experiment were able to colonize young maize (seedling) when inoculated by seed immersion treatment, and a percentage of leaves colonized by endophytic-entomopathogenic fungi was high at 14 days than at 7 days after inoculation (Table 2). They were confirmed as endophytic fungi. The seed immersion treatment resulted leaves of treated young maize grown on to the SDA medium were overgrown with the fungal isolates (Fig. 3). No fungal growth was found on the leaves of untreated maize on the last rinse water. This confirmed that the fungal isolates used in this study were endophytic fungi and it also showed that the surface-sterilization of maize tissues eliminated the epiphytic microorganisms and the fungi growing out of the leaf surface were the endophytic fungi originating from within the maize tissues.

The larvae that consumed leaves of colonized maize exhibited distinctive symptoms, namely smaller body, shrivels, hardens like a mummy, but the healthy larvae of the control were longer and bigger than treated larvae. The cadavers were covered by mycelia and conidia and their colors depending of the fungal species (Fig. 4). The color of cadavers from the larvae that consumed leaves colonized by *B. bassiana* and *M. anisopliae* was white and green, respectively. Re-isolation of the fungus from the cadavers showed that the same fungal isolates found from the larvae that died after feeding on the leaves of the plants where seed treatment was given (Fig 4).

Feeding on leaves of fungal colonized maize significantly increased developmental time of the second, third, fourth, fifth, and sixth larval instars ($P<0.0001$) (Table 3). However, there was non-significant difference in the developmental time of first instar larvae of treated and untreated maize (control). This fungal colonized maize also increased egg and pupal development time, but decreased female and male adult longevity. The lifespan of *S. frugiperda* fed on leaves of fungal colonized maize was significantly longer than those fed on leaves of non-colonized maize (Table 4). The longest lifespan of *S. frugiperda* occurred on the individuals fed on leaves of *B. bassiana* colonized maize.

The fungal colonized young maize significantly increased mortality of all larval instars than the non-colonized one (Table 5). The last larval instar mortality treated with *B. bassiana* (JGTP240521A isolates) (51.33%) was the highest among other treatments and did not significantly differ from each of the *B. bassiana* (WTTJC260521A and WTTJC290521A isolates) (45.33 and 44.67%, respectively). Feeding on leaves of fungal colonized maize significantly decreased the percentage of the last larval instar becoming pupal stage and adult emergence (Table 6). The young maize colonized with fungi significantly reduced eggs laid by the adults (fecundity), but did not affect the sex ratio of *S. frugiperda*. Percentage of hatched eggs significantly decreased on the treatment of *B. bassiana* (JGTP240521A, WTTJC260521A, WTTJC290521A, and WTTJC290521B isolates) (Table 6).

Table 2 Effect of fungal isolates on mean colonization (%) or percentage of leaves colonized by endophytic-entomopathogenic fungi at 7 and 14 days after inoculation

		Mean colonization (%)	
Isolate	Species	7 days after inoculation	14 days after inoculation
Control	-	0.00b	0.00c
JGTP240521A	*Beauveria bassiana*	100.00a	100.00a
WTTJC260521A	*Beauveria bassiana*	93.33a	100.00a
WTTJC260521B	*Metarhizium anisopliae*	26.67b	60.00b
WTTJC290521A	*Beauveria bassiana*	100.00a	100.00a
WTTJC290521B	*Beauveria bassiana*	80.00a	100.00a
JGNT300521	*Beauveria bassiana*	80.00a	100.00a
F-value		26.31^{**}	168.50^{**}
P-value		3.07×10^{-6}	7.16×10^{-11}
HSD value		32.16	13.07

Table 3 Length of different developmental stages of instar larvae of *Spodoptera frugiperda* fed on leaves of endophytic fungi colonized (seed treated) and non-colonized (control) young maize

		Length of different developmental larval instars (days)					
Isolate	Species	1st	2nd	3rd	4th	5th	6th
Control	-	2.67	3.34c	2.36d	2.27c	3.26b	3.23b
JGTP240521A	*Beauveria bassiana*	2.71	3.66b	5.70a	4.45a	3.95a	3.86ab
WTTJC260521A	*Beauveria bassiana*	2.59	3.71b	4.00b	4.60a	2.99b	3.76ab
WTTJC260521B	*Metarhizium anisopliae*	2.63	3.68b	2.66d	3.71b	3.65ab	3.51ab
WTTJC290521A	*Beauveria bassiana*	2.63	3.71b	3.63a	4.46a	3.69ab	4.28a
WTTJC290521B	*Beauveria bassiana*	2.60	4.28a	5.46a	3.79b	3.28ab	3.13b
JGNT300521	*Beauveria bassiana*	2.65	3.64b	3.62c	2.27c	3.37ab	3.57ab
F-value		2.37^{ns}	23.40^{*}	292.73^{*}	296.38^{*}	4.26^{*}	5.22^{*}
P-value		0.10	<0.0001	<0.0001	<0.0001	0.02	0.007
HSD value		-	0.07	0.90	0.07	0.22	0.21

Note: ns = not significantly different * = 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

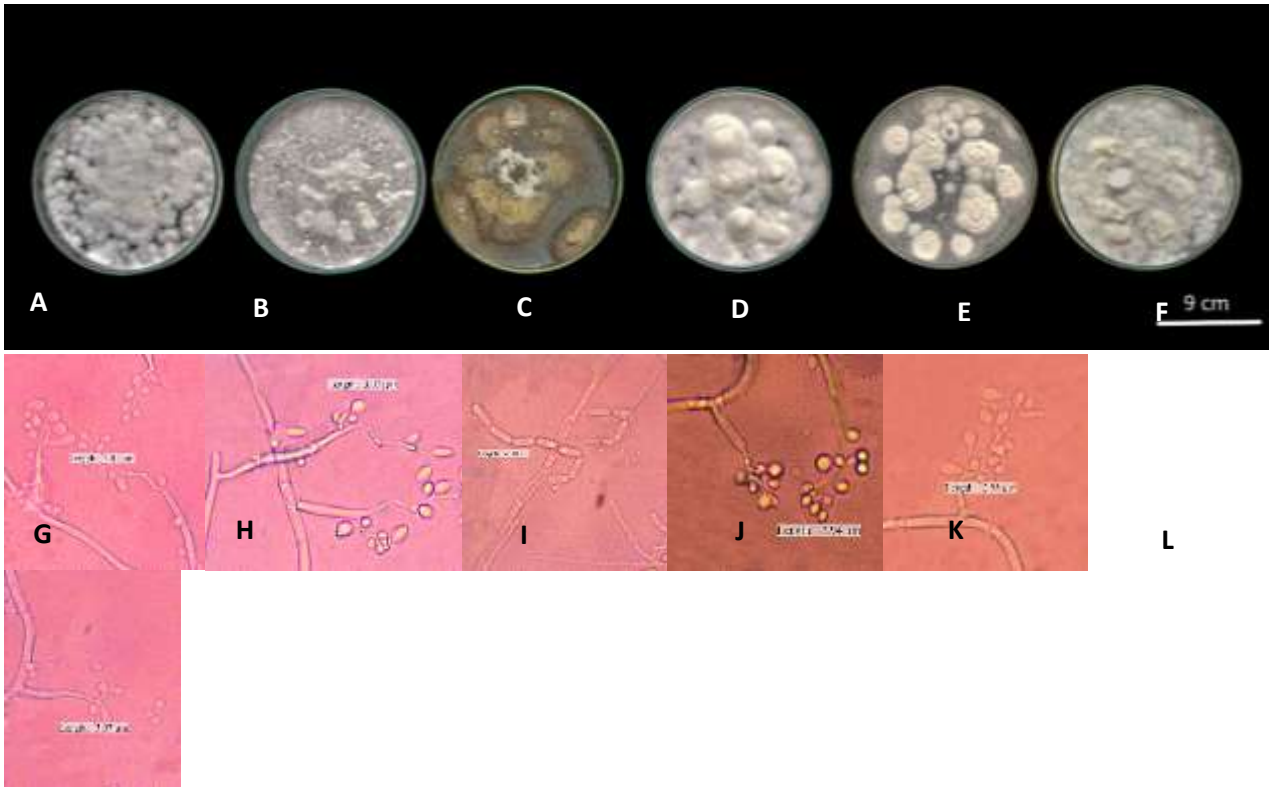
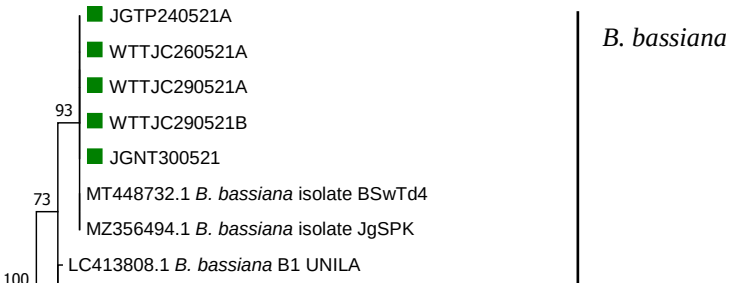


Fig. 1. Colony morphology of endophytic fungi cultured on PDA media (above) and the conidial and hyphal morphology (below) of the fungi: *Beauveria bassiana* of JGTP240521 isolate (A and G) and WTTJC260521A isolate (B and H), *Metarhizium anisoplae* WTTJC260521B isolate (C and I), *Beauveria bassiana* of WTTJC290521A isolate (D and J), WTTJC290521B isolate (E and K), and JGNT300521 isolate (F and L)

Tabel 4 Length of different developmental stages of pupae and adults of *Spodoptera frugiperda* fed on leaves of endophytic fungi colonized (seed treated) and non-colonized (control) young maize

		Length of different developmental stages (days)					
Isolate	Species	Prepupae	Pupae	Female adult	Male adult	Egg	Total lifespan
Control	-	3.61	6.95b	4.82a	4.40a	2.59c	32.51d
JGTP240521A	*Beauveria bassiana*	3.68	9.71a	4.37ab	3.28b	3.22abc	41.62a
WTTJC260521A	*Beauveria bassiana*	3.11	10.24a	4.21ab	3.40b	2.74bc	39.21ab
WTTJC260521B	*Metarhizium anisopliae*	3.65	7.43b	4.52ab	3.63b	2.84bc	35.44c
WTTJC290521A	*Beauveria bassiana*	3.79	9.85a	3.99b	4.34a	3.39ab	40.05a
WTTJC290521B	*Beauveria bassiana*	3.22	10.58a	4.23ab	3.52b	3.27abc	40.57a
JGNT300521	*Beauveria bassiana*	3.71	9.49a	4.93a	4.58a	3.65a	37.25bc
F-value		0.95^{ns}	22.43^{*}	5.12^{*}	49.86^{*}	6.39^{*}	34.57^{*}
P-value		0.50	<0.0001	0.008	<0.0001	0.003	<0.0001
HSD value		-	1.47	0.17	0.09	0.19	0.22

Note: ns = not significantly differen * = 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



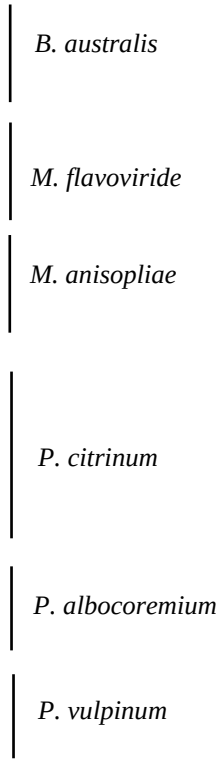


Fig. 2. Phylogenetic tree developed based on Internal Transcribed Spacer (ITS) region by Maximum Likelihood (Tamura-Nei model). The six investigated isolates were placed within group of *Beauveria bassiana* (5 isolates) and *Metarhizium anisopliae* (1 isolate). T= Type isolate

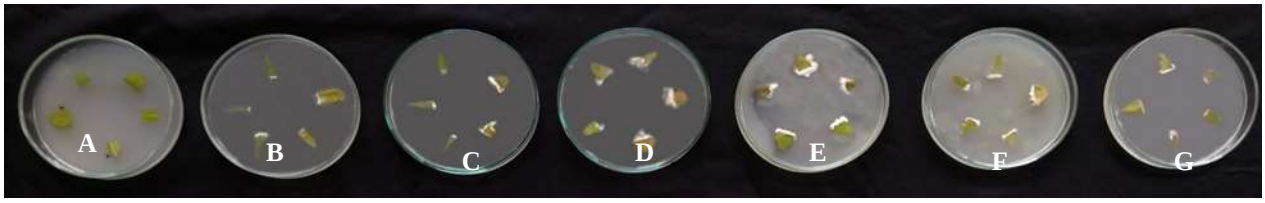


Fig. 3. Colony morphology of endophytic fungi from the leaves of maize where seed treatment was given and control (untreated seeds): Control (A), *Beauveria bassiana* of JGTP240521 isolate (B) and WTTJC260521A isolate (C), *Metarhizium anisoplae* WTTJC260521B isolate (D), *Beauveria bassiana* of WTTJC290521A isolate (E), WTTJC290521B isolate (F), and JGNT300521 isolate (G)

Fig. 4. The morphology of healthy larvae (control) (A), and the cadavers from larvae feeding on leaves colonized by fungi (above) and the conidial and hyphal morphology of fungi from cadaver re-isolation (below): *Beauveria bassiana* of JGTP240521 isolate (B and H) and WTTJC260521A isolate (C and I), *Metarhizium anisoplae* WTTJC260521B isolate (D and J), *Beauveria bassiana* of WTTJC290521A isolate (E and K), WTTJC290521B isolate (F and L), and JGNT300521 isolate (G and M)

Table 5 Mean of mortality of different larval instars of *Spodoptera frugiperda* fed on leaves of endophytic fungi colonized (seed treated) and non-colonized (control) young maize

Isolate	Species	Mean of mortality of different larval instars (%)					
		1st	2nd	3rd	4th	5th	6th
Control	-	2.67	6.00b	6.67e	6.67c	6.67c	6.67e
JGTP240521A	*Beauveria bassiana*	14.67	24.00a	43.33a	48.67a	51.33a	51.33a
WTTJC260521A	*Beauveria bassiana*	8.00	21.33a	36.67ab	39.33ab	42.00ab	45.33ab
WTTJC260521B	*Metarhizium anisopliae*	2.67	5.33b	8.00de	12.00c	15.33c	24.67d
WTTJC290521A	*Beauveria bassiana*	7.33	11.33ab	20.00cd	30.67b	36.00b	44.67ab
WTTJC290521B	*Beauveria bassiana*	8.67	16.67ab	22.67bc	27.33b	29.33b	38.67bc

JGNT300521	*Beauveria bassiana*	8.00	16.00ab	21.33bc	26.67b	28.00b	32.67c
F-value		2.93^{ns}	7.74*	22.96*	27.02*	35.02*	176.07*
P-value		0.053	0.001	<0.0001	<0.0001	<0.0001	<0.0001
HSD value		-	10.95	10.00	9.64	8.74	3.90

Note: ns = not significantly different * = 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

Table 6 Mean of percentage of pupae and adults emergence, sex ratio, egg laid, and viable eggs of *Spodoptera frugiperda* fed on leaves of endophytic fungi colonized (seed treated) and non-colonized (control) young maize

Isolates	Fungal species	Pupae emergence (%)	Adult emergence (%)	Sex ratio	Eggs laid per female	Viable (hatched) eggs (%)
Control		93.33a	93.33a	0.56a	68.28a	99.92a
JGTP240521A	*Beauveria bassiana*	46.00e	42.00e	0.83a	15.91c	88.86d
WTTJC260521A	*Beauveria bassiana*	52.67de	48.00de	0.84a	15.31c	90.93cd
WTTJC260521B	*Metarhizium anisopliae*	74.67b	71.33b	0.47a	42.89b	99.72a
WTTJC290521A	*Beauveria bassiana*	54.00d	47.33de	0.74a	27.86bc	95.91b
WTTJC290521B	*Beauveria bassiana*	59.33cd	54.00cd	0.87a	17.50c	92.98bc
JGNT300521	*Beauveria bassiana*	65.33c	58.67c	0.51a	39.36b	99.31a
F-value		134.80*	95.08*	3.67*	34.26*	75.47*
P-value		<0.0001	<0.0001	0.026	<0.0001	<0.0001
HSD value		6.85	9.03	0.19	1.37	4.16

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

Discussion

The results of identification based on the morphological characteristics of five isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) showed that they had similar morphology of the colony, hypha, and mycelia and the conidial shape. All belong to species of *B. bassiana*. These characteristics matched to *B. bassiana* described by Herlinda *et al.* (2021). The isolate of WTTJC260521B belonged to the species, *M. anisopliae*. The isolate morphology of the colony, and the hyphal, mycelia, and conidial of shape matched to *M. anisopliae* described by Herlinda *et al.* (2020).

The five fungal isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) found in this study had an rDNA sequence similarity value of 100% to the reference species (BLAST), JgSPK (Acc. No. MZ356494.1) an endophytic fungi isolated from maize (Herlinda *et al.* 2021), except one isolate (JGNT300521). If the similarity value is 100%, It means that they are the same strain (Henry *et al.* 2000). These isolates shared 99.80% of similarity with JGNT300521 as well as with BSwTd4 (Acc. No. MT448732.1) which was isolated from oil palm rhizosphere (Herlinda *et al.* 2020). JGNT300521 isolate shared 99.61% of similarity with BSwTd4. All the isolates shared 98.83% of similarity with B1 UNILA (=NKPT) (Acc. No. LC413808.1) which was isolated from maize rhizosphere (Fitriana *et al.* 2021) and type strain on *B. bassiana* (ARSEF 1564.T., Acc. No. HQ880761.1). The isolate of WTTJC260521B had an rDNA sequence similarity value of more than 99% to the BLAST (reference species). The similarity value of 99-100% indicated that the isolates were the same

species (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). If the similarity value of the isolates is 89–99%, it means that the isolates are of the same genus (Henry *et al.* 2000).

All fungal isolates of the *B. bassiana* and *M. anisopliae* tested in this study were able to colonize leaves of young maize when inoculated by seed immersion treatment. From the leaves of young maize treated by the fungi were overgrown with the fungal mycelia, but no fungal mycelia were found on the leaves of untreated seeds. The finding showed that both species of fungi from seed treatment were able to colonize the leaves. In addition to, the fungi of *B. bassiana* and *M. anisopliae* not only can colonize maize by seed treatment, but they also can colonize maize by foliar spray and root deeping and the fungi can systemically colonized leaves, stems, and roots of plants (Russo *et al.* 2020). *B. bassiana* inoculated by foliar spray can penetrate the leaf surface and move within the maize vascular (Wagner and Lewis 2000). *M. anisopliae* was often reported to be restricted to plant roots (Russo *et al.* 2020), however the present study reported that the strain of *M. anisopliae* was able to colonize the leaves of young maize. The extent and persistence of plant colonization by the fungi were influenced by fungal species/strain, inoculation method, and host plant species (Russo *et al.* 2020). The fungi used for seed treatment in this study is easier to be applied by soaking the seeds in fungal suspension before planting, or when the seeds stored, dry conidia of the fungi can be covered on the seeds.

Obtained findings reported that *B. bassiana* and *M. anisopliae* from South Sumatra (Indonesia) in seed treated corn seedlings had negative effects on development of *S. frugiperda*. This is first report that the fungi as an endophyte could decrease the female and male adult longevity of *S. frugiperda* and increased the larval mortality. The young maize colonized with the *B. bassiana* and *M. anisopliae* also reduced the percentage of the last instar becoming pupal stage and adult emergence, and decreased the eggs laid by the adults and the percentage of hatched eggs. Previous study reported that *B. bassiana* and *M. anisopliae* in foliar treated caused adverse effects on *S. frugiperda* development and survival (Russo *et al.* 2020). These adverse effects of endophytic fungi against *S. frugiperda* were caused by fungal production of secondary metabolites and mycosis (Vidal and Jaber 2015). The fungal secondary metabolites are produced by blastospores in insect hemolymph and disrupted the normal cell metabolism (Mancillas-Paredes *et al.* 2019) and then the toxins produced by the metabolites kill the insects (El-Ghany 2015). Our previous study showed that the endophytic fungi could decrease the leaf consumption by the *S. frugiperda* larvae resulting in larval weight loss and low survival (Gustianingtyas *et al.* 2021). The reduction in leaf area consumed by the larvae treated with the endophytes is caused by antifeedant or deterrent properties of *in planta*-produced *B. bassiana* metabolites (Russo *et al.* 2020). The corn plants colonized with *B. bassiana* may enhance levels of terpenoid defense compounds against *S. frugiperda* (Russo *et al.* 2020). The endophytic fungi could produce secondary metabolites *in planta* resulting antibiosis and feeding deterrence for the insects (Jaber and Ownley 2018).

These adverse effects of endophytic fungi against *S. frugiperda* were also caused by mycosis (Vidal and Jaber 2015). The present study found that the mycosis found on the cadavers of *S. frugiperda* treated with the fungi. The mycosis was evidenced by fungal mycelia and spores emerging from the cadavers of treated insects. However, no fungal mycelia and spores were found on the cadavers of untreated insects. Some previous studies have similar reported insect

mycosis feeding on fungal-endophytically colonized plants by *S. frugiperda* (Herlinda *et al.* 2021).

This study also showed that the fungal colonized young maize increased eggs, larvae, pupal developmental time, and lifespan of *S. frugiperda.* In contrast to the previous study of Russo *et al.* (2020) that these fungal species could decreased the development time of *S. frugiperda*. However, obtained findings are in agreement with previous study of Hussain *et al.* (2009) which showed that the lepidopteran, *Ocinara varians* treated with *B. bassiana* and *M. anisopliae* extended the developmental time of treated insects as compared to untreated ones (control) and the conversion of digested food and ingested food declined in treated insects compared to untreated insects, and stimulated the larvae to develop more slowly.

Conclusions

The results of molecular identification showed that the fungal species found were *B. bassiana* of five fungal isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) and *M. anisopliae* of an isolate (WTTJC260521B). *B. bassiana* and *M. anisopliae* colonized young maize significantly increased mortality of all larval instars of FAW compared to non-colonized ones. The larval mortality treated with *B. bassiana* (JGTP240521A isolates) was the highest among other treatments. Feeding on leaves of fungal colonized maize significantly decreased the percentage of the last larval instar becoming pupal stage and the adult emergence and the eggs laid, and the percentage of hatched eggs and increased the larval mortality. This is the first report that the *B. bassiana* and *M. anisopliae* from South Sumatra (Indonesia) in seed treated corn seedlings that had negative effects on development of *S. frugiperda.* Finally, these results highlight the potential of endophytic entomopathogenic fungi to protect corn plants against *S. frugiperda.*

List of abbreviations

ANOVA: analysis of variance; BLAST: Basic Local Alignment Search Tool; CTAB: cetyltrimethylammonium bromide; DNA: Deoxyribonucleic acid; EtOH: Ethyl alcohol; FAW: fall armyworm; 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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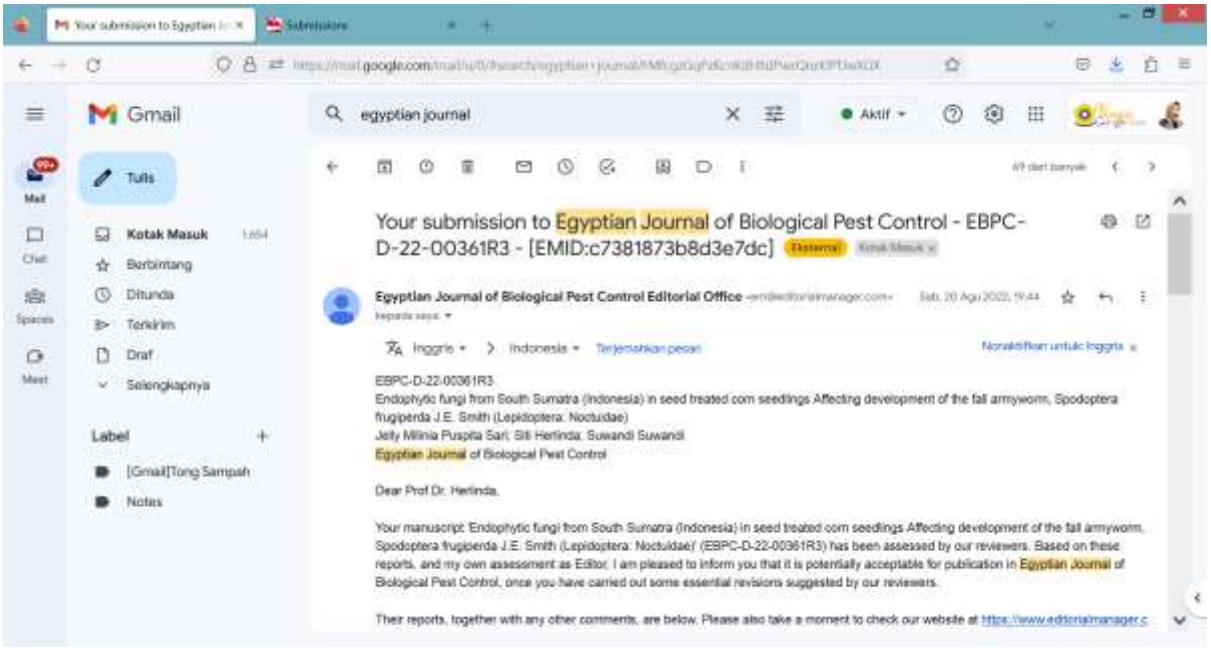
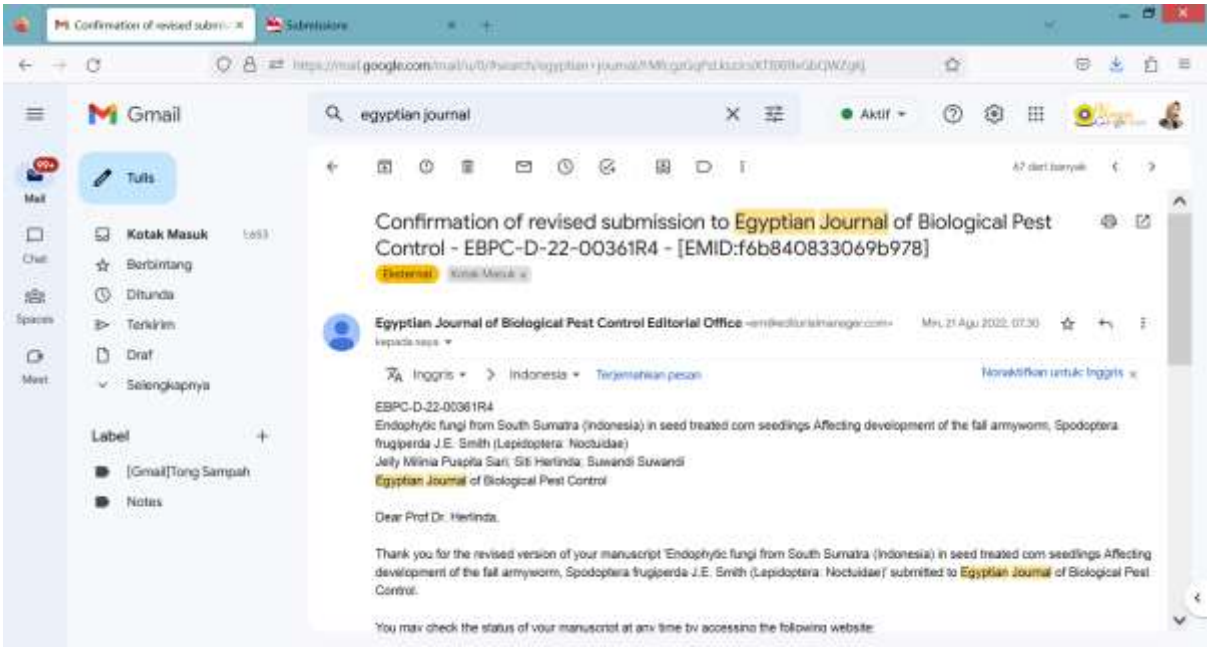
4. Bukti konfirmasi review ketiga dan hasil revisi ketiga





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Endophytic fungi from South Sumatra (Indonesia) in seed treated corn seedlings Affecting development of the fall armyworm, Spodoptera frugiperda J.E. Smith (Lepidoptera: Noctuidae)

--Manuscript Draft--

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Abstract:	Background: Topical application of the entomopathogenic fungi (EPFs) against Spodoptera frugiperda J.E. Smith (Lepidoptera: Noctuidae) larvae is less effective due to larvae hiding in the corn midribs in the field. To control the larvae, the fungi colonize in plant tissues or endophytic fungi are needed. There is no information on the pathogenicity of the endophytic fungi from Indonesia on the development of S. frugiperda. The endophytic fungi isolated from infected-host cadavers from South Sumatra (Indonesia) were identified morphologically and molecularly and the effect of seed treated corn seedlings with the fungi on S. frugiperda development was evaluated. The fungal identification was based on morphological and molecular characteristics. Bioassay of the endophytic fungal species in seed treated young maize was performed against the neonate larvae (hatching within 24 hrs.) of first instar and their development were observed. Results: The results of molecular identification showed that the fungal species were Beauveria bassiana of five fungal isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) and Metarhizium anisopliae of an isolate (WTTJC260521B). The lifespan of S. frugiperda fed on leaves of fungal colonized maize was significantly longer than those fed on leaves of non-colonized maize. The fungal colonized young maize significantly increased mortality rate of all larval instars than non-colonized one. The last instar larvae mortality treated with B. bassiana (JGTP240521A isolates) (51.33%) was the highest among other treatments and did not significantly differ from each of B. bassiana of WTTJC260521A and WTTJC290521A isolates 45.33% and 44.67%, respectively. Feeding on leaves of fungal colonized maize significantly decreased the percentage of the last instar larvae development to the pupal stage, the adult emergence, the eggs laid, and the percentage of hatched eggs. This is first report that B. bassiana and M. anisopliae from South Sumatra (Indonesia) in seed treated corn seedlings had negative effects on development of S. frugiperda. Conclusions: Finally, these results highlight the potential of endophytic EPFs to protect corn plants against S. frugiperda. Keywords: Spodoptera frugiperda, Beauveria bassiana, Metarhizium anisopliae, Endophyte, Entomopathogens,
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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

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Abstract

Background: Topical application of the entomopathogenic fungi (EPFs) against *Spodoptera frugiperda* J.E. Smith (Lepidoptera: Noctuidae) larvae is less effective due to larvae hiding in the corn midribs in the field. To control the larvae, the fungi colonize in plant tissues or endophytic fungi are needed. There is no information on the pathogenicity of the endophytic fungi from Indonesia on the development of *S. frugiperda*. The endophytic fungi isolated from infected-host cadavers from South Sumatra (Indonesia) were identified morphologically and molecularly and the effect of seed treated corn seedlings with the fungi on *S. frugiperda* development was evaluated. The fungal identification was based on morphological and molecular characteristics. Bioassay of the endophytic fungal species in seed treated young maize was performed against the neonate larvae (hatching within 24 hrs.) of first instar and their development were observed.

Results: The results of molecular identification showed that the fungal species were *Beauveria bassiana* of five fungal isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) and *Metarhizium anisopliae* of an isolate (WTTJC260521B). The lifespan of *S. frugiperda* fed on leaves of fungal colonized maize was significantly longer than those fed on leaves of non-colonized maize. The fungal colonized young maize significantly increased mortality rate of all larval instars than non-colonized one. The last instar larvae mortality treated with *B. bassiana* (JGTP240521A isolates) (51.33%) was the highest among other treatments and did not significantly differ from each of *B. bassiana* of WTTJC260521A and WTTJC290521A isolates 45.33% and 44.67%, respectively. Feeding on leaves of fungal colonized maize significantly decreased the percentage of the last instar larvae development to the pupal stage, the adult emergence, the eggs laid, and the percentage of hatched eggs. This is first report that *B. bassiana* and *M. anisopliae* from South Sumatra (Indonesia) in seed treated corn seedlings had negative effects on development of *S. frugiperda.*

Conclusions: Finally, these results highlight the potential of endophytic EPFs to protect corn plants against *S. frugiperda.*

Keywords: *Spodoptera frugiperda*, *Beauveria bassiana*, *Metarhizium anisopliae*, Endophyte, Entomopathogens,

Background

Spodoptera frugiperda J.E. Smith (Lepidoptera: Noctuidae) or fall armyworm (FAW) is one of the most important noctuid pests of corn in the world. The FAW is a migratory and polyphagous pest that can attack 353 host plant species from 76 plant families (Montezano *et al.* 2018). The pest is native to the neotropics of the Americas and has spread throughout the world (Otim *et al.* 2018). More recently, the FAW becomes a new invasive pest in many parts of Africa (Niassy *et al.* 2021) and Asia (Lamsal *et al.* 2020), including Indonesia (Herlinda *et al.* 2021). This pest is commonly controled using synthetic insecticides (Kumela *et al.* 2018), however the resistances of the FAW to many insecticides, such as pyrethroid, spinosad, and organophosphorus insecticides have occured (Zhang *et al.* 2021). In addition, the insecticide

application negatively affects the human health and the environment (Harrison *et al.* 2019). An alternative more sustainable and eco-friendly control methods against *S. frugiperda* is urgently needed.

Biological control based on utilizing EPFs is the preferred control option for FAW (Mantzoukas and Eliopoulos 2020). Topical application (direct contact) of the EPF, *Beauveria bassiana* (Balsamo) Vuillemin (Deuteromycotina: Hypomycetes) killed more than 80% of *S. frugiperda* larvae (Ramanujam *et al.* 2020). *Metarhizium anisopliae* (Metsch.) Sorok. (Deuteromycotina: Hypomycetes) could kill 75% of *S. frugiperda* larvae (Ramos *et al.* 2020). However, in the field, the larvae occured on the surface of leaves or maize stalks only in the morning but at daylight up to night, they hide in the corn midribs (Herlinda *et al.* 2021). So, topical application of the fungus against the *S. frugiperda* larvae is less effective (Gustianingtyas *et al.* 2021). To control such hiding larvae in the field, the fungi colonizing in plant tissues or endophytic fungi are needed (Ramos *et al.* 2020). The endophytic fungi associate mutually with their host plants (Lira *et al.* 2020) and can stimulate the plant growth but suppress the insect pest growth (Russo *et al.* 2020).

Previous studies have demonstrated that the endophytic fungal *B. bassiana* from corn root from Indonesia applied topically caused 29.33% of the FAW larval mortality (Gustianingtyas *et al.* 2021). The endophytic fungal *B. bassiana* used as seed treatment caused up to 22.67% of the FAW larval mortality (Herlinda *et al.* 2021). An experiment has previously also reported that endophytic *B. bassiana* sprayed on leaves has a high ability to colonize corn plants and the fungus caused significant reductions in the growth and development of *S. frugiperda* (Russo *et al.* 2020). There is no information on the pathogenicity of the endophytic fungi from Indonesia on the development of *S. frugiperda*. In addition, the potential of the fungi isolated from the infected-host cadaver as endophytic entomopathogens needs to be investigated. In this study, the fungi isolated from infected-host cadavers from South Sumatra (Indonesia) were identified morphologically and molecularly and the effect of seed treated corn seedlings with the fungi on *S. frugiperda* development was evaluated.

Methods

Exploration of the fungi was performed by collecting infected-host insect cadavers from crops in South Sumatra, Indonesia from May until June 2021. Purification and isolation of the fungi were carried out from June to July 2021. The morphological identification was carried out in the Laboratory of Entomology, Faculty of Agriculture, Universitas Sriwijaya in July 2021 and the molecular identification was performed from August to December 2021 at the Laboratory of Agricultural Biotechnology (accredited according to the ISO/IEC 17025 standard), Department of Plant Protection, Faculty of Agriculture, Universitas Lampung, Indonesia. Experimental design used for bioassay was a completely randomized block designs consisted of seven treatments (six fungal isolates and control), and the experiment was repeated three times.

Fungal Exploration, isolation, and purification

Fungal exploration from the infected-host cadavers using the method of Ab Majid *et al.* (2015) by collecting infected-host insects or cadaver infected with the fungi from the fields. The exploration was carried out in Tanjung Pering, Ogan Ilir, South Sumatra (3°13′23″S104°38′27″E), Tanjung Cermin, Pagar Alam, South Sumatra (4°02′23″S103°13″14″E), and Nendagung, Pagar Alam, South Sumatra (3°56′22″S103°12′15″E) (Table 1). The infected

insects or cadavers were first surface sterilized with 70% EtOH (Ethyl alcohol) and 1% NaOCl (Sodium hypochlorite), then rinsed 3 times (Elfita *et al.* 2019). After that, the sample cadavers were cultured aseptically onto SDA (Sabouraud Dextrose Agar) medium (Russo *et al.* 2020). The fungal culture was purified to make an isolate per sample. The fungal macroscopic and microscopic characteristics, such as the colonial color and shape, the conidial shape and size, and the conidiophores were observed (Herlinda *et al.* 2021) and then molecular identification was performed.

DNA extraction, PCR amplification, and sequencing

DNA was extracted according to the method of Swibawa *et al.* (2020) and carried out on fungal conidia of 7 days old fungus. As much as 10 ml of conidia suspension was centrifuged using CF15RXII for 10 min at a speed of 14,000 rpm. Then, 1 ml of 70% ethanol was added to the centrifuge tube and centrifuged again for 10 min. The supernatant was removed and 1 ml of extraction buffer (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) was added. The suspension was incubated at -40 °C for 24 hrs. The frozen suspension was crushed until pulverized. A total of 500 µl of pellet suspension was put into a 1.5 ml tube and 400 µl of 2% CTAB (cetyltrimethylammonium bromide) was added, homogenized and heated at 65 °C for an hour using a water bath (Brookfield TC 550 MX-230, USA). After the incubation, 500 µl of PCI (Phenol Chloroform Isoamyl alcohol) (25: 24: 1) was added, homogenized and centrifuged at 14,000 rpm for 10 minutes at 14,000 rpm for 10 min. A total of 600 µl supernatant was transferred to a new 1.5 ml tube, and 600 µl Chloroform Isoamyl Alcohol (24:1) was added, homogenized, and centrifuged (Microspin12; Biosan, Latvia) again at 14,000 rpm for 10 minutes. A total of 400 µl of supernatant was then put into to a new 1.5 ml tube and 400 µl of cold isopropanol was homogenized and incubated at -40 ºC for 20 minutes. Then, the suspension was centrifuged at 14,000 rpm for 15 min. The supernatant was then discarded, and the pellet was added with 500 µl of 70% cold ethanol and centrifuged at 14,000 rpm for 5 minutes. The supernatant was then discarded and the pellets obtained were incubated at room temperature for 24 hrs to dry. After drying, the pellets were 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 ITS (the Internal Transcribed Spacer) using ITS1 and ITS4 primers (White *et al.* 1990). The DNA amplification stage consisted of 1 initiation cycle at 95 ºC for 5 min, 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. Then, the PCR results were 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 under taken in 1x TBE buffer solution at 50 volts for 70 min, and the results were visualized using a DigiDoc UV transilluminator (UVP, USA).

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

strains used as a reference in this study were obtained from NCBI (https://www.ncbi.nlm.nih.gov/).

Mass-rearing of *Spodoptera frugiperda*

The mass-rearing of *S. frugiperda* was performed using the method of Herlinda *et al.* (2020). The eggs of *S. frugiperda* were obtained from the Laboratory of Entomology, Faculty of Agriculture, Universitas Sriwijaya. They were reared in laboratory for more than 5 generations at 28–29°C temperature, and 82–83% RH and the lighting set to photoperiod 12:12 (L:D) hrs. In the laboratory, the larvae of *S. frugiperda* were maintained individually due to cannibal behaviours and reared using fresh maize leaves. The prepupae and pupae were replaced 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. Emerged adults were used for bioassays.

Assessing endophytic fungal colonization

Fungal inoculation for maize seeds treated was carried out to assess the ability of the fungal colonization into the maize seedling tissue and to ensure that the fungi used in this experiment were truly endophytic. All the isolates used were grown in SDA medium incubated for 14 days, then the SDA fungal culture was transferred to the broth medium (SDB, Sabouraud Dextrose Broth) following the method of Gustianingtyas *et al.* (2020) and incubated for 7 days on the shaker and 7 days unshaken position. The 45 corn seeds for an isolate were surface sterilized by using (Russo *et al.* 2020) method. The seeds were immersed in 10 ml of fungal suspension (1 x 10^{10} conidia ml^{-1}) for 24 hrs, while for the control only 10 ml of sterilized water was treated for the seeds. Then, the seeds were grown in the hydroponic medium, following the method of Novianti *et al.* (2020) and incubated for 14 days and this treatment was repeated 3 times for each isolate. The tip leaves of 14-day old maize seedlings (young maize) were cut of 5 x 5 mm^2 to be grown onto the SDA medium to detect the mycelia of the endophytic fungi. The leaf materials were first surface-sterilized by using method of (Russo *et al.* 2020) before grown onto the SDA medium. The leaf material surface-sterilized was carried out by immersion in 70% ethanol, then followed by sodium hypochlorite for 2 minutes, and rinsed twice in sterile distilled water and the final rinse water was grown onto SDA and incubated for 10 days. The rest or remaining leaves were used for bioassays as described below.

Bioassay for assessing effect of corn seed treatment on *S. frugiperda* development

The bioassay for assessing the effect of corn seed treatment on *S. frugiperda* growth and development, followed the method of Russo *et al.* (2020). The 14-day old maize seedlings already inoculated with the endophytic fungi as described above were given to be consumed to the first instar neonate larvae of *S. frugiperda*, while for control treatment, the larvae were provided the non-inoculated young maize and this experiment was repeated three times. The 50 neonate larvae (hatching within 24 hrs.) of first larvae instar were allowed to feed on the treated young maize and untreated ones (control) for 6 hrs. or until the leaves eaten up and this treatment was replicated three times for each isolate and the control. Then, the larvae were individually kept in a porous plastic cup (Ø 6.5 cm, height 4.6 cm) and were fed on healthy non-inoculated leaves measuring 2 cm x 5 cm per day per larvae and replaced with a fresh new one every day. The treatments of this experiment consisted of the six fungal isolates and the control

(water) and used the completely randomized block designs. The variables were recorded were development time of each stage (egg, larval, pupal, and adult), and mortality of each stage. The larval and pupal mortality were recorded daily, and the adults emerging were observed every day. The sex of adults emerged were recorded and the adults were placed in the wire mesh cage for copulation with fresh maize leaves inside to allow egg-laying. Egg collection and 10% honey bee solution replacement for adults were carried out every day. The adult longevity was also observed until the adult death.

Data analysis

The differences in the length of different stages (egg, larval, pupal, and adult), mortality of each stage, adult longevity, eggs laid, and sex ratio of each treatment were analyzed by analysis 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

Identification of the endophytic fungal isolates

Five fungal isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) had the same macroscopic and microscopic characteristics, while the isolate (WTTJC260521B) had different characteristics (Fig. 1). The WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A isolates had a white colonial, hyphal, and mycelia color, and non-septate and globose conidia or spores. These characteristics were placed the isolates within the group of *B. bassiana* (Fig. 2). The isolates were deposited in the GenBank with the accession number ON631784 (JGTP240521A isolate), ON631780 (WTTJC260521A isolate), ON631783 (WTTJC290521A isolate), ON631782 (WTTJC290521B isolate), ON631778 (JGNT300521 isolate) (Table 1). The isolate of WTTJC260521B had a white young colony, then the older colony turned greenish white to dark green, and the isolate had the green hyphae and mycelia, the isolate had the non-septation, clear, and cylindrical conidia. The isolate was placed within the group of *M. anisopliae* (Fig. 2). The WTTJC260521B isolate was deposited in the GenBank with the accession number ON631793 (WTTJC260521B isolate) (Table 1).

The result of BLAST search revealed that five fungal isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) shared 100% of similarity each other as well as with JgSPK (Acc. No. MZ356494.1). All the isolates shared 99.80% of similarity with JGNT300521 as well as with BSwTd4 (Acc. No. MT448732.1). JGNT300521 isolate shared 99.61% of similarity with BSwTd4. All the isolates shared 98.83% of similarity with B1 UNILA (=NKPT) (Acc. No. LC413808.1) and type strain on *B. bassiana* (ARSEF1564.T., Acc. No. HQ880761.1). Isolate of WTTJC260521B shared 99.992% of similarity with reference isolate of IPPM010202 (Acc. No. KY437678.1), 99.994% of similarity with strain MSwTp3 (Acc. No. MT448733.1), and 99.985% of similarity with the type strain of *M. anisopliae* ARSEF 7487.T (Acc. No. HQ331446.1). So, there were two 2 species from the 6 investigated isolates of the endophytic fungi found in this study. The five isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) were in the group of *B. bassiana* and one isolate (WTTJC260521B) was in the group of *M. anisopliae.*

Table 1: Origin of isolates of endophytic-entomopathogenic 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	*Spodoptera frugiperda*	35.0	*Beauveria bassiana*	JGTP240521A	ON631784
Tanjung Cermin, Pagar Alam	Lepidoptera	817.2	*Beauveria bassiana*	WTTJC260521A	ON631780
Tanjung Cermin, Pagar Alam	Lepidoptera	817.2	*Metarhizium anisopliae*	WTTJC260521B	ON631793
Tanjung Cermin, Pagar Alam	Lepidoptera	817.2	*Beauveria bassiana*	WTTJC290521A	ON631783
Tanjung Cermin, Pagar Alam	Lepidoptera	817.2	*Beauveria bassiana*	WTTJC290521B	ON631782
Nendagung, Pagar Alam	*Spodoptera frugiperda*	802.6	*Beauveria bassiana*	JGNT300521	ON631778

Development of *Spodoptera frugiperda* fed on young maize colonized and non-colonized by fungi

Six fungal isolates found and used in this experiment were able to colonize young maize (seedling) when inoculated by seed immersion treatment, and a percentage of leaves colonized by endophytic-entomopathogenic fungi was high at 14 days than at 7 days after inoculation (Table 2). They were confirmed as endophytic fungi. The seed immersion treatment resulted leaves of treated young maize grown on to the SDA medium were overgrown with the fungal isolates (Fig. 3). No fungal growth was found on the leaves of untreated maize on the last rinse water. This confirmed that the fungal isolates used in this study were endophytic fungi and it also showed that the surface-sterilization of maize tissues eliminated the epiphytic microorganisms and the fungi growing out of the leaf surface were the endophytic fungi originating from within the maize tissues.

The larvae that consumed leaves of colonized maize exhibited distinctive symptoms, namely smaller body, shrivels, hardens like a mummy, but the healthy larvae of the control were longer and bigger than treated larvae. The cadavers were covered by mycelia and conidia and their colors depending of the fungal species (Fig. 4). The color of cadavers from the larvae that consumed leaves colonized by *B. bassiana* and *M. anisopliae* was white and green, respectively. Re-isolation of the fungus from the cadavers showed that the same fungal isolates found from the larvae that died after feeding on the leaves of the plants where seed treatment was given (Fig 4).

Feeding on leaves of fungal colonized maize significantly increased developmental time of the second, third, fourth, fifth, and sixth larval instars ($P<0.0001$) (Table 3). However, there was non-significant difference in the developmental time of first instar larvae of treated and untreated maize (control). This fungal colonized maize also increased egg and pupal development time, but decreased female and male adult longevity. The lifespan of *S. frugiperda* fed on leaves of fungal colonized maize was significantly longer than those fed on leaves of non-colonized maize (Table 4). The longest lifespan of *S. frugiperda* occurred on the individuals fed on leaves of *B. bassiana* colonized maize.

The fungal colonized young maize significantly increased mortality of all larval instars than the non-colonized one (Table 5). The mortality of last instar larvae treated with *B. bassiana* (JGTP240521A isolates) (51.33%) was the highest among other treatments and did not significantly differ from each of the *B. bassiana* (WTTJC260521A and WTTJC290521A isolates) (45.33 and 44.67%, respectively). Feeding on leaves of fungal colonized maize significantly decreased the percentage of the last larval instar becoming pupal stage and adult emergence (Table 6). The young maize colonized with fungi significantly reduced eggs laid by the adults (fecundity), but did not affect the sex ratio of *S. frugiperda*. Percentage of hatched eggs significantly decreased on the treatment of *B. bassiana* (JGTP240521A, WTTJC260521A, WTTJC290521A, and WTTJC290521B isolates) (Table 6).

Table 2 Effect of fungal isolates on mean colonization (%) or percentage of leaves colonized by endophytic-entomopathogenic fungi at 7 and 14 days after inoculation

		Mean colonization (%)	
Isolate	Species	7 days after inoculation	14 days after inoculation
Control	-	0.00b	0.00c
JGTP240521A	*Beauveria bassiana*	100.00a	100.00a
WTTJC260521A	*Beauveria bassiana*	93.33a	100.00a
WTTJC260521B	*Metarhizium anisopliae*	26.67b	60.00b
WTTJC290521A	*Beauveria bassiana*	100.00a	100.00a
WTTJC290521B	*Beauveria bassiana*	80.00a	100.00a
JGNT300521	*Beauveria bassiana*	80.00a	100.00a
F-value		26.31^{**}	168.50^{**}
P-value		3.07×10^{-6}	7.16×10^{-11}
HSD value		32.16	13.07

Table 3 Length of different developmental stages of instar larvae of *Spodoptera frugiperda* fed on leaves of endophytic fungi colonized (seed treated) and non-colonized (control) young maize

		Length of different developmental larval instars (days)					
Isolate	Species	1st	2nd	3rd	4th	5th	6th
Control	-	2.67	3.34c	2.36d	2.27c	3.26b	3.23b
JGTP240521A	*Beauveria bassiana*	2.71	3.66b	5.70a	4.45a	3.95a	3.86ab
WTTJC260521A	*Beauveria bassiana*	2.59	3.71b	4.00b	4.60a	2.99b	3.76ab
WTTJC260521B	*Metarhizium anisopliae*	2.63	3.68b	2.66d	3.71b	3.65ab	3.51ab
WTTJC290521A	*Beauveria bassiana*	2.63	3.71b	3.63a	4.46a	3.69ab	4.28a
WTTJC290521B	*Beauveria bassiana*	2.60	4.28a	5.46a	3.79b	3.28ab	3.13b
JGNT300521	*Beauveria bassiana*	2.65	3.64b	3.62c	2.27c	3.37ab	3.57ab
F-value		2.37^{ns}	23.40^{*}	292.73^{*}	296.38^{*}	4.26^{*}	5.22^{*}
P-value		0.10	<0.0001	<0.0001	<0.0001	0.02	0.007
HSD value		-	0.07	0.90	0.07	0.22	0.21

Note: ns = not significantly different * = 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

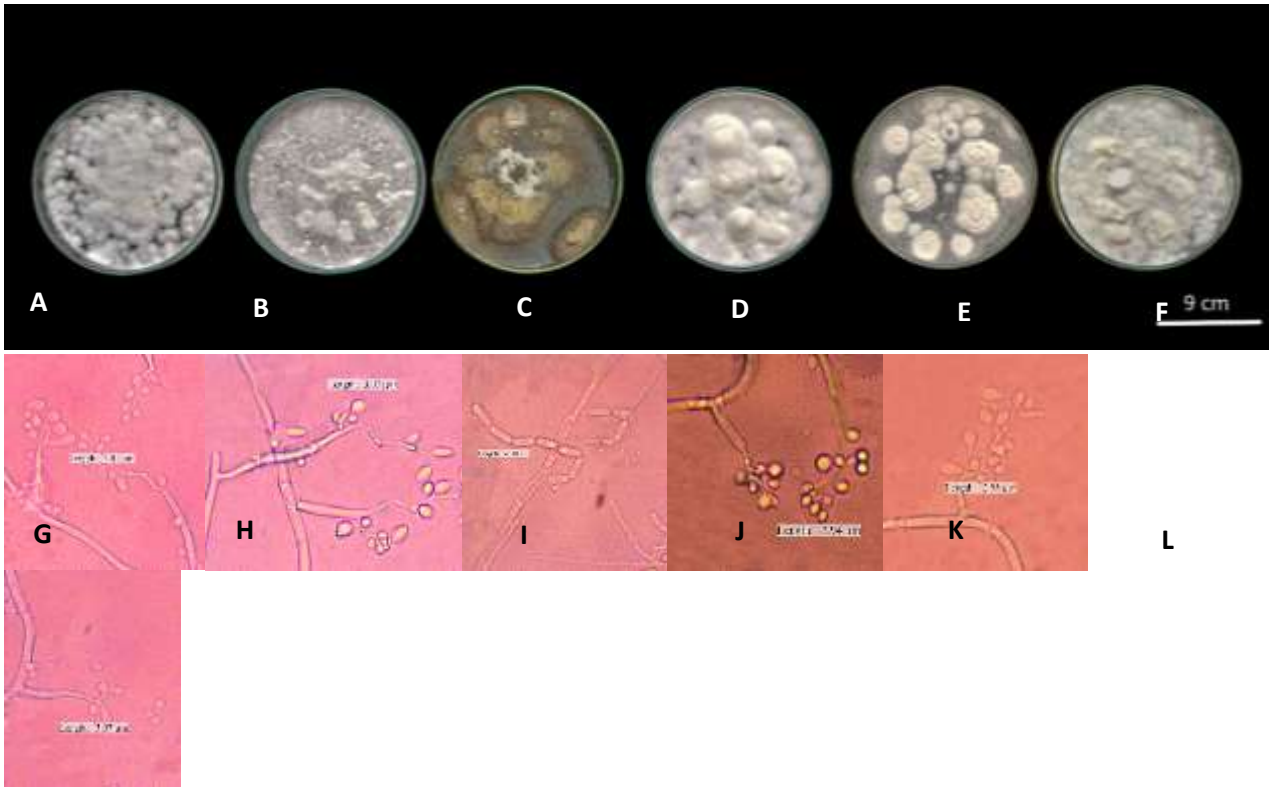
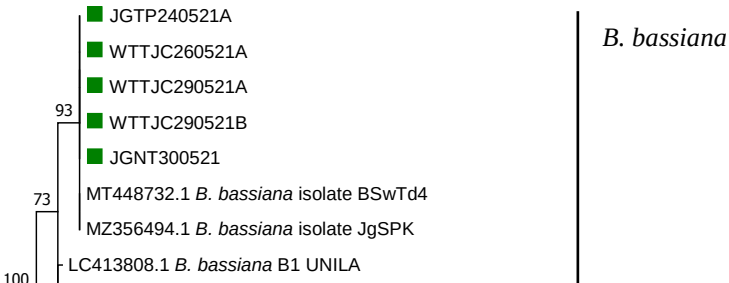


Fig. 1. Colony morphology of endophytic fungi cultured on PDA media (above) and the conidial and hyphal morphology (below) of the fungi: *Beauveria bassiana* of JGTP240521 isolate (A and G) and WTTJC260521A isolate (B and H), *Metarhizium anisoplae* WTTJC260521B isolate (C and I), *Beauveria bassiana* of WTTJC290521A isolate (D and J), WTTJC290521B isolate (E and K), and JGNT300521 isolate (F and L)

Tabel 4 Length of different developmental stages of pupae and adults of *Spodoptera frugiperda* fed on leaves of endophytic fungi colonized (seed treated) and non-colonized (control) young maize

		Length of different developmental stages (days)					
Isolate	Species	Prepupae	Pupae	Female adult	Male adult	Egg	Total lifespan
Control	-	3.61	6.95b	4.82a	4.40a	2.59c	32.51d
JGTP240521A	*Beauveria bassiana*	3.68	9.71a	4.37ab	3.28b	3.22abc	41.62a
WTTJC260521A	*Beauveria bassiana*	3.11	10.24a	4.21ab	3.40b	2.74bc	39.21ab
WTTJC260521B	*Metarhizium anisopliae*	3.65	7.43b	4.52ab	3.63b	2.84bc	35.44c
WTTJC290521A	*Beauveria bassiana*	3.79	9.85a	3.99b	4.34a	3.39ab	40.05a
WTTJC290521B	*Beauveria bassiana*	3.22	10.58a	4.23ab	3.52b	3.27abc	40.57a
JGNT300521	*Beauveria bassiana*	3.71	9.49a	4.93a	4.58a	3.65a	37.25bc
F-value		0.95^{ns}	22.43^{*}	5.12^{*}	49.86^{*}	6.39^{*}	34.57^{*}
P-value		0.50	<0.0001	0.008	<0.0001	0.003	<0.0001
HSD value		-	1.47	0.17	0.09	0.19	0.22

Note: ns = not significantly differen * = 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



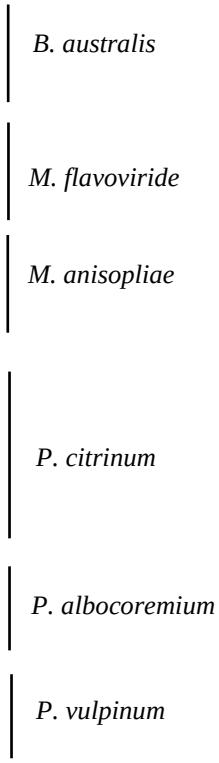


Fig. 2. Phylogenetic tree developed based on Internal Transcribed Spacer (ITS) region by Maximum Likelihood (Tamura-Nei model). The six investigated isolates were placed within group of *Beauveria bassiana* (5 isolates) and *Metarhizium anisopliae* (1 isolate). T= Type isolate

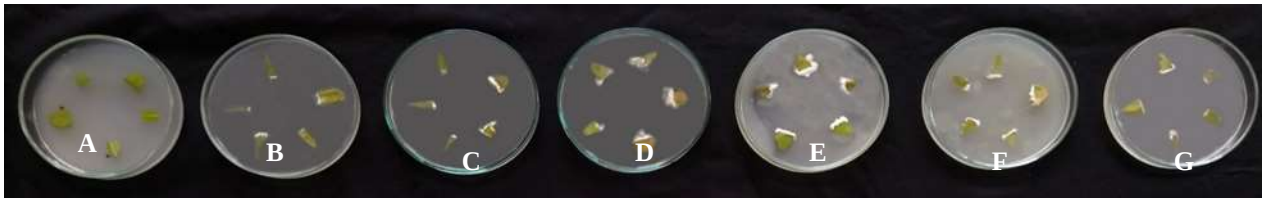


Fig. 3. Colony morphology of endophytic fungi from the leaves of maize where seed treatment was given and control (untreated seeds): Control (A), *Beauveria bassiana* of JGTP240521 isolate (B) and WTTJC260521A isolate (C), *Metarhizium anisoplae* WTTJC260521B isolate (D), *Beauveria bassiana* of WTTJC290521A isolate (E), WTTJC290521B isolate (F), and JGNT300521 isolate (G)

Fig. 4. The morphology of healthy larvae (control) (A), and the cadavers from larvae feeding on leaves colonized by fungi (above) and the conidial and hyphal morphology of fungi from cadaver re-isolation (below): *Beauveria bassiana* of JGTP240521 isolate (B and H) and WTTJC260521A isolate (C and I), *Metarhizium anisoplae* WTTJC260521B isolate (D and J), *Beauveria bassiana* of WTTJC290521A isolate (E and K), WTTJC290521B isolate (F and L), and JGNT300521 isolate (G and M)

Table 5 Mean of mortality of different larval instars of *Spodoptera frugiperda* fed on leaves of endophytic fungi colonized (seed treated) and non-colonized (control) young maize

Isolate	Species	Mean of mortality of different larval instars (%)					
		1st	2nd	3rd	4th	5th	6th
Control	-	2.67	6.00b	6.67e	6.67c	6.67c	6.67e
JGTP240521A	*Beauveria bassiana*	14.67	24.00a	43.33a	48.67a	51.33a	51.33a
WTTJC260521A	*Beauveria bassiana*	8.00	21.33a	36.67ab	39.33ab	42.00ab	45.33ab
WTTJC260521B	*Metarhizium anisopliae*	2.67	5.33b	8.00de	12.00c	15.33c	24.67d
WTTJC290521A	*Beauveria bassiana*	7.33	11.33ab	20.00cd	30.67b	36.00b	44.67ab
WTTJC290521B	*Beauveria bassiana*	8.67	16.67ab	22.67bc	27.33b	29.33b	38.67bc

JGNT300521	*Beauveria bassiana*	8.00	16.00ab	21.33bc	26.67b	28.00b	32.67c
F-value		2.93^{ns}	7.74*	22.96*	27.02*	35.02*	176.07*
P-value		0.053	0.001	<0.0001	<0.0001	<0.0001	<0.0001
HSD value		-	10.95	10.00	9.64	8.74	3.90

Note: ns = not significantly different * = 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

Table 6 Mean of percentage of pupae and adults emergence, sex ratio, egg laid, and viable eggs of *Spodoptera frugiperda* fed on leaves of endophytic fungi colonized (seed treated) and non-colonized (control) young maize

Isolates	Fungal species	Pupae emergence (%)	Adult emergence (%)	Sex ratio	Eggs laid per female	Viable (hatched) eggs (%)
Control		93.33a	93.33a	0.56a	68.28a	99.92a
JGTP240521A	*Beauveria bassiana*	46.00e	42.00e	0.83a	15.91c	88.86d
WTTJC260521A	*Beauveria bassiana*	52.67de	48.00de	0.84a	15.31c	90.93cd
WTTJC260521B	*Metarhizium anisopliae*	74.67b	71.33b	0.47a	42.89b	99.72a
WTTJC290521A	*Beauveria bassiana*	54.00d	47.33de	0.74a	27.86bc	95.91b
WTTJC290521B	*Beauveria bassiana*	59.33cd	54.00cd	0.87a	17.50c	92.98bc
JGNT300521	*Beauveria bassiana*	65.33c	58.67c	0.51a	39.36b	99.31a
F-value		134.80*	95.08*	3.67*	34.26*	75.47*
P-value		<0.0001	<0.0001	0.026	<0.0001	<0.0001
HSD value		6.85	9.03	0.19	1.37	4.16

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

Discussion

The results of identification based on the morphological characteristics of five isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) showed that they had similar morphology of the colony, hypha, and mycelia and the conidial shape. All belong to species of *B. bassiana*. These characteristics matched to *B. bassiana* described by Herlinda *et al.* (2021). The isolate of WTTJC260521B belonged to the species, *M. anisopliae*. The isolate morphology of the colony, and the hyphal, mycelia, and conidial of shape matched to *M. anisopliae* described by Herlinda *et al.* (2020).

The five fungal isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) found in this study had an rDNA sequence similarity value of 100% to the reference species (BLAST), JgSPK (Acc. No. MZ356494.1) an endophytic fungi isolated from maize (Herlinda *et al.* 2021), except one isolate (JGNT300521). If the similarity value is 100%, It means that they are the same strain (Henry *et al.* 2000). These isolates shared 99.80% of similarity with JGNT300521 as well as with BSwTd4 (Acc. No. MT448732.1) which was isolated from oil palm rhizosphere (Herlinda *et al.* 2020). JGNT300521 isolate shared 99.61% of similarity with BSwTd4. All the isolates shared 98.83% of similarity with B1 UNILA (=NKPT) (Acc. No. LC413808.1) which was isolated from maize rhizosphere (Fitriana *et al.* 2021) and type strain on *B. bassiana* (ARSEF 1564.T., Acc. No. HQ880761.1). The isolate of WTTJC260521B had an rDNA sequence similarity value of more than 99% to the BLAST (reference species). The similarity value of 99-100% indicated that the isolates were the same

species (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). If the similarity value of the isolates is 89–99%, it means that the isolates are of the same genus (Henry *et al.* 2000).

All fungal isolates of the *B. bassiana* and *M. anisopliae* tested in this study were able to colonize leaves of young maize when inoculated by seed immersion treatment. From the leaves of young maize treated by the fungi were overgrown with the fungal mycelia, but no fungal mycelia were found on the leaves of untreated seeds. The finding showed that both species of fungi from seed treatment were able to colonize the leaves. In addition to, the fungi of *B. bassiana* and *M. anisopliae* not only can colonize maize by seed treatment, but they also can colonize maize by foliar spray and root deeping and the fungi can systemically colonized leaves, stems, and roots of plants (Russo *et al.* 2020). *B. bassiana* inoculated by foliar spray can penetrate the leaf surface and move within the maize vascular (Wagner and Lewis 2000). *M. anisopliae* was often reported to be restricted to plant roots (Russo *et al.* 2020), however the present study reported that the strain of *M. anisopliae* was able to colonize the leaves of young maize. The extent and persistence of plant colonization by the fungi were influenced by fungal species/strain, inoculation method, and host plant species (Russo *et al.* 2020). The fungi used for seed treatment in this study is easier to be applied by soaking the seeds in fungal suspension before planting, or when the seeds stored, dry conidia of the fungi can be covered on the seeds.

Obtained findings reported that *B. bassiana* and *M. anisopliae* from South Sumatra (Indonesia) in seed treated corn seedlings had negative effects on development of *S. frugiperda.* This is first report that the fungi as an endophyte could decrease the female and male adult longevity of *S. frugiperda* and increased the larval mortality. The young maize colonized with the *B. bassiana* and *M. anisopliae* also reduced the percentage of the last instar becoming pupal stage and adult emergence, and decreased the eggs laid by the adults and the percentage of hatched eggs. Previous study reported that *B. bassiana* and *M. anisopliae* in foliar treated caused adverse effects on *S. frugiperda* development and survival (Russo *et al.* 2020). These adverse effects of endophytic fungi against *S. frugiperda* were caused by fungal production of secondary metabolites and mycosis (Vidal and Jaber 2015). The fungal secondary metabolites are produced by blastospores in insect hemolymph and disrupted the normal cell metabolism (Mancillas-Paredes *et al.* 2019) and then the toxins produced by the metabolites kill the insects (El-Ghany 2015). Our previous study showed that the endophytic fungi could decrease the leaf consumption by the *S. frugiperda* larvae resulting in larval weight loss and low survival (Gustianingtyas *et al.* 2021). The reduction in leaf area consumed by the larvae treated with the endophytes is caused by antifeedant or deterrent properties of *in planta*-produced *B. bassiana* metabolites (Russo *et al.* 2020). The corn plants colonized with *B. bassiana* may enhance levels of terpenoid defense compounds against *S. frugiperda* (Russo *et al.* 2020). The endophytic fungi could produce secondary metabolites *in planta* resulting antibiosis and feeding deterrence for the insects (Jaber and Ownley 2018).

These adverse effects of endophytic fungi against *S. frugiperda* were also caused by mycosis (Vidal and Jaber 2015). The present study found that the mycosis found on the cadavers of *S. frugiperda* treated with the fungi. The mycosis was evidenced by fungal mycelia and spores emerging from the cadavers of treated insects. However, no fungal mycelia and spores were found on the cadavers of untreated insects. Some previous studies have similar reported insect

mycosis feeding on fungal-endophytically colonized plants by *S. frugiperda* (Herlinda *et al.* 2021).

This study also showed that the fungal colonized young maize increased eggs, larvae, pupal developmental time, and lifespan of *S. frugiperda.* In contrast to the previous study of Russo *et al.* (2020) that these fungal species could decreased the development time of *S. frugiperda*. However, obtained findings are in agreement with previous study of Hussain *et al.* (2009) which showed that the lepidopteran, *Ocinara varians* treated with *B. bassiana* and *M. anisopliae* extended the developmental time of treated insects as compared to untreated ones (control) and the conversion of digested food and ingested food declined in treated insects compared to untreated insects, and stimulated the larvae to develop more slowly.

Conclusions

The results of molecular identification showed that the fungal species found were *B. bassiana* of five fungal isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) and *M. anisopliae* of an isolate (WTTJC260521B). *B. bassiana* and *M. anisopliae* colonized young maize significantly increased mortality of all larval instars of FAW compared to non-colonized ones. The larval mortality treated with *B. bassiana* (JGTP240521A isolates) was the highest among other treatments. Feeding on leaves of fungal colonized maize significantly decreased the percentage of the last larval instar becoming pupal stage and the adult emergence and the eggs laid, and the percentage of hatched eggs and increased the larval mortality. This is the first report that the *B. bassiana* and *M. anisopliae* from South Sumatra (Indonesia) in seed treated corn seedlings that had negative effects on development of *S. frugiperda.* Finally, these results highlight the potential of endophytic entomopathogenic fungi to protect corn plants against *S. frugiperda.*

List of abbreviations

ANOVA: analysis of variance; BLAST: Basic Local Alignment Search Tool; CTAB: cetyltrimethylammonium bromide; DNA: Deoxyribonucleic acid; EtOH: Ethyl alcohol; FAW: fall armyworm; 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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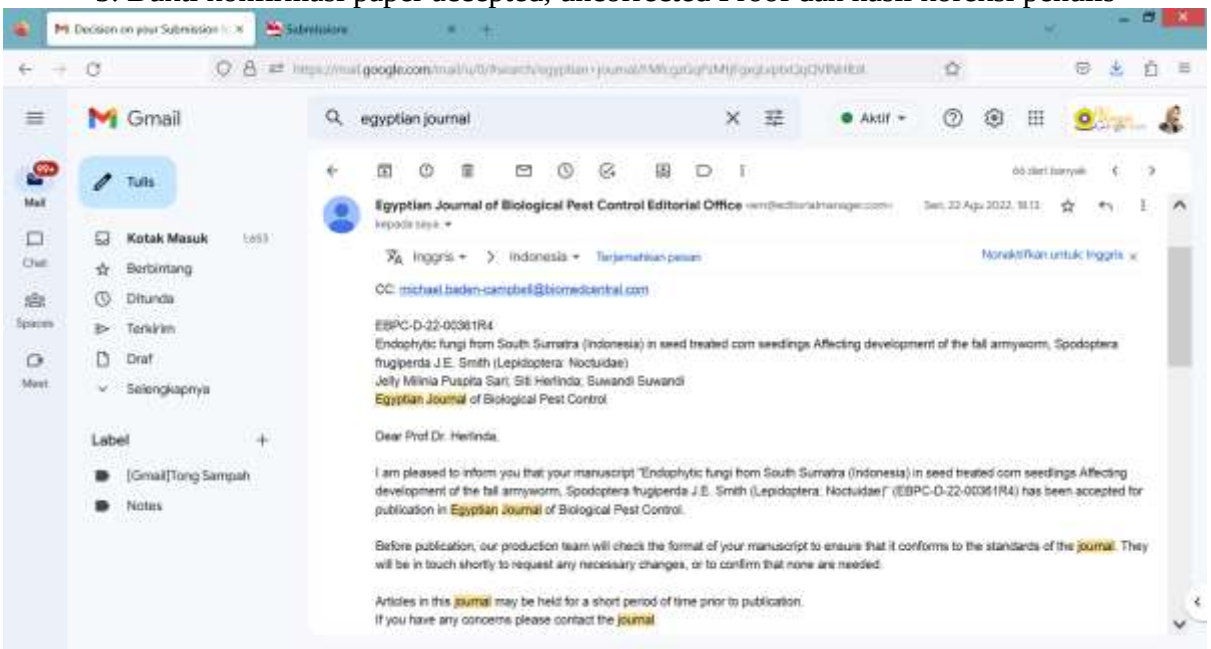

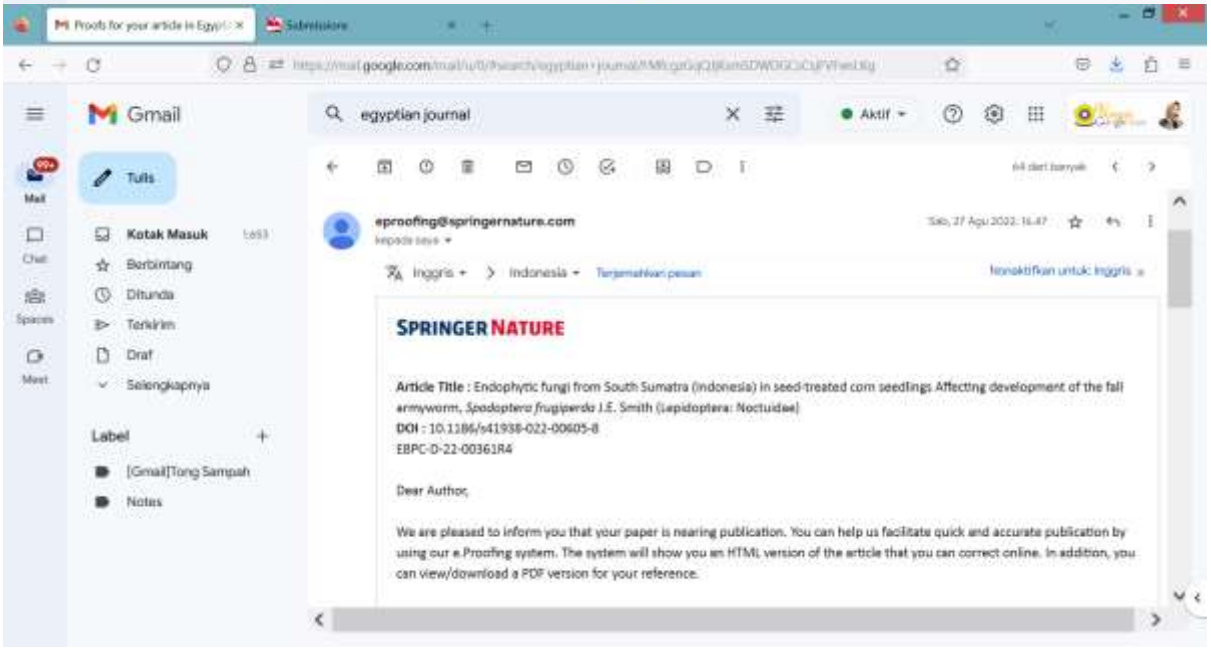

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Research

Endophytic fungi from South Sumatra (Indonesia) in seed-treated corn seedlings Affecting development of the fall armyworm, *Spodoptera frugiperda* J.E. Smith (Lepidoptera: Noctuidae)

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Abstract
Background

Topical application of the entomopathogenic fungi (EPFs) against *Spodoptera frugiperda* J.E. Smith (Lepidoptera: Noctuidae) larvae is less effective due to larvae hiding in the corn midribs in the field. To control the larvae, the fungi colonize in plant tissues or endophytic fungi are needed. There is no information on the pathogenicity of the endophytic fungi from Indonesia on the development of *S. frugiperda*. The endophytic fungi isolated from infected-host cadavers from South Sumatra (Indonesia) were identified morphologically and molecularly, and the effect of seed-treated corn seedlings with the fungi on *S. frugiperda* development was evaluated. **AQ1** The fungal identification was based on morphological and molecular characteristics. Bioassay of the endophytic fungal species in seed-treated young maize was performed against the neonate larvae (hatching within 24 h.) of first instar, and their development was observed. **AQ2**

Results

The results of molecular identification showed that the fungal species were *Beauveria bassiana* of five fungal isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) and *Metarhizium anisopliae* of an isolate (WTTJC260521B). The life span of *S. frugiperda* fed on leaves of fungal-colonized maize was significantly longer than those fed on leaves of non-colonized maize. The fungal-colonized young maize significantly increased mortality rate of all larval instars than non-colonized one. The last instar larvae mortality treated with *B. bassiana* (JGTP240521A isolates) (51.33%) was the highest among other treatments and did not significantly differ from each of *B. bassiana* of WTTJC260521A and WTTJC290521A isolates 45.33% and 44.67%, respectively. Feeding on leaves of fungal-colonized maize significantly decreased the percentage of the last instar larvae development to the pupal stage, the adult emergence, the eggs laid, and the percentage of hatched eggs. This is the first report that *B. bassiana* and *M. anisopliae* from South Sumatra (Indonesia) in seed-treated corn seedlings had negative effects on development of *S. frugiperda*.

Conclusions

Finally, these results highlight the potential of endophytic EPFs to protect corn plants against *S. frugiperda*.

Keywords

Spodoptera frugiperda
Beauveria bassiana
Metarhizium anisopliae
Endophyte
Entomopathogens

Abbreviations

ANOVA Analysis of variance
BLAST Basic local alignment search tool
CTAB Cetyltrimethylammonium bromide
DNA Deoxyribonucleic acid
EtOH Ethyl alcohol
FAW Fall armyworm
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

Background

Spodoptera frugiperda J.E. Smith (Lepidoptera: Noctuidae) or fall armyworm (FAW) is one of the most important noctuid pests of corn in the world. The FAW is a migratory and polyphagous pest that can attack 353 host plant species from 76 plant families (Montezano et al. 2018). The pest is native to the neotropics of the Americas and has spread throughout the world (Otim et al. 2018). More recently, the FAW becomes a new invasive pest in many parts of Africa (Niassy et al. 2021) and Asia (Lamsal et al. 2020), including Indonesia (Herlinda et al. 2021). This pest is commonly controlled using synthetic insecticides (Kumela et al. 2018); however, the resistances of the FAW to many insecticides, such as pyrethroid, spinosad, and organophosphorus insecticides have occurred (Zhang et al. 2021). In addition, the insecticide application negatively affects the human health and the environment (Harrison et al. 2019). An alternative more sustainable and eco-friendly control methods against *S. frugiperda* is urgently needed.

Biological control based on utilizing EPFs is the preferred control option for FAW (Mantzoukas and Eliopoulos 2020). Topical application (direct contact) of the EPF, *Beauveria bassiana* (Balsamo) Vuillemin (Deuteromycotina: Hyphomycetes), killed more than 80% of *S. frugiperda* larvae (Ramanujam et al. 2020). *Metarhizium anisopliae* (Metsch.) Sorok. (Deuteromycotina: Hyphomycetes) could kill 75% of *S. frugiperda* larvae (Ramos et al. 2020). However, in the field, the larvae occurred on the surface of leaves or maize stalks only in the morning, but at daylight up to night, they hide in the corn midribs (Herlinda et al. 2021). So, topical application of the fungus against the *S. frugiperda* larvae is less effective (Gustianingtyas et al. 2021). To control such hiding larvae in the field, the fungi colonizing in plant tissues or endophytic fungi are needed (Ramos et al. 2020). The endophytic

growth (Russo et al. 2020).

Previous studies have demonstrated that the endophytic fungal *B. bassiana* from corn root from Indonesia applied topically caused 29.33% of the FAW larval mortality (Gustianingtyas et al. 2021). The endophytic fungal *B. bassiana* used as seed treatment caused up to 22.67% of the FAW larval mortality (Herlinda et al. 2021). An experiment has previously also reported that endophytic *B. bassiana* sprayed on leaves has a high ability to colonize corn plants and the fungus caused significant reductions in the growth and development of *S. frugiperda* (Russo et al. 2020). There is no information on the pathogenicity of the endophytic fungi from Indonesia on the development of *S. frugiperda*. In addition, the potential of the fungi isolated from the infected-host cadaver as endophytic entomopathogens needs to be investigated. In this study, the fungi isolated from infected-host cadavers from South Sumatra (Indonesia) were identified morphologically and molecularly and the effect of seed-treated corn seedlings with the fungi on *S. frugiperda* development was evaluated.

Methods

Exploration of the fungi was performed by collecting infected-host insect cadavers from crops in South Sumatra, Indonesia, from May until June 2021. Purification and isolation of the fungi were carried out from June to July 2021. The morphological identification was carried out in the Laboratory of Entomology, Faculty of Agriculture, Universitas Sriwijaya, in July 2021, and the molecular identification was performed from August to December 2021 at the Laboratory of Agricultural Biotechnology (accredited according to the ISO/IEC 17025 standard), Department of Plant Protection, Faculty of Agriculture, Universitas Lampung, Indonesia. Experimental design used for bioassay was a completely randomized block designs consisted of seven treatments (six fungal isolates and control), and the experiment was repeated three times.

Fungal exploration, isolation, and purification

Fungal exploration from the infected-host cadavers using the method of Ab Majid et al. (2015) by collecting infected-host insects or cadaver infected with the fungi from the fields. The exploration was carried out in Tanjung Pering, Ogan Ilir, South Sumatra (3°13'23"S104°38'27"E), Tanjung Cermin, Pagar Alam, South Sumatra (4°02'23"S103°13"14"E), and Nendagung, Pagar Alam, South Sumatra (3°56'22"S103°12'15"E) (Table 1). The infected insects or cadavers were first surface-sterilized with 70% EtOH (Ethyl alcohol) and 1% NaOCl (Sodium hypochlorite), then rinsed 3 times (Elfita et al. 2019). After that, the sample cadavers were cultured aseptically onto SDA (Sabouraud Dextrose Agar) medium (Russo et al. 2020). The fungal culture was purified to make an isolate per sample. The fungal macroscopic and microscopic characteristics, such as the colonial color and shape, the conidial shape and size, and the conidiophores were observed (Herlinda et al. 2021), and then, molecular identification was performed.

Table 1

Origin of isolates of endophytic entomopathogenic 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	*Spodoptera frugiperda*	35.0	*Beauveria bassiana*	JGTP240521A	ON631784
Tanjung Cermin, Pagar Alam	Lepidoptera	817.2	*Beauveria bassiana*	WTTJC260521A	ON631780
Tanjung Cermin, Pagar Alam	Lepidoptera	817.2	*Metarhizium anisopliae*	WTTJC260521B	ON631793
Tanjung Cermin, Pagar Alam	Lepidoptera	817.2	*Beauveria bassiana*	WTTJC290521A	ON631783
Tanjung Cermin, Pagar Alam	Lepidoptera	817.2	*Beauveria bassiana*	WTTJC290521B	ON631782
Nendagung, Pagar Alam	*Spodoptera frugiperda*	802.6	*Beauveria bassiana*	JGNT300521	ON631778

DNA extraction, PCR amplification, and sequencing

DNA was extracted according to the method of Swibawa et al. (2020) and carried out on fungal conidia of 7-day-old fungus. As much as 10 ml of conidia suspension was centrifuged using CF15RXII for 10 min at a speed of 14,000 rpm. Then, 1 ml of 70% ethanol was added to the centrifuge tube and centrifuged again for 10 min. The supernatant was removed, and 1 ml of extraction buffer (0.5 ml Tris HCl, 1 mL SDS 1% + 2.8 mL NaCl, 0.2 ml mercaptoethanol, 2 ml EDTA, 3.5 ml sterile water) was added. The suspension was incubated at -40 °C for 24 h. The frozen suspension was crushed until pulverized. A total of 500 µl of pellet suspension was put into a 1.5 ml tube, and 400 µl of 2% CTAB (cetyltrimethylammonium bromide) was added, homogenized, and heated at 65 °C for an hour using a water bath (Brookfield TC 550 MX-230, USA). After the incubation, 500 µl of PCI (phenol/chloroform/isoamyl alcohol) (25:24:1) was added, homogenized, and centrifuged at 14,000 rpm for 10 min at 14,000 rpm for 10 min. A total of 600 µl supernatant was transferred to a new 1.5 ml tube, and 600 µl chloroform/isoamyl alcohol (24:1) was added, homogenized, and centrifuged (Microspin12; Biosan, Latvia) again at 14,000 rpm for 10 min. A total of 400 µl of supernatant was then put into to a new 1.5 ml tube, and 400 µl of cold isopropanol was homogenized and incubated at − 40 °C for 20 min. Then, the suspension was centrifuged at 14,000 rpm for 15 min. The supernatant was then discarded, and the pellet was added with 500 µl of 70% cold ethanol and centrifuged at 14,000 rpm for 5 min. The supernatant was then discarded, and the pellets obtained were incubated at room temperature for 24 h to dry. After drying, the pellets were 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 ITS (the Internal Transcribed Spacer) using ITS1 and ITS4 primers (White et al. 1990). The DNA amplification stage consisted of 1 initiation cycle at 95 °C for

5 min, 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. Then, the PCR results were 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 under taken in 1 × TBE buffer solution at 50 V for 70 min, and the results were visualized using a DigiDoc UV transilluminator (UVP, USA).

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

Mass-rearing of *Spodoptera frugiperda*

The mass-rearing of *S. frugiperda* was performed using the method of Herlinda et al. (2020a, b). The eggs of *S. frugiperda* were obtained from the Laboratory of Entomology, Faculty of Agriculture, Universitas Sriwijaya. They were reared in laboratory for more than 5 generations at 28–29 °C temperature, and 82–83% RH and the lighting set to photoperiod 12:12 (L:D) hrs. In the laboratory, the larvae of *S. frugiperda* were maintained individually due to cannibal behaviors and reared using fresh maize leaves. The prepupae and pupae were replaced 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. Emerged adults were used for bioassays.

Assessing endophytic fungal colonization

Fungal inoculation for maize seeds treated was carried out to assess the ability of the fungal colonization into the maize seedling tissue and to ensure that the fungi used in this experiment were truly endophytic. All the isolates used were grown in SDA medium incubated for 14 days, and then, the SDA fungal culture was transferred to the broth medium (SDB, Sabouraud Dextrose Broth) following the method of Gustianingtyas et al. (2020) and incubated for 7 days on the shaker and 7 days unshaken position. The 45 corn seeds for an isolate were surface-sterilized by using (Russo et al. 2020) method. The seeds were immersed in 10 ml of fungal suspension (1 × 10^{10} conidia ml^{-1}) for 24 h, while for the control only 10 ml of sterilized water was treated for the seeds. Then, the seeds were grown in the hydroponic medium, following the method of Novianti et al. (2020) and incubated for 14 days, and this treatment was repeated 3 times for each isolate. The tip leaves of 14-day-old maize seedlings (young maize) were cut of 5 × 5 mm^2 to be grown onto the SDA medium to detect the mycelia of the endophytic fungi. The leaf materials were first surface-sterilized by using method of (Russo et al. 2020) before grown onto the SDA medium. The leaf material surface-sterilized was carried out by immersion in 70% ethanol, then followed by sodium hypochlorite for 2 min, and rinsed twice in sterile distilled water, and the final rinse water was grown onto SDA and incubated for 10 days. The rest or remaining leaves were used for bioassays as described below.

Bioassay for assessing effect of corn seed treatment on *S. frugiperda* development

The bioassay for assessing the effect of corn seed treatment on *S. frugiperda* growth and development followed the method of Russo et al. (2020). The 14-day-old maize seedlings already inoculated with the endophytic fungi as described above were given to be consumed to the first instar neonate larvae of *S. frugiperda*, while for control treatment, the larvae were provided the non-inoculated young maize and this experiment was repeated three times. The 50 neonate larvae (hatching within 24 h) of first larvae instar were allowed to feed on the treated young maize and untreated ones (control) for 6 h or until the leaves eaten up, and this treatment was replicated three times for each isolate and the control. Then, the larvae were individually kept in a porous plastic cup (Ø 6.5 cm, height 4.6 cm) and were fed on healthy non-inoculated leaves measuring 2 cm × 5 cm per day per larvae and replaced with a fresh new one every day. The treatments of this experiment consisted of the six fungal isolates and the control (water) and used the completely randomized block designs. The variables recorded were development time of each stage (egg, larval, pupal, and adult) and mortality of each stage. The larval and pupal mortality were recorded daily, and the adults emerging were observed every day. The sex of adults emerged was recorded, and the adults were placed in the wire mesh cage for copulation with fresh maize leaves inside to allow egg-laying. Egg collection and 10% honey bee solution replacement for adults were carried out every day. The adult longevity was also observed until the adult death.

Data analysis

The differences in the length of different stages (egg, larval, pupal, and adult), mortality of each stage, adult longevity, eggs laid, and sex ratio of each treatment were analyzed by analysis 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

Identification of the endophytic fungal isolates

Five fungal isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) had the same macroscopic and microscopic characteristics, while the isolate (WTTJC260521B) had different characteristics (Fig. 1). The

WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A isolates had a white colonial, hyphal, and mycelia color, and non-septate and globose conidia or spores. These characteristics were placed the isolates within the group of *B. bassiana* (Fig. 2). The isolates were deposited in the GenBank with the accession number ON631784 (JGTP240521A isolate), ON631780 (WTTJC260521A isolate), ON631783 (WTTJC290521A isolate), ON631782 (WTTJC290521B isolate), ON631778 (JGNT300521 isolate) (Table 1). The isolate of WTTJC260521B had a white young colony, then the older colony turned greenish white to dark green, and the isolate had the green hyphae and mycelia, and the isolate had the non-septation, clear, and cylindrical conidia. The isolate was placed within the group of *M. anisopliae* (Fig. 2). The WTTJC260521B isolate was deposited in the GenBank with the accession number ON631793 (WTTJC260521B isolate) (Table 1).

Fig. 1

Colony morphology of endophytic fungi cultured on PDA media (above) and the conidial and hyphal morphology (below) of the fungi: *Beauveria bassiana* of JGTP240521 isolate (**A** and **G**) and WTTJC260521A isolate (**B** and **H**), *Metarhizium anisopliae* WTTJC260521B isolate (**C** and **I**), *Beauveria bassiana* of WTTJC290521A isolate (**D** and **J**), WTTJC290521B isolate (**E** and **K**), and JGNT300521 isolate (F and L)

Fig. 2

Phylogenetic tree developed based on internal transcribed spacer (ITS) region by maximum likelihood (Tamura–Nei model). The six investigated isolates were placed within group of *Beauveria bassiana* (5 isolates) and *Metarhizium anisopliae* (1 isolate). T = Type isolate

The result of BLAST search revealed that five fungal isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) shared 100% of similarity each other as well as with JgSPK (Acc. No. MZ356494.1). All the isolates shared 99.80% of similarity with JGNT300521 as well as with BSwTd4 (Acc. No. MT448732.1). JGNT300521 isolate shared 99.61% of similarity with BSwTd4. All the isolates shared 98.83% of similarity with B1 UNILA (= NKPT) (Acc. No. LC413808.1) and type strain on *B. bassiana* (ARSEF1564.T., Acc. No. HQ880761.1). Isolate of WTTJC260521B shared 99.992% of similarity with reference isolate of IPPM010202 (Acc. No. KY437678.1), 99.994% of similarity with strain MSwTp3 (Acc. No. MT448733.1), and 99.985% of similarity with the type strain of *M. anisopliae* ARSEF 7487.T (Acc. No. HQ331446.1). So, there were two 2 species from the 6 investigated isolates of the endophytic fungi found in this study. The five isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) were in the group of *B. bassiana*, and one isolate (WTTJC260521B) was in the group of *M. anisopliae*.

Development of *Spodoptera frugiperda* fed on young maize colonized and non-colonized by fungi

Six fungal isolates found and used in this experiment were able to colonize young maize (seedling) when inoculated by seed immersion treatment, and a percentage of leaves colonized by endophytic entomopathogenic fungi was high at 14 days than at 7 days after inoculation (Table 2). They were confirmed as endophytic fungi. The seed immersion treatment resulted leaves of treated young maize grown on to the SDA medium were overgrown with the fungal isolates (Fig. 3). No fungal growth was found on the leaves of untreated maize on the last rinse water. This confirmed that the fungal isolates used in this study were endophytic fungi and it also showed that the surface sterilization of maize tissues eliminated the epiphytic microorganisms and the fungi growing out of the leaf surface were the endophytic fungi originating from within the maize tissues.

Table 2

Effect of fungal isolates on mean colonization (%) or percentage of leaves colonized by endophytic entomopathogenic fungi at 7 and 14 days after inoculation

Isolate	Species	Mean colonization (%)	
		7 days after inoculation	14 days after inoculation
Control	–	0.00b	0.00c
JGTP240521A	*Beauveria bassiana*	100.00a	100.00a
WTTJC260521A	*Beauveria bassiana*	93.33a	100.00a
WTTJC260521B	*Metarhizium anisopliae*	26.67b	60.00b
WTTJC290521A	*Beauveria bassiana*	100.00a	100.00a
WTTJC290521B	*Beauveria bassiana*	80.00a	100.00a
JGNT300521	*Beauveria bassiana*	80.00a	100.00a
F-value		26.31**	168.50**
P-value		3.07×10^{-6}	7.16×10^{-11}
HSD value		32.16	13.07

Fig. 3

Colony morphology of endophytic fungi from the leaves of maize where seed treatment was given and control (untreated seeds): Control (**A**), *Beauveria bassiana* of JGTP240521 isolate (**B**) and WTTJC260521A isolate (**C**), *Metarhizium anisopliae* WTTJC260521B isolate (**D**), *Beauveria bassiana* of WTTJC290521A isolate (**E**), WTTJC290521B isolate (**F**), and JGNT300521 isolate (**G**)

The larvae that consumed leaves of colonized maize exhibited distinctive symptoms, namely smaller body, shrivels, hardens like a mummy, but the healthy larvae of the control were longer and bigger than treated larvae. The cadavers were covered by mycelia and conidia and their colors depending of the fungal species (Fig. 4). The color of cadavers from the larvae that consumed leaves colonized by *B. bassiana* and *M. anisopliae* was white and green, respectively. Re-isolation of the fungus from the cadavers showed the same fungal isolates found from the larvae that died after feeding on the leaves of the plants where seed treatment was given (Fig. 4).

Fig. 4

The morphology of healthy larvae (control) (**A**) and the cadavers from larvae feeding on leaves colonized by fungi (above) and the

and WTTJC260521A isolate (**C** and **I**), *Metarhizium anisopliae* WTTJC260521B isolate (**D** and **J**), *Beauveria bassiana* of WTTJC290521A isolate (**E** and **K**), WTTJC290521B isolate (**F** and **L**), and JGNT300521 isolate (**G** and **M**)

Feeding on leaves of fungal-colonized maize significantly increased developmental time of the second, third, fourth, fifth, and sixth larval instars ($P < 0.0001$) (Table 3). However, there was non-significant difference in the developmental time of first instar larvae of treated and untreated maize (control). This fungal-colonized maize also increased egg and pupal development time, but decreased female and male adult longevity. The life span of *S. frugiperda* fed on leaves of fungal-colonized maize was significantly longer than those fed on leaves of non-colonized maize (Table 4). The longest life span of *S. frugiperda* occurred on the individuals fed on leaves of *B. bassiana*-colonized maize.

Table 3

Length of different developmental stages of instar larvae of *Spodoptera frugiperda* fed on leaves of endophytic fungi colonized (seed treated) and non-colonized (control) young maize

Isolate	Species	Length of different developmental larval instars (days)					
		1st	2nd	3rd	4th	5th	6th
Control	–	2.67	3.34c	2.36d	2.27c	3.26b	3.23b
JGTP240521A	*Beauveria bassiana*	2.71	3.66b	5.70a	4.45a	3.95a	3.86ab
WTTJC260521A	*Beauveria bassiana*	2.59	3.71b	4.00b	4.60a	2.99b	3.76ab
WTTJC260521B	*Metarhizium anisopliae*	2.63	3.68b	2.66d	3.71b	3.65ab	3.51ab
WTTJC290521A	*Beauveria bassiana*	2.63	3.71b	3.63a	4.46a	3.69ab	4.28a
WTTJC290521B	*Beauveria bassiana*	2.60	4.28a	5.46a	3.79b	3.28ab	3.13b
JGNT300521	*Beauveria bassiana*	2.65	3.64b	3.62c	2.27c	3.37ab	3.57ab
F-value		2.37^{ns}	23.40*	292.73*	296.38*	4.26*	5.22*
P-value		0.10	< 0.0001	< 0.0001	< 0.0001	0.02	0.007
HSD value		–	0.07	0.90	0.07	0.22	0.21

Note: ns = not significantly different * = 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

Table 4

Length of different developmental stages of pupae and adults of *Spodoptera frugiperda* fed on leaves of endophytic fungi colonized (seed treated) and non-colonized (control) young maize

Isolate	Species	Length of different developmental stages (days)					
		Prepupae	Pupae	Female adult	Male adult	Egg	Total life span
Control	–	3.61	6.95b	4.82a	4.40a	2.59c	32.51d
JGTP240521A	*Beauveria bassiana*	3.68	9.71a	4.37ab	3.28b	3.22abc	41.62a
WTTJC260521A	*Beauveria bassiana*	3.11	10.24a	4.21ab	3.40b	2.74bc	39.21ab
WTTJC260521B	*Metarhizium anisopliae*	3.65	7.43b	4.52ab	3.63b	2.84bc	35.44c
WTTJC290521A	*Beauveria bassiana*	3.79	9.85a	3.99b	4.34a	3.39ab	40.05a
WTTJC290521B	*Beauveria bassiana*	3.22	10.58a	4.23ab	3.52b	3.27abc	40.57a
JGNT300521	*Beauveria bassiana*	3.71	9.49a	4.93a	4.58a	3.65a	37.25bc
F-value		0.95^{ns}	22.43*	5.12*	49.86*	6.39*	34.57*

ns = not significantly different * = significantly different; values within a column followed by the same letters were not significantly different

Isolate	Species	Length of different developmental stages (days)					
		Prepupae	Pupae	Female adult	Male adult	Egg	Total life span
P-value		0.50	< 0.0001	0.008	< 0.0001	0.003	< 0.0001
HSD value		–	1.47	0.17	0.09	0.19	0.22

ns = not significantly different * = 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 fungal-colonized young maize significantly increased mortality of all larval instars than the non-colonized one (Table 5). The mortality of last instar larvae treated with *B. bassiana* (JGTP240521A isolates) (51.33%) was the highest among other treatments and did not significantly differ from each of the *B. bassiana* (WTTJC260521A and WTTJC290521A isolates) (45.33 and 44.67%, respectively). Feeding on leaves of fungal-colonized maize significantly decreased the percentage of the last larval instar becoming pupal stage and adult emergence (Table 6). The young maize colonized with fungi significantly reduced eggs laid by the adults (fecundity), but did not affect the sex ratio of *S. frugiperda*. Percentage of hatched eggs significantly decreased on the treatment of *B. bassiana* (JGTP240521A, WTTJC260521A, WTTJC290521A, and WTTJC290521B isolates) (Table 6).

Table 5

Mean of mortality of different larval instars of *Spodoptera frugiperda* fed on leaves of endophytic fungi colonized (seed treated) and non-colonized (control) young maize

Isolate	Species	Mean of mortality of different larval instars (%)					
		1st	2nd	3rd	4th	5th	6th
Control	–	2.67	6.00b	6.67e	6.67c	6.67c	6.67e
JGTP240521A	*Beauveria bassiana*	14.67	24.00a	43.33a	48.67a	51.33a	51.33a
WTTJC260521A	*Beauveria bassiana*	8.00	21.33a	36.67ab	39.33ab	42.00ab	45.33ab
WTTJC260521B	*Metarhizium anisopliae*	2.67	5.33b	8.00de	12.00c	15.33c	24.67d
WTTJC290521A	*Beauveria bassiana*	7.33	11.33ab	20.00cd	30.67b	36.00b	44.67ab
WTTJC290521B	*Beauveria bassiana*	8.67	16.67ab	22.67bc	27.33b	29.33b	38.67bc
JGNT300521	*Beauveria bassiana*	8.00	16.00ab	21.33bc	26.67b	28.00b	32.67c
F-value		2.93 ns	7.74*	22.96*	27.02*	35.02*	176.07*
P-value		0.053	0.001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
HSD value		–	10.95	10.00	9.64	8.74	3.90

ns = not significantly different * = 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

Table 6

Mean of percentage of pupae and adults emergence, sex ratio, egg laid, and viable eggs of *Spodoptera frugiperda* fed on leaves of endophytic fungi colonized (seed treated) and non-colonized (control) young maize

Isolates	Fungal species	Pupae emergence (%)	Adult emergence (%)	Sex ratio	Eggs laid per female	Viable (hatched) eggs (%)
Control		93.33a	93.33a	0.56a	68.28a	99.92a
JGTP240521A	*Beauveria bassiana*	46.00e	42.00e	0.83a	15.91c	88.86d
WTTJC260521A	*Beauveria bassiana*	52.67de	48.00de	0.84a	15.31c	90.93 cd
WTTJC260521B	*Metarhizium anisopliae*	74.67b	71.33b	0.47a	42.89b	99.72a
WTTJC290521A	*Beauveria bassiana*	54.00d	47.33de	0.74a	27.86bc	95.91b
WTTJC290521B	*Beauveria bassiana*	59.33 cd	54.00 cd	0.87a	17.50c	92.98bc
JGNT300521	*Beauveria bassiana*	65.33c	58.67c	0.51a	39.36b	99.31a
F-value		134.80*	95.08*	3.67*	34.26*	75.47*
P-value		< 0.0001	< 0.0001	0.026	< 0.0001	< 0.0001
HSD value		6.85	9.03	0.19	1.37	4.16

* = 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

Discussion

The results of identification based on the morphological characteristics of five isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) showed that they had similar morphology of the colony, hypha, and mycelia

al. (2021). The isolate of WTTJC260521B belonged to the species, *M. anisopliae*. The isolate morphology of the colony and the hyphal, mycelia, and conidial of shape matched to *M. anisopliae* described by Herlinda et al. (2020a, b).

The five fungal isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) found in this study had an rDNA sequence similarity value of 100% to the reference species (BLAST), JgSPK (Acc. No. MZ356494.1) an endophytic fungi isolated from maize (Herlinda et al. 2021), except one isolate (JGNT300521). If the similarity value is 100%, it means that they are the same strain (Henry et al. 2000). These isolates shared 99.80% of similarity with JGNT300521 as well as with BSwTd4 (Acc. No. MT448732.1) which was isolated from oil palm rhizosphere (Herlinda et al. 2020a, b). JGNT300521 isolate shared 99.61% of similarity with BSwTd4. All the isolates shared 98.83% of similarity with B1 UNILA (= NKPT) (Acc. No. LC413808.1) which was isolated from maize rhizosphere (Fitriana et al. 2021) and type strain on *B. bassiana* (ARSEF 1564.T., Acc. No. HQ880761.1). The isolate of WTTJC260521B had an rDNA sequence similarity value of more than 99% to the BLAST (reference species). The similarity value of 99–100% indicated that the isolates were the same species (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). If the similarity value of the isolates is 89–99%, it means that the isolates are of the same genus (Henry et al. 2000).

All fungal isolates of the *B. bassiana* and *M. anisopliae* tested in this study were able to colonize leaves of young maize when inoculated by seed immersion treatment. The leaves of young maize treated by the fungi were overgrown with the fungal mycelia, but no fungal mycelia were found on the leaves of untreated seeds. The finding showed that both species of fungi from seed treatment were able to colonize the leaves. In addition, the fungi of *B. bassiana* and *M. anisopliae* not only can colonize maize by seed treatment, but they also can colonize maize by foliar spray and root deeping and the fungi can systemically colonize leaves, stems, and roots of plants (Russo et al. 2020). *B. bassiana* inoculated by foliar spray can penetrate the leaf surface and move within the maize vascular (Wagner and Lewis 2000). *M. anisopliae* was often reported to be restricted to plant roots (Russo et al. 2020); however, the present study reported that the strain of *M. anisopliae* was able to colonize the leaves of young maize. The extent and persistence of plant colonization by the fungi were influenced by fungal species/strain, inoculation method, and host plant species (Russo et al. 2020). The fungi used for seed treatment in this study are easier to be applied by soaking the seeds in fungal suspension before planting, or when the seeds stored, dry conidia of the fungi can be covered on the seeds. **AQ3**

Obtained findings reported that *B. bassiana* and *M. anisopliae* from South Sumatra (Indonesia) in seed-treated corn seedlings had negative effects on development of *S. frugiperda*. This is the first report that the fungi as an endophyte could decrease the female and male adult longevity of *S. frugiperda* and increased the larval mortality. The young maize colonized with the *B. bassiana* and *M. anisopliae* also reduced the percentage of the last instar becoming pupal stage and adult emergence and decreased the eggs laid by the adults and the percentage of hatched eggs. Previous study reported that *B. bassiana* and *M. anisopliae* in foliar treated caused adverse effects on *S. frugiperda* development and survival (Russo et al. 2020). These adverse effects of endophytic fungi against *S. frugiperda* were caused by fungal production of secondary metabolites and mycosis (Vidal and Jaber 2015). The fungal secondary metabolites are produced by blastospores in insect hemolymph and disrupted the normal cell metabolism (Mancillas-Paredes et al. 2019), and then, the toxins produced by the metabolites kill the insects (El-Ghany 2015). Our previous study showed that the endophytic fungi could decrease the leaf consumption by the *S. frugiperda* larvae resulting in larval weight loss and low survival (Gustianingtyas et al. 2021). The reduction in leaf area consumed by the larvae treated with the endophytes is caused by antifeedant or deterrent properties of *in planta*-produced *B. bassiana* metabolites (Russo et al. 2020). The corn plants colonized with *B. bassiana* may enhance levels of terpenoid defense compounds against *S. frugiperda* (Russo et al. 2020). The endophytic fungi could produce secondary metabolites *in planta* resulting in antibiosis and feeding deterrence for the insects (Jaber and Ownley 2018).

These adverse effects of endophytic fungi against *S. frugiperda* were also caused by mycosis (Vidal and Jaber 2015). The present study found the mycosis found on the cadavers of *S. frugiperda* treated with the fungi. The mycosis was evidenced by fungal mycelia and spores emerging from the cadavers of treated insects. However, no fungal mycelia and spores were found on the cadavers of untreated insects. Some previous studies have similar reported insect mycosis feeding on fungal-endophytically colonized plants by *S. frugiperda* (Herlinda et al. 2021).

This study also showed that the fungal-colonized young maize increased eggs, larvae, pupal developmental time, and life span of *S. frugiperda*. In contrast to the previous study of Russo et al. (2020), these fungal species could decrease the development time of *S. frugiperda*. However, obtained findings are in agreement with previous study of Hussain et al. (2009) which showed that the lepidopteran, *Ocinara varians* treated with *B. bassiana* and *M. anisopliae*, extended the developmental time of treated insects as compared to untreated ones (control) and the conversion of digested food and ingested food declined in treated insects compared to untreated insects and stimulated the larvae to develop more slowly.

Conclusions

The results of molecular identification showed that the fungal species found were *B. bassiana* of five fungal isolates (WTTJC290521B, WTTJC290521A, JGTP240521A, JGNT300521, and WTTJC260521A) and *M. anisopliae* of an isolate (WTTJC260521B). *B. bassiana*- and *M. anisopliae*-colonized young maize significantly increased mortality of all larval instars of FAW compared to non-colonized ones. The larval mortality treated with *B. bassiana* (JGTP240521A isolates) was the highest among other treatments. Feeding on leaves of fungal-colonized maize significantly decreased the percentage of the last larval instar becoming pupal stage and the adult emergence and the eggs laid and the percentage of hatched eggs and increased the larval mortality. This is the first report that the *B. bassiana* and *M. anisopliae* from South Sumatra (Indonesia) in seed-treated corn seedlings had negative effects on development of *S. frugiperda*. Finally, these results highlight the potential of endophytic entomopathogenic fungi to protect corn plants against *S. frugiperda*.

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Authors' contributions

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